

# Construction and Expression of Hepatitis B Surface Antigen Escape Variants within the "a" Determinant by Site Directed Mutagenesis

Forough Golsaz Shirazi<sup>1</sup>, Mohammad Mehdi Amiri<sup>1</sup>, Hamed Mohammadi<sup>1</sup>, Ali Ahmad Bayat<sup>2</sup>, Azam Roohi<sup>1,3</sup>, Jalal Khoshnoodi<sup>1</sup>, Amir Hassan Zarnani<sup>4</sup>, Mahmood Jeddi-Tehrani<sup>2</sup>, Gholam Ali Kardar<sup>4</sup>, Fazel Shokri<sup>1,2\*</sup>

<sup>1</sup>Department of Immunology, School of Public Health, Tehran University of Medical Sciences, <sup>2</sup>Monoclonal Antibody Research Center, <sup>3</sup>Department of Molecular Medicine, School of Advanced Medical Technologies, Tehran University of Medical Sciences, <sup>4</sup>Reproductive Immunology Research Center, Avicenna Research Institute, ACECR; <sup>4</sup>Immunology, Asthma and Allergy Research Institute, Tehran University of Medical Sciences, Tehran, Iran

## ABSTRACT

**Background:** The antibody response to hepatitis B surface antigen (HBsAg) controls hepatitis B virus infection. The "a" determinant of HBsAg is the most important target for protective antibody response, diagnosis and immunoprophylaxis. Mutations in this area may induce immune escape mutants and affect the performance of HBsAg assays. **Objectives:** To construct clinically relevant recombinant mutant forms of HBsAg and assessment of their reactivity with anti-HBs monoclonal antibodies (MAbs). **Methods:** Wild type (wt) and mutant (mt) HBsAg genes were constructed by site directed mutagenesis and SEOing PCR. The amplified genes were inserted into pCMV6-neo plasmid and transfected in CHO cell line. The expression of wt- and mtHBsAg was assessed by commercial ELISA assays and stable cells were established and cloned by limiting dilution. The recombinant mutants were further characterized using a panel of anti-HBs monoclonal antibodies (MAbs) and the pattern of their reactivity was assessed by ELISA. **Results:** Ten HBsAg mutants having single mutation within the "a" determinant including P120E, T123N, Q129H, M133L, K141E, P142S, D144A, G145R, N146S and C147S together with a wt form were successfully constructed and expressed in CHO cells. Reactivity of anti-HBs MAbs with mtHBsAg displayed different patterns. The effect of mutations on antibody binding differed depending on the amino acid involved and its location within the "a" determinant. Mutation at amino acids 123 and 145 resulted in either complete loss or significant reduction of binding to all anti-HBs MAbs. **Conclusion:** Our panel of mtHBsAg is a valuable tool for assessment of the antibody response to HBV escape mutants and may have substantial implications in HBV immunological diagnostics.

Golsaz Shirazi F, et al. *Iran J Immunol.* 2013; 10(3):127-138

**Keywords:** "a" Determinant, HBs Ag, Monoclonal Antibody

\*Corresponding authors: Professor Fazel Shokri, Monoclonal Antibody Research Center, Avicenna Research Institute, ACECR, Tehran, Iran, Tel: (+) 98 21 22432020, Fax: (+) 98 21 22432021, e-mail: fshokri@tums.ac.ir  
Dr. Gholam Ali Kardar, Immunology, Asthma and Allergy Research Institute, Tehran University of Medical Sciences, Tehran, Iran, Tel: (+) 98 21 66919589, Fax: (+) 98 21 66428998, e-mail: gakardar@tums.ac.ir

## INTRODUCTION

Hepatitis B virus (HBV) infection is a major health problem, which is currently estimated to affect 350-400 million people worldwide. As many as one third of the world's population have evidence of exposure to this virus (1). Moreover, chronic hepatitis B infection is associated with the risk of developing liver cirrhosis and hepatocellular carcinoma (2-3).

Since HBV uses reverse transcriptase to replicate through an RNA intermediate, mutant viral genomes and quasi-species are generated leading to emergence of viral mutants during naturally occurring infection (4). Emergence of vaccine-induced escape mutant form of HBV was first described in 1990 in a child who received passive-active post-exposure immunization (5). This escape mutant contained a single mutation at the amino acid position 145 of the hepatitis B surface antigen (HBsAg) that translated into a change from a glycine to an arginine. Recognition of other mutations within the "a" determinant raised concerns about the success of vaccination program, and also diagnostic assays, which depend on the epitopes recognized by the assay reagent configuration (6-7). Besides, prevention and treatment strategies such as vaccination, hepatitis B immune globulin (HBIG) and anti-viral drugs exert evolutionary pressures to select mutants, which are termed escape mutants (4). The mutants could also be selected by pressure from the humoral or cellular immune response (8).

In fact, mutations within S gene could foster complications such as occult hepatitis B infection, reactivation of hepatitis B, diagnostic assay failure and reinfection in HBV-infected recipients of orthotopic liver transplantations (4). These mutations are stable and can be transmitted horizontally and vertically. Mutations detected in patients with chronic hepatitis, are mainly clustered inside the "a" determinant; which is located between amino acids 124 and 147 of HBsAg and placed within the major hydrophilic region (MHR) of the S gene (4,9). According to recent publications, prevalence of mutant HBsAg variants (mtHBsAg) among random chronic carriers is estimated to be between 6 to 12% (7). Since spread of escape HBV mutants to other successfully vaccinated individuals is possible, control of mutant forms is a matter of great concern (7). Over the past two decades, new treatment regimens, with successfully reduced overall HBV infection rates, have been developed. These therapeutic protocols, however, have also exerted powerful selection pressures in favor of the emergence of HBV mutants. These treatments include immunotherapy (vaccination, administration of HBIG) and nucleoside analogues to inhibit polymerase activity, which could suppress wild-type HBV to undetectable levels, allowing a mutant HBV strain to emerge as the predominant form (6).

Escape mutants with amino acid changes at the "a" determinant might be transmitted or carried undetected by conventional HBsAg screening tests and lead to misdiagnosis of infectious patients and blood donor screening, unreliability of epidemiological investigation and threatening the success of hepatitis B immunization in long term (10-11). Since these mutants may reduce the efficiency of the current diagnostic and treatment protocols, diagnostic and healthcare industry needs to increase their awareness (6).

In the present study, we constructed and expressed 10 common recombinant mtHBsAg and studied their pattern of reactivity with a panel of anti-HBs monoclonal antibodies. These mutants are a valuable tool for immune-screening of escape variants of HBV and assessment of the antibody response to HBV mutants.

## MATERIALS AND METHODS

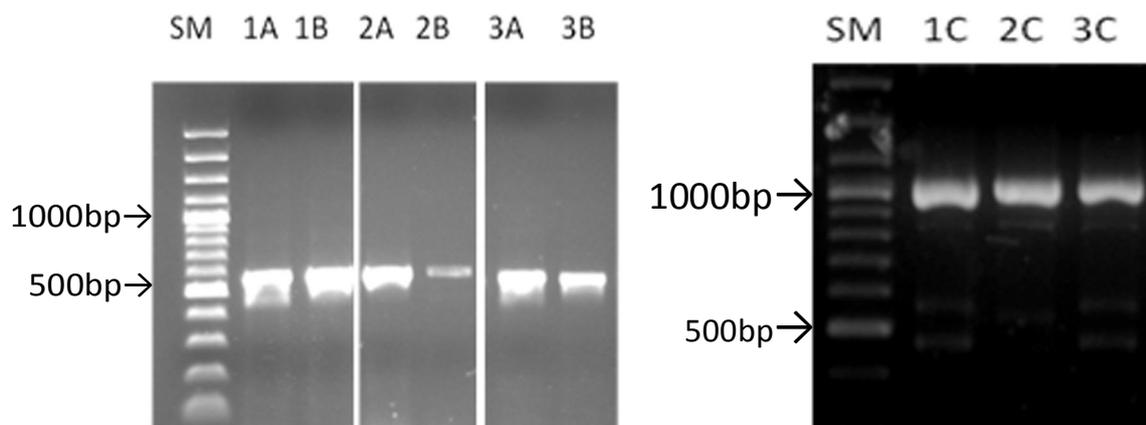
**Construction of Plasmids Encoding Recombinant Wild and Mutant Forms of HBsAg.** Plasmid DNA (pRK5) of un-mutated HBsAg, genotype A2, subtype Adw2 (12) was used as a backbone to construct several point-mutated DNA sequences with primer-specific site-directed mutagenesis method based on splicing overlap extension polymerase chain reaction (SOEing PCR). Exhb-f and Exhb-r primers recognize approximately 150 bp upstream and downstream region of wild type HBsAg (wtHBsAg) in pRK5 vector. In order to construct the mutant forms of HBsAg, this wtHBsAg was used as a template. Two rounds of PCR were carried out employing two "flanking" primers and two internal mutagenic primers containing the desired base substitution. The primers are listed in Table 1.

**Table 1. Specific primers used for construction of mutant variants of HBsAg by site directed mutagenesis.**

Forward	5'→3'	Reverse	5'→3'
Exhb-f	GAGGTCTATATAAGCAGAGCTCGTTTAGTG	Exhb-r	GTCTGACTAGGTGTCCTTCTATAATATTATGG
P120E	CCAGTACGGGAGAATGCAAAACCTG	P120E	CCAGTACGGGAGAATGCAAAACCTG
T123N	GCAAAAACCTGCACGACTCCTGCTC	T123N	GCAAAAACCTGCACGACTCCTGCTC
Q129H	ACGACTCCTGCTCACGGCAACTCTATG	Q129H	CTCCTGCTCACGGCAACTCTATGTTTCC
M133L	CTCAAGGCAACTCTCTGTTCCCTCATG	M133L	CAAGGCAACTCTCTGTTCCCTCATGTTG
K141E	CTCATGTTGCTGTACAGAACCTACGGATG	K141E	GTTGCTGTACAGAACCTACGGATGGAAAT
P142S	CATGTTGCTGTACAAAATCTACGGATGG	P142S	CTGTACAAAATCTACGGATGGAAATTGC
D144A	GTACAAAACCTACGGCCGGAAATTGCAC	D144A	CAAAACCTACGGCCGGAAATTGCAC
G145R	CAAAACCTACGGATCGCAATTGCACC	G145R	CTACGGATCGCAATTGCACCTGTATTC
N146S	CCTACGGATGGAAGCTGCACCTGTATTC	N146S	CTACGGATGGAAGCTGCACCTGTATTC
C147S	CCTACGGATGGAAATAGCACCTGTATTC	C147S	GGATGGAAATAGCACCTGTATTCATC

All amplifications were carried out using high-fidelity Pfu DNA polymerase (Fermentas, Life Sciences, Burlington, Canada). The first round of PCR (Figure 1A) was performed with mutagenic internal primer and the first forward flanking primer (Exhb-f) and the second round (Figure 1B) with the other mutagenic internal primer and the second reverse flanking primer (Exhb-r). PCR conditions for both amplifications were the same: 30 cycles of 94°C for 35 seconds, 65°C for 35 seconds, and 72°C for 45 seconds followed by a final 7 min at 72°C extension time. Finally a third PCR (SEOing PCR) was run to join the product of two PCR together (Figure 1C) under the following condition (this step of PCR was run without the presence of primers): 3 min of initial denaturation at 94°C, 5 cycles of 45s at 95°C, 45s at 68°C and 45s at 72°C followed by

1 min at 94°C. At this step, the flanking primers were added to the PCR tube and the procedure was completed at the same conditions for 30 cycles, and a final 7 min 72°C extension time.



**Figure 1.** Representative PCR amplification of mtHBsAg constructs by site directed mutagenesis and SOEing PCR. Upstream (A) and downstream (B) segments of HBsAg gene were first amplified and subsequently joined together by SOEing (C) PCR. Two rounds of PCR were carried out employing two flanking primers (Exhb-f and Exhb-r) and two internal mutagenic primers containing the desired base substitution.

SM: size marker; lanes 1, 2 and 3: D144A, Q129H, K141E mtHBsAg, respectively.

The PCR products of the following single point mutations were electrophoresed on 1% agarose gel and the HBsAg fragments: P120E, T123N, Q129H, M133L, K141E, P142S, D144A, G145R, N146S and C147S, were extracted using the gel extraction kit (Fermentas Life Sciences). PCR products were digested with HindIII and EcoRI enzymes (New England Biolabs, Ipswich, MA, USA) and cloned into the mammalian cell expression vector pCMV6-neo plasmid (Origene, Rockville MD, USA). Plasmid DNA was propagated by transformation of *E coli* strain JM109 (CinnaGen, Iran) by heat-shock method. In summary, appropriate amount of plasmid DNA was mixed with JM109, incubated on ice for 20-30 minutes and subsequently the cells were heat shocked at 42°C for 90 sec and immediately placed on ice for 2 min. LB Medium (200 µl) was added to the cells and incubated at 37°C for 60 min. This sample was plated on LB agar plates supplemented with 100 µg/ml ampicillin and incubated overnight at 37°C. Plasmid DNA was subsequently purified by Plasmid Miniprep kit (Fermentas Life Sciences, Burlington, Canada).

**Transfection of Expression Plasmids Containing Mutant HBsAg Genes.** CHO cells (provided by National Cell Bank of Iran, Pasteur Institute of Iran, Tehran) were cultured in 12-well plates in RPMI-1640 medium supplemented with 10% fetal calf serum (FBS), under the condition of 5% CO<sub>2</sub>, 37°C. Transfection of pCMV6-neo vector that contains wt- and mtHBsAg, was conducted using JetPEI reagents (PolyPlus-Transfection Co., Illkirch, France). The amount of DNA was adjusted in all experiments to 1 µg/well in a 12-well plate. 500 µl of growth medium was added to sub-confluent monolayer of CHO cells. Plasmid DNA was diluted in 50 µl of 150 mM NaCl. Then 4

$\mu$ l JET-PEI transfection reagent, pre-diluted in 50  $\mu$ l of 150 mM NaCl, was added to dilute DNA and kept at room temperature for 20-30 min. The complex was added to the cells and the cells were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Following 3 h incubation, 500  $\mu$ l of growth medium was added to the cells.

**Detection of Mutant Forms of HBsAg in Supernatant of CHO Transfected Cells.**

Two days after transfection of CHO cells with pCMV6-neo vector the culture medium was harvested and secretion of wtHBsAg and mtHBsAg was evaluated with home-made and commercial Enzyme Linked Immunoassays (ELISA). Commercial ELISA kit (BioELISA HBsAg, Biokit, Barcelona, Spain) employs polyclonal antibodies for both capture and detection layers and recognizes mtHBsAg with high specificity and sensitivity. The quantity of the antigen in the supernatant was assessed with this kit and also it was used for the standardization of HBsAg. Supernatant of mock-transfected CHO cells was used as negative control. In order to establish stable cell lines, which secrete wt- and mtHBsAg, transfected cells, were subcloned four times under the pressure of G418 700  $\mu$ g/ml (GIBCO-BRL, Gaithersburg, Md, USA) by limiting dilution assay (13).

**Production of Anti-HBs Monoclonal Antibodies.** Five anti-HBs MAbs were employed in this study. Two MAbs (S11A and S7A) were produced and characterized previously (14). Three MAbs (2C5, 6F9 and 4A3) were produced in this study based on the same hybridoma methodology. Briefly, female BALB/c mice (6-8 weeks old) were immunized intraperitoneally with 1 $\mu$ g of commercial recombinant HBsAg vaccine, adw2 subtype, (Heberbiovac, Heberbiotec, Cuba) emulsified in complete Freund's adjuvant. Five additional injections of the same amount of antigen, emulsified in incomplete Freund's adjuvant, were followed at biweekly intervals.

During immunization intervals, the serum antibody titer was tested using ELISA. Two days before fusion, an intravenous injection was given without adjuvant. The mouse was then killed and spleen cells were fused with myeloma SP2/0 cells (National Cell Bank of Iran, Pasture Institute, Tehran, Iran) in the presence of polyethylene glycol (PEG, MW 1500, Sigma, St. Louis, MO, USA). Cells were seeded in 96-well plates and incubated at 37°C, 5% CO<sub>2</sub>, 95% humidity. After 7-10 days of culture in RPMI-1640 containing 20% heat-inactivated fetal calf serum, supplemented with hypoxanthine ( $1 \times 10^{-4}$  M), aminopterin ( $4 \times 10^{-7}$  M), and thymidine ( $1.6 \times 10^{-5}$  M; HAT; Sigma) growing hybridomas were selected and screened by an indirect ELISA assay. Positive hybridomas were cloned by limiting dilution at least three times to obtain single clones (13).

**Screening of Anti-HBs Antibody Producing Hybridomas.** Recombinant HBsAg (1  $\mu$ g/ml) was coated on microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) overnight at 4°C. After blocking with 3% skim milk for 2 h, supernatant of growing hybridomas was added and plates were incubated for 1 h at 37°C. After washing with PBS-T (PBS containing 0.05% Tween 20), a 1:10000 dilution of HRP-conjugated goat anti-mouse Ig antibody (Sigma) was added for 1 hour at 37°C. Following 3 times washing, 3,3',5,5' Tetramethylbenzidine (TMB) substrate solution (Pishtaz Teb, Tehran, Iran) was added. The reaction was stopped by addition of 3 N H<sub>2</sub>SO<sub>4</sub> and plates were read at 450 nm by an ELISA reader (Organon Teknika, Bostel, Netherlands).

**Isotype Determination of MAbs.** Goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 (ISO-2 kit, Sigma) at 1/1000 dilution were coated at microtiter plates for 1 h at 37°C. After blocking with 3% skim milk and 3 times washing, supernatant of the hybridoma cells was added to the wells and incubated for 1 h. Following washing with PBS-T,

appropriate dilution of HRP-conjugated goat anti-mouse Ig (prepared in our lab) was added. Finally the reaction was revealed with TMB substrate and OD measured as described above.

**SDS-PAGE and Immunoblotting.** One microgram HBsAg under reduced (sample buffer contains 8% of 2-mercaptoethanol) and non-reduced conditions were loaded onto each well of 12% discontinuous polyacrylamide gel. At the end of electrophoresis, the gel was transblotted to a PVDF membrane (Amersham Pharmacia, Glattbrugg, Switzerland) using the Bio-Rad electroblot system (Bio-Rad, Hercules, CA, USA). The blotted membranes were blocked with 5% skim milk and then immunoprobed with anti-HBs MAbs at room temperature for 2 h. Subsequently, secondary anti-mouse IgG horseradish peroxidase coupled antibody was used as the secondary antibody for 2 h at RT. Finally, the blots were developed with enhanced chemiluminescence (ECL) detection system (Pierce, Rockford, IL, USA).

**Reactivity Pattern of Anti-HBs MAbs with Mutant HBsAg by Sandwich ELISA.** The reactivity of mtHBsAg with anti-HBs MAbs was tested by ELISA assay. Five MAbs: S11A, 2C5, S7A, 4A3 and 6F9 were used in this study. All MAbs except S11A, S7A were dissolved in PBS at a final concentration of 5 µg/ml. The latter two MAbs were employed at 10 and 80 µg/ml, respectively. Supernatants of cultured CHO cells transfected with vectors encoding mutant forms of HBsAg were added to the solid phase and incubated for 1 h at 37°C. After washing, sheep anti-HBs biotinylated conjugate (Pishtaz Teb, Tehran, Iran) was added and incubated for 1 h at 37°C. Streptavidin horseradish peroxidase conjugated antibody (Invitrogen) was added at 1/2000 dilution and incubated for 1 h at 37°C. After adding TMB substrate solution, the OD was measured in an automated plate reader at 450 nm.

## RESULTS

**Construction of the Wild and Mutant Forms of HBsAg.** Two fragments of approximately 600 bp mtHBsAg gene were generated by two steps of PCR with Exhb-f, Exhb-r and two mutagenic internal primers (Figure 1A, B). The products of these two PCR reactions were used as template for the third PCR (SEOing PCR) to join these two segments together (Figure 1C). The length of SEOing PCR product corresponded to 634 bp of HBsAg and around 300 bp of upstream and downstream region of pRK5 vector. After cleavage of the latter fragment by Hind III and EcoRI, mtHBsAg genes were inserted into the multiple cloning site of pCMV6-neo vector and eventually their sequences was verified. The nucleic and amino acid sequences of the inserts are shown in Figure 2.

**Expression of Recombinant Mutant HBsAg proteins.** All constructs including ten mutants and one unmutated standard sequences were successfully expressed in CHO cells. Secreted HBsAg was measured using a commercial polyclonal antibody based ELISA kit. The levels of secreted wt and mtHBsAg in the supernatant were measured using different concentrations of the standard adw HBsAg to construct the standard curve. The absorbance (measured at 450 nm) of wild type and mutant samples and their concentrations are presented in Table 2.

	119	158
<b>adw</b>	GPKKTCTTPA QGNSMFPSCC CTKPTDGNCT CIPIPSSWAF	
<b>P120E</b>	<b><u>G</u></b> ECKTCTTPA QGNSMFPSCC CTKPTDGNCT CIPIPSSWAF	
<b>T123N</b>	GPCK <b><u>N</u></b> CTTPA QGNSMFPSCC CTKPTDGNCT CIPIPSSWAF	
<b>Q129H</b>	GPCKTCTTPA <b><u>H</u></b> GNSMFPSCC CTKPTDGNCT CIPIPSSWAF	
<b>M133L</b>	GPCKTCTTPA QGNS <b><u>L</u></b> FPSCC CTKPTDGNCT CIPIPSSWAF	
<b>K141E</b>	GPCKTCTTPA QGNSMFPSCC C <b><u>T</u></b> <b><u>E</u></b> PTDGNCT CIPIPSSWAF	
<b>P142S</b>	GPCKTCTTPA QGNSMFPSCC CTK <b><u>S</u></b> TDGNCT CIPIPSSWAF	
<b>D144A</b>	GPCKTCTTPA QGNSMFPSCC CTKPT <b><u>A</u></b> GNCT CIPIPSSWAF	
<b>G145R</b>	GPCKTCTTPA QGNSMFPSCC CTKPTD <b><u>R</u></b> NCT CIPIPSSWAF	
<b>N146S</b>	GPCKTCTTPA QGNSMFPSCC CTKPTD <b><u>G</u></b> SCT CIPIPSSWAF	
<b>C147S</b>	GPCKTCTTPA QGNSMFPSCC CTKPTDGN <b><u>S</u></b> T CIPIPSSWAF	
	358	417
<b>adw</b>	CCATGCAAAACCTGCACGACTCCTGCTCAAGGCAACTCTATGTTTTCCCTCATGTTGCTGT	
<b>P120E</b>	<b><u>GA</u></b> ATGCAAAACCTGCACGACTCCTGCTCAAGGCAACTCTATGTTTTCCCTCATGTTGCTGT	
<b>T123N</b>	CCATGCAAAA <b><u>A</u></b> ACTGCACGACTCCTGCTCAAGGCAACTCTATGTTTTCCCTCATGTTGCTGT	
<b>Q129H</b>	CCATGCAAAACCTGCACGACTCCTGCTCA <b><u>C</u></b> GGCAACTCTATGTTTTCCCTCATGTTGCTGT	
<b>M133L</b>	CCATGCAAAACCTGCACGACTCCTGCTCA <b><u>C</u></b> TGTTTTCCCTCATGTTGCTGT	
<b>K141E</b>	CCATGCAAAACCTGCACGACTCCTGCTCAAGGCAACTCTATGTTTTCCCTCATGTTGCTGT	
<b>P142S</b>	CCATGCAAAACCTGCACGACTCCTGCTCAAGGCAACTCTATGTTTTCCCTCATGTTGCTGT	
<b>D144A</b>	CCATGCAAAACCTGCACGACTCCTGCTCAAGGCAACTCTATGTTTTCCCTCATGTTGCTGT	
<b>G145R</b>	CCATGCAAAACCTGCACGACTCCTGCTCAAGGCAACTCTATGTTTTCCCTCATGTTGCTGT	
<b>N146S</b>	CCATGCAAAACCTGCACGACTCCTGCTCAAGGCAACTCTATGTTTTCCCTCATGTTGCTGT	
<b>C147S</b>	CCATGCAAAACCTGCACGACTCCTGCTCAAGGCAACTCTATGTTTTCCCTCATGTTGCTGT	
	418	476
<b>adw</b>	ACAAAACCTACGGATGGAAATTGCACCTGTATTCCCATCCCATCGTCCTGGGCTTTTCGCA	
<b>P120E</b>	ACAAAACCTACGGATGGAAATTGCACCTGTATTCCCATCCCATCGTCCTGGGCTTTTCGCA	
<b>T123N</b>	ACAAAACCTACGGATGGAAATTGCACCTGTATTCCCATCCCATCGTCCTGGGCTTTTCGCA	
<b>Q129H</b>	ACAAAACCTACGGATGGAAATTGCACCTGTATTCCCATCCCATCGTCCTGGGCTTTTCGCA	
<b>M133L</b>	ACAAAACCTACGGATGGAAATTGCACCTGTATTCCCATCCCATCGTCCTGGGCTTTTCGCA	
<b>K141E</b>	AC <b><u>G</u></b> AAACCTACGGATGGAAATTGCACCTGTATTCCCATCCCATCGTCCTGGGCTTTTCGCA	
<b>P142S</b>	AC <b><u>A</u></b> AAAT <b><u>T</u></b> CTACGGATGGAAATTGCACCTGTATTCCCATCCCATCGTCCTGGGCTTTTCGCA	
<b>D144A</b>	ACAAAACCTACGG <b><u>CC</u></b> GGAAATTGCACCTGTATTCCCATCCCATCGTCCTGGGCTTTTCGCA	
<b>G145R</b>	ACAAAACCTACGGAT <b><u>CGC</u></b> AATTGCACCTGTATTCCCATCCCATCGTCCTGGGCTTTTCGCA	
<b>N146S</b>	ACAAAACCTACGGATGGAA <b><u>GC</u></b> TGCACCTGTATTCCCATCCCATCGTCCTGGGCTTTTCGCA	
<b>C147S</b>	ACAAAACCTACGGATGGAAAT <b><u>AG</u></b> CACCTGTATTCCCATCCCATCGTCCTGGGCTTTTCGCA	

**Figure 2.** The nucleotide and amino acid substitutions of constructed mutant S genes. Wt and mtHBsAg genes were inserted into the multiple cloning site of pCMV6-neo vector and eventually their sequences were verified. Amino acid (positions 119 to 158) and nucleotide (positions 358 to 476) sequences of wt and 10 forms of mtHBsAg constructs are shown. Mutation sites are shown as bold underlined letters.

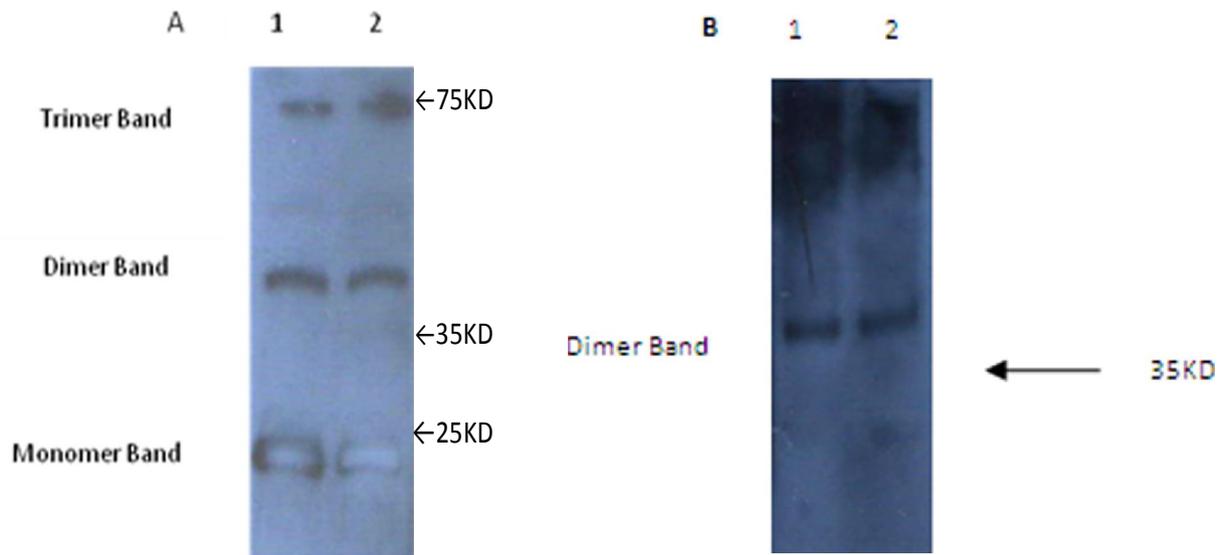
**Table 2. Detection and quantification of wt and mtHBsAg in culture supernatants of CHO transfected cells by ELISA.**

Construct	A <sub>450</sub>	Concentration
adw	1.3	54.7
P120E	1.8	89.8
T123N	1.6	291
Q129H	1.8	170
M133L	1.7	154
K141E	1.4	29.8
P142S	0.842	58.8
D144A	1.3	213
G145R	2	218
N146S	1.8	174
C147S	1.2	97.8

Results are expressed as absorbance measured at 450 nm (OD) and concentration presented as ng/mL. The OD belongs to different optimal working dilutions of each supernatant which are: adw (1/2), P120E (1/2), T123N (1/8), Q129H (1/4), M133L (1/4), K141E (Neat), P142S (1/4), D144A (1/8), G145R (1/4), N146S (1/4) and C147S (1/4). The OD value of negative control was subtracted from OD value of each sample

**Characteristics of Anti-HBsAg MAbs.** MAbs were further characterized by Western blot analysis using purified reduced and non-reduced forms of wtHBsAg. Representative results are shown in Figure 3. The major part of non-reduced HBsAg was polymerized, thus neither entered the gel nor transferred to PVDF membrane. Only a small proportion of the antigen was present in a dimer form. Once the sample was reduced, the polymerised antigen was mostly converted to monomer, dimer and trimer bands (Figure 3). The monomer band corresponds to the MW size (~24KD) of the glycosylated HBsAg.

**Reactivity of Anti-HBs MAbs with HBsAg Mutants.** We further tested the reactivity of mtHBsAg in ELISA with our MAbs to assess the effect of the amino acid substitutions. Supernatants of untransfected cells or cells transfected with pCMV-6 vector alone were used as background and negative control respectively. Our results showed that the amino acid substitution of T123N or G145R led to either complete loss or a strong reduction (less than 40%) of reactivity with all MAbs relative to the wild type form.



**Figure 3.** Representative immunoblotting profile of two MAbs with reduced (A) and non-reduced (B) HBsAg. As it is obvious in part B, the major part of non-reduced HBsAg was polymerized and could not enter the resolving gel properly. Only a small proportion of the antigen was present in a dimer form.  
1: 2C5, 2: 4A3

All antibodies lost their ability to recognize HBsAg after T123N mutation, while they all could recognize M133L, Q129H and P142S. Interestingly, reactivity of these mutants with some MAbs was improved as evidenced by the ratio of the mutant OD to that of the wtHBsAg. The remaining mutants displayed different reactivity patterns (Table 3).

**Table 3. Reactivity of anti-HBs MAbs with mutant forms of HBsAg.**

	P120E	T123N	Q129H	M133L	K141E	P142S	D144A	G145R	N146S	C147S
S11A	-	-	++	+	-	+	-	-	-	-
S7A	+		++	+	+++	+	+	+	+	++
6F9	+	-	++	++	++	+	+	-	+	+
2C5	+	-	++	+	++	+	+	-	+	+
4A3	+	-	++	+	++	+	+	+	+	+
PAb	++	++	++	++	+++	++	+++	++	++	++

Results are expressed as the ratio of OD obtained for each MAb with mutant HBsAg to that of the wild type HBsAg and presented as:  
- :< 40%, +:40-80%, ++:80-120%, +++:>120; PAb: data refers to the pattern of reactivity of polyclonal anti-HBs antibody as a control.

## DISCUSSION

In this study we constructed a panel of ten recombinant mutant forms of HBsAg. Seven of these mutants have already been reported to be clinically important as immune escape mutants; M133L, G145R (15), Q129H (16), K141E (17), D144A (16,18), T123N (19), P142S (16,18). We also assessed the impact of these mutations on anti-HBs MAb reactivity. Plasmids containing wtHBsAg and mtHBsAg were constructed and transfected into mammalian CHO cells. The expressed HBsAg levels produced by these constructs were evaluated by a polyclonal antibody based commercial detection assay to avoid the influence of these single mutations on their quantification.

Appearance of anti-HBs antibodies leads to protection against HBV infection, therefore altered antigenicity of surface mutants could affect most clinical aspects of hepatitis B: chronicity, vaccine efficacy, effectiveness of HBIG immunoprophylaxis post-transplantation and diagnostic accuracy (20).

HBV exhibits a mutation rate more than 10-fold higher than other DNA viruses due to lack of proofreading function of reverse transcriptase (21). On the other hand immune pressure could cause HBV mutation (22), which may lead to HBsAg conformational changes and failure of detection by commercial HBsAg diagnostic assays (23).

The major envelope protein of HBV is S protein, which consists of 226 amino acids (24). There is a hydrophilic region designated "a" determinant (aa 124 to 147) in this area which is common to all HBV subtypes. The "a" determinant is a conformational epitope and projects out from the surface of HBV particle. This projection is made up by special two-loop structure kept by the disulfide bonds between Cys124 and Cys137, Cys139 and Cys147, respectively (24). During the course of initial immune response in acute hepatitis, this determinant is one of the main targets of anti-HBs antibodies (15).

Since most HBsAg immunoassays use MAbs against "a" determinant, amino acid substitution in this region may account for false-negative results in immunoassays (25). Lots of reports have mentioned the importance of mutations in specific residues such as 123, 144 and 145 for binding of many of anti-HBs MAbs (26-28). Besides the effect of mutations inside the first and second loops of the "a" determinant, mutations of residues which are mostly located outside these loops such as P120E and T123N could be crucial for the antigenicity of hepatitis B surface antigen (28). Our results also confirm the importance of T123N in antigenicity of HBsAg, since all of our MAbs lost their reactivity (less than 40%) with T123N mutant. According to our results the most influential mutations are thought to be T123N and G145R. The reactivity of mtHBsAg with amino acid substitution K141E was reduced to a level of almost 50% of the reactivity of wtHBsAg (Table 2), perhaps suggesting impaired secretion and/or weak recognition by anti-HBs antibody. The effect of K141 mutation in decreasing the secretion of HBsAg and also HBV virions has previously been reported by others (29-30). However, the same effect for other mutants such as G145R and P142S has also been reported.

Our findings indicate that HBsAg mutants can escape detection by current HBsAg detection methods, which use MAbs in the coating and detection layers. This situation suggests a need for closer HBV surveillance monitoring since these mutant forms may spread undetected to the general population through horizontal transmission or through blood supplies and cause liver diseases (6,31). Inclusion of the mutant forms of HBsAg within the current standard HBV vaccines might be essential as the new generation

vaccines. Even though HBIG is an effective prophylaxis therapy, liver-graft infection with HBV still occurs in about 30% of patients receiving HBIG (20).

Additionally, HBIG therapy for long-term can provoke emergence of genetic HBV mutants, which may cause the virus to become resistant to neutralization (32). Therefore, a combination of MAbs with ability to recognize all common escape mutant forms of the virus could be a proper alternative therapy. The mtHBsAg variants used in this study were expressed at relatively high levels. But clinical samples may have these mtHBsAg at lower levels, therefore may not be recognized in HBsAg detection immunoassays. Construction of mutant forms of HBsAg could have important clinical implications for immune-screening and diagnosis of HBV infection and more importantly for designing of new generation of recombinant HBV vaccines.

## ACKNOWLEDGEMENTS

We thank Dr. Boutorabi and Pishtaz Teb Diagnostics for providing anti HBsAg conjugate. This study was supported in part by grants from Avicenna Research Institute and the National Science Foundation of Iran. F.Golsaz Shirazi, MM. Amiri and H. Mohammadi contributed equally to this manuscript.

## REFERENCES

- 1 Dufour DR. Hepatitis B surface antigen (HBsAg) assays- are they good enough for their current uses? *Clin Chem.* 2006; 52:1457-9.
- 2 Nassal M, Schaller H. Hepatitis B virus replication-an update. *J Viral Hepat.* 1996; 3:217-26.
- 3 Lam W, Li Y, Liou JY, Dutschman GE, Cheng YC. Reverse transcriptase activity of hepatitis B virus (HBV) DNA polymerase within core capsid: interaction with deoxynucleoside triphosphates and anti-HBV 1-deoxynucleoside analog triphosphates. *Mol Pharmacol.* 2004; 65:400-6.
- 4 Purdy MA. Hepatitis B virus S gene escape mutants. *Asian J Transfus Sci.* 2007; 1:62-70.
- 5 Carman WF, Zanetti AR, Karayiannis P, Waters J, Manzillo G, Tanzi E, et al. Vaccine-induced escape mutant of hepatitis B virus. *Lancet.* 1990; 336: 325-9.
- 6 Coleman PF. Detecting hepatitis B surface antigen mutants. *Emerg Infect Dis.* 2006; 12:198-203.
- 7 Horvat RT. Diagnostic and Clinical Relevance of HBV Mutations. *Lab Med.* 2011; 42:488-96.
- 8 Carman WF, Trautwein C, van Deursen FJ, Colman K, Dornan E, McIntyre G, et al. Hepatitis B virus envelope variation after transplantation with and without hepatitis B immune globulin prophylaxis. *Hepatology.* 1996; 24:489-93.
- 9 Shizuma T, Hasegawa K, Ishikawa K, Naritomi T, Iizuka A, Kanai N, et al. Molecular analysis of antigenicity and immunogenicity of a vaccine-induced escape mutant of hepatitis B virus. *J Gastroenterol.* 2003; 38:244-53.
- 10 Chang MH. Breakthrough HBV infection in vaccinated children in Taiwan: surveillance for HBV mutants. *Antivir Ther.* 2010; 15:463-9.
- 11 Li F, Zhang C, Liu J, Zhang X, Yan B, Zhang B, et al. Preparation, identification, and clinical application of anti-HBs monoclonal antibody that binds both wild-type and immune escape mutant HBsAgs. *Front Med China.* 2009; 3:277-83.
- 12 Roohi A, Yazdani Y, Khoshnoodi J, Jazayeri SM, Carman WF, Chamankhah M, et al. Differential reactivity of mouse monoclonal anti-HBs antibodies with recombinant mutant HBs antigens. *World J Gastroenterol.* 2006; 12:5368-74.
- 13 Zola H. *Monoclonal antibodies: the second generation.* 1st ed. Oxford: BIOS Scientific Publishers; 1995.
- 14 Roohi A, Khoshnoodi J, Zarnani A, Shokri F. Epitope mapping of recombinant hepatitis B surface antigen by murine monoclonal antibodies. *Hybridoma.* 2005; 24:71-7.

- 15 Lada O, Benhamou Y, Poynard T, Thibault V. Coexistence of hepatitis B surface antigen (HBsAg) and anti-HBs antibodies in chronic hepatitis B virus carriers: influence of “a” determinant variants. *J Virol.* 2006; 80:2968-75.
- 16 Caligiuri P, Sticchi L, Cerruti R, Icardi G, Bruzzone B. Presence of HBV S-gene mutants in immunocompromised patients. *The new microbiologica.* 2012; 35:97-8.
- 17 Peng XM, Gu L, Chen XJ, Li JG, Huang YS, Gao ZL. Optimization of competitively differentiated polymerase chain reaction in detection of HBV basal core promoter mutation. *World J Gastroenterol.* 2005; 11:3614-8.
- 18 Torresi J. Hepatitis B antiviral resistance and vaccine escape: two sides of the same coin. *Antivir Ther.* 2008; 13:337-40.
- 19 Ie S, Thedja MD, Roni M, Muljono DH. Prediction of conformational changes by single mutation in the hepatitis B virus surface antigen (HBsAg) identified in HBsAg-negative blood donors. *Virology.* 2010; 7:326.
- 20 Cooreman MP, Van Roosmalen MH, Te Morsche R, Sünnen CM, Schoondermark-van de Ven EM, Jansen JB, et al. Characterization of the reactivity pattern of murine monoclonal antibodies against wild-type hepatitis B surface antigen to g145r and other naturally occurring “a” loop escape mutations. *Hepatology.* 1999; 30:1287-92.
- 21 Bartholomeusz A, Locarnini S. Hepatitis B virus mutants and fulminant hepatitis B: fitness plus phenotype. *Hepatology.* 2001; 34:432-5.
- 22 Weber B. Genetic variability of the S gene of hepatitis B virus: clinical and diagnostic impact. *J Clin Virol.* 2005; 32:102-12.
- 23 Amini-Bavil-Olyae S, Vuçur M, Luedde T, Trautwein C, Tacke F. Differential impact of immune escape mutations G145R and P120T on the replication of lamivudine-resistant hepatitis B virus e antigen-positive and-negative strains. *J Virol.* 2010; 84:1026-33.
- 24 Chen HB, Fang DX, Li FQ, Jing HY, Tan WG, Li SQ. A novel hepatitis B virus mutant with A-to-G at nt551 in the surface antigen gene. *World J Gastroenterol.* 2003; 9:304-8.
- 25 Ly TD, Servant-Delmas A, Bagot S, Gonzalo S, Férey M-P, Ebel A, et al. Sensitivities of four new commercial hepatitis B virus surface antigen (HBsAg) assays in detection of HBsAg mutant forms. *J Clin Microbiol.* 2006; 44:2321-6.
- 26 Carman WF. The clinical significance of surface antigen variants of hepatitis B virus. *J Viral Hepat.* 1997; 4:11-20.
- 27 Protzer-Knolle U, Naumann U, Bartenschlager R, Berg T, Hopf U, Meyer zum Büschenfelde KH, et al. Hepatitis B virus with antigenically altered hepatitis B surface antigen is selected by high-dose hepatitis B immune globulin after liver transplantation. *Hepatology.* 1998; 27:254-63.
- 28 Tian Y, Xu Y, Zhang Z, Meng Z, Qin L, Lu M, et al. The amino acid residues at positions 120 to 123 are crucial for the antigenicity of hepatitis B surface antigen. *J Clin Microbiol.* 2007; 45:2971-8.
- 29 Huang CH, Yuan Q, Chen PJ, Zhang YL, Chen CR, Zheng QB, et al. Influence of mutations in hepatitis B virus surface protein on viral antigenicity and phenotype in occult HBV strains from blood donors. *J Hepatol.* 2012; 57:720-9.
- 30 Kwei K, Tang X, Lok AS, Sureau C, Garcia T, Li J, et al. Impaired virion secretion by hepatitis B virus immune escape mutants and its rescue by wild-type envelope proteins or a second-site mutation. *J Virol.* 2013; 87:2352-7.
- 31 Jongerius JM, Wester M, Cuypers HT, Oostendorp WR, Lelie PN, van der Poel CL, et al. New hepatitis B virus mutant form in a blood donor that is undetectable in several hepatitis B surface antigen screening assays. *Transfusion.* 1998; 38:56-9.
- 32 Katz L, Paul M, Guy D, Tur-Kaspa R. Prevention of recurrent hepatitis B virus infection after liver transplantation: hepatitis B immunoglobulin, antiviral drugs, or both? Systematic review and meta-analysis. *Transpl Infect Dis.* 2010;12:292-308.