# Immune Escape Variants in Chronic Hepatitis B – A Clinicopathological Correlation with Sequencing of MHR Region of S Gene Characterizing Influence of 'A' Determinant

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## ABSTRACT

**Introduction**: Hepatitis B surface antigen (HBsAg) and Anti-HBs antibodies can coexist in 10-25% cases. Major reason is buildup of mutations in the Major Hydrophilic Region (MHR) of the S gene especially within the "a" determinant region leading to change in antigenicity and escape from the host immune system. The aim of this study was to analyze influence of 'a' determinant of S gene on creation of these immune escape mutants in CHB by sequencing.

**Material and methods**: 100 cases of CHB reporting for treatment or review were enrolled. HBsAg and anti-HBs was performed on samples using enzyme immunoassay along with determination of biochemical profile. Sequencing of MHR region was done on identified Immune escape variants to determine influence of 'a' determinant.

**Results**: Eleven percent CHB patients showed coexistence of HBsAg and Anti-HBs. Seven out of eleven patients were positive for HBV-DNA and sequencing of MHR region of S gene showed common accumulation of mutations seen at position 127. Other common sites of mutation were at positions 128 and 118 in 'a' determinant we found point mutations at positions 118, 120, 124 and137 which have not been described.

**Conclusion:** Accumulation of residue changes within the MHR, including the highly conformational and cysteine-rich loops of "a" determinant can be a possible mechanism for coexistence of HBsAg and anti-HBs antibodies in CHB. Such cases increase the problem of transmission of these variants and can cause widespread vaccination failure and are thus public health concern as these individuals may unknowingly transmit the infection.


**Keywords:** hepatitis B, immune escape variants, MHR

## INTRODUCTION

Hepatitis B virus (HBV) infection and its long term sequelae, which include chronic hepatitis B (CHB), cirrhosis and hepatocellular carcinoma are foremost public health problems throughout the world.[1] Approximately one third of all cases of cirrhosis and 50% of cases of hepatocellular carcinoma are attributable to chronic HBV infection. Overall 30% deaths in these patients are linked directly to consequences of HBV infection.[2] Majority of the patients, however, are known to recover from illness and recovery is characterized by loss of hepatitis B surface antigen (HBsAg) and acquisition of Anti HBs antibodies.

Although, occurrence of Anti-HBs is a favorable outcome in HBV infection, several authors have pointed out that presence of Anti-HBs antibodies (Ab) does not always mean loss of HBsAg and they can coexist in as many as 10-25% of cases.[3,4] Though the simultaneous presence of anti-HBs in HBsAg positive cases is perplexing, this phenomenon is known since 1976, still the exact cause of presence of both HBsAg and Anti-HBs antibodies in spite of replicative disease in patients is unknown.[5,6] Major reason cited by many authors is selection of HBsAg immune variants and buildup of residual changes in the Major Hydrophilic Region (MHR) of the S gene especially within the "a" determinant region, which is the main target of anti-HBs, could alter the structure of surface antigen, leading to change in antigenicity and escape from recognition by the host immune system.[7] Antibodies against the group specific "a" determinant, which is a complex antigenic structure with multiple immunogenic epitopes, normally neutralize virus and confer cross protective immunity to all HBV subtypes. Historically, the secondary structure of this epitope is a double loop formed via disulfide bridges between cysteine residues between 124 and 137 and residues 139 and 147. Nucleotide substitution that leads to change in amino acid sequence can lead to decreased binding and failure to detect HBsAg in diagnostic assays using monoclonal and polyclonal antibodies.[7,8]

The co-existence of HBsAg and anti-HBsAb are feared to be associated with important clinical concerns. Indeed, such HBsAg-mutated HBV strains may not be fully susceptible to vaccine-induced anti-HBs antibody with the potential risk of vaccine failure including contamination of seemingly protected vaccinated individuals.[6,7]

The detection of such non-protective anti-HBs antibody may also lead to misdiagnosis of chronic HBV infection if detection of HBsAg is not carried out concomitantly. Furthermore, due to the frame-shifted overlap flanked by the open reading frames of HBsAg and HBV polymerase genes, mutations within HBsAg gene might cause structural and functional alterations in the HBV reverse transcriptase (RT) with potential influence on viral replicative capacity and efficacy of antiviral drugs.[1]

In view of these potential implications, the frequency, clinical settings and significance of the presence of concurrent HBsAg and anti-HBsAb in serum remain largely unknown to date. Thus the aim of this study was to analyze the clinic-pathological profile of CHB patients with special reference to Immune escape variants (coexistence of both HBsAg and Anti HBs) and

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determine by sequencing influence of 'a' determinant of S gene on creation of these immune escape mutants.

## MATERIAL AND METHODS

**Study population:** The study comprised of 100 HBsAg positive patients attending Gastroenterology OPD of a tertiary care hospital clinically diagnosed as CHB and enrolled for treatment. The study group consisted of predominantly young males. Age group was 20 to 65 years. All subjects in this study were male. Informed consent and clearance from Institutional ethical committee was taken. A performa with detailed history of past medical and surgical illness, risk factors and predisposing factors was filled for all subjects prior to conduct of study along with routine medical examination.

**Screening**: All subjects were screened for HBsAg (Hepacard Biomed Industries, Parwanoo (HP) - one step cassette style HBsAg detection test, India)

**Biochemical profile:** Routine biochemical parameters of liver function including serum bilirubin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was performed on Siemen Dimension EXL 200 analyser using Siemen kits. Quality control protocol was strictly adhered to.

**Virological markers** of CHB infection including Anti HBs antibodies (M.B.S-S.R.L. Medical Biological Service, Milano, Italy), HBeAg (Adatis Italia S.P.A,Bologna Italy) and anti HBcAb (M.B.S-S.R.L. Medical Biological Service, Milano, Italy) were performed on clinical samples using enzyme immunoassay. Negative and positive controls were run simultaneously to ascertain validity of the test.

**Sequencing of MHR region of S gene:** DNA was extracted using QIAamp DNA Mini Kit from QIAGEN Diagnostics. Sequencing HBV PCR products was carried out using ABI PRISM 3100 Genetic Analyzer which is a multi-colour fluorescence-based DNA analysis system using technology of capillary electrophoresis with 16 capillaries operating in parallel.

## RESULTS

### Age profile

Forty were in age group 20-30, 39 in 30-40, 16 in 40-50, four in 50-60 and one in 60-70 years age group. Detailed clinical history revealed that 63% patients did not have any particular risk factor, 11% gave history of exposure to commercial sex workers, 8% gave history of minor surgery, 8% had multiple sexual partners, 7% gave history of blood transfusion and 3% attributed their disease to professional hazard as they were Health Care Workers.

Most of the patients were young and healthy and except for CHB they did not have any other co morbidity. 6% had associated hypertension, 4% had Type-2 diabetes mellitus, 3% gave history of pulmonary tuberculosis and one each were known cases of Non-Hodgkins Lymphoma, Alcoholic Liver cirrhosis, Gilbert's syndrome and rheumatic heart disease.

### Serological markers

Eleven (11%) out of the one hundred patients had coexistence of Anti HBs antibodies while thirteen (13%) were positive for anti hepatitis B core antibody (HBcAb) and nineteen (19%) were positivity for hepatitis B e antigen (HBeAg). Anti-HBs titre of these 11 cases is summarized in Table-1.

### Clinical profile of immune escape variants

All the eleven patients with co-existence of HBsAg and Anti HBs were asymptomatic with none showing signs and symptoms of chronic active hepatitis. There was no associated co -morbidity in these patients. History of risk factors revealed one patient had received blood transfusion, five had history of exposure to commercial sex worker and one patient each smoked tobacco and consumed alcohol.

Serological and biochemical profile of immune-escape variants

Out of these 11 immune escape mutants three were positive for HBeAg and two were positive for Anti HBc antibody. These 11 patients had, however, normal levels of serum AST, ALT and bilirubin levels except one patient who showed mildly increased AST and ALT.

### Sequencing of MHR region of S gene

Seven samples showed HBV- DNA positivity out of 11 samples, Five samples (1, 6, 10, 22 and 39) out of seven showed same mutation of replacement of proline by threonine at amino acid position 127. Four samples (sample no. 1,10,22,39) showed replacement of threonine by valine at amino acid position 118. Four other samples (sample no. 1,10,22,39) showed replacement of alanine by valine. Sample 14 showed a solitary mutation at amino acid position 137 with replacement of cysteine by arginine. Sample number six showed mutation in amino acid position 125 with replacement of threonine by methionine in addition to mutation at amino acid position 127. Sample number 55 out of these seven samples did not show any mutation in MHR region. All mutations detected were therefore in the MHR region (amino acid sequence 118-137). No mutation was identified in the "C" terminal or "N" terminal of S gene. These are summarized in Table-2 and figure-1.

## DISCUSSION

Several authors have described point mutations resulting in amino acid changes in the S protein antigenic loops in vaccines and hepatitis B immune globulin recipients.[7,8] However, HBV escape mutants may also arise naturally in CHB virus carriers due to increased pressure of the host immune system.[9,10]

The clinical significance of the co-existence of HBsAg and protective levels of anti-HBs antibodies is not well known since clinical data are lacking in most studies. Previous studies have pointed that this profile could be associated with chronic active hepatitis.[7,8] In our study, in contrast to Western data, all

| S.No | Sample No | Anti HBs titers |
|---|---|---|
| 1 | 1 | 192 |
| 2 | 6 | 133 |
| 3 | 10 | 128.5 |
| 4 | 14 | 251.11 |
| 5 | 22 | 214.44 |
| 6 | 39 | 130.15 |
| 7 | 43 | 234.4 |
| 8 | 55 | 24.97 |
| 9 | 62 | 42.379 |
| 10 | 78 | 55.8 |
| 11 | 88 | 161.1 |

**Table-1:** Anti-HBs titres of 11 cases (more than 100 mIU/ml is considered protective)[7]

these eleven patients were asymptomatic with none showing any signs and symptoms suggestive of chronic active hepatitis. These eleven patients had normal levels of serum AST, ALT and bilirubin levels except one patient who showed mildly increased liver enzymes.

In many Western studies this serological profile usually associated with HBV replication despite the presence of anti-HBsAb at a protective level though in our study in contrast to western data only 3 out of these 11 patients were positive for HBeAg. Median age of patients in this study was lower as compared to Western studies (34 years vs 43-45 years in the West) but this bias in this study could have arisen because the study subjects comprised of young serving soldiers who were comparatively young and 80 out of 100 patients were between 20 and 40 years.[6-8]

Detailed clinical history revealed that 63% patient's did not have history of any particular risk factor. Maximum patients from the group with no associated risk gave history of injections from reusable syringes without proper sterilization for minor illness in their home town. This profile is seen normally in the West in association with orthotropic liver transplant, myeloproliferative disorders and chemotherapy although in our study none of the eleven patients had any such co morbidity.[3] No noteworthy association of smoking and alcohol was seen associated with this study population.

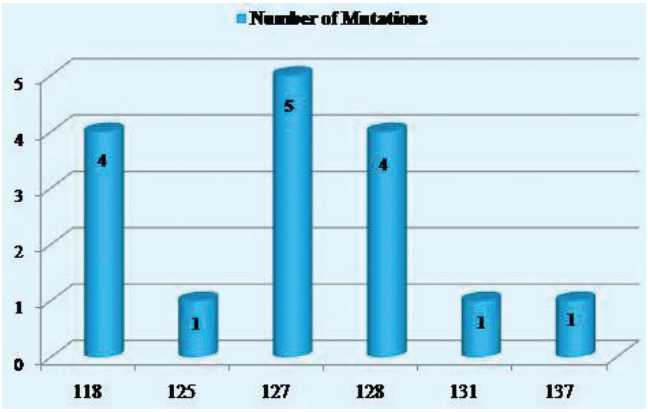


**Figure-1:** Number of mutations present in MHR region; X axis – serial no of AA sequence; Y axis- no of mutations documented

It must also be emphasized in context of immune suppression which is a main feature associated with this profile and quoted as high as 69% in other studies carried out in South East Asia and Western countries, it was not apparent in any of the 11 immune escape variants detected in this study.[7,11] However, detailed immunological evaluation was not undertaken in this study and, thus, this factor cannot be commented upon with conviction.

In comparing HBsAg sequences with the reference sequence, it was striking to notice an accumulation of residue changes in viral strains isolated from those with immune escape variants. Moreover, the distribution of these changes in amino acid sequences was not uniformly distributed along the protein but were accumulated within the MHR, including the highly conformational and cysteine-rich "a" determinant region.

This antigenic loop of HBsAg region which is classically described as the main target of the humoral response and any change of its primary sequence will alter its antigenicity and would render any anti- HBs humoral defense against this region less effective.[1] Previous studies have characterized most prevalent residues affected by such changes.[7,11] Thus, positions 145, 144, 129, 126, and 130 were the most likely targets of point mutation but in contrast to Western studies, we found mutations in positions 118, 120,124, 125, 127, 131 and137 with maximum mutations in position 127. Although an Indian study carried out by Kumarvelu et al showed presence of mutations at positions 125, 126, 127, 131,134 and 136 in two immunized children, but point mutation at positions 118, 120, 124 and137 has not been described.[12] Western and South Eastern studies have also demonstrated changes in HBsAg sub region 4 but no such mutation was found in their study

The description of these residue changes, particularly the G145R change, is remarkable because these substitutions also match up to common mutations described for HBV vaccine

| | Standard | Sample 1 | Sample 6 | Sample 10 | Sample 14 | sample 22 | sample 39 | sample 55 |
|---|---|---|---|---|---|---|---|---|
| 118 | Threonine | Valine | Threonine | Valine | Threonine | Valine | Valine | Threonine |
| 119 | Glycine | Glycine | Glycine | Glycine | Glycine | Glycine | Glycine | Glycine |
| 120 | Proline | Proline | Proline | Proline | Proline | Proline | Proline | Proline |
| 121 | Cysteine | Cysteine | Cysteine | Cysteine | Cysteine | Cysteine | Cysteine | Cysteine |
| 122 | Arginine | Arginine | Arginine | Arginine | Arginine | Arginine | Arginine | Arginine |
| 123 | Threonine | Threonine | Threonine | Threonine | Threonine | Threonine | Threonine | Threonine |
| 124 | Cysteine | Cysteine | Cysteine | Cysteine | Cysteine | Cysteine | Cysteine | Cysteine |
| 125 | Threonine | Threonine | Methionine | Threonine | Threonine | Threonine | Threonine | Threonine |
| 126 | Threonine | Threonine | Threonine | Threonine | Threonine | Threonine | Threonine | Threonine |
| 127 | Proline | Threonine | Threonine | Threonine | Proline | Threonine | Threonine | Proline |
| 128 | Alanine | Valine | Alanine | Valine | Alanine | Valine | Valine | Alanine |
| 129 | Glutamine | Glutamine | Glutamine | Glutamine | Glutamine | Glutamine | Glutamine | Glutamine |
| 130 | Glycine | Glycine | Glycine | Glycine | Glycine | Glycine | Glycine | Glycine |
| 131 | Threonine | Threonine | Threonine | Threonine | Threonine | Threonine | Alanine | Threonine |
| 132 | Serine | Serine | Serine | Serine | Serine | Serine | Serine | Serine |
| 133 | Methionine | Methionine | Methionine | Methionine | Methionine | Methionine | Methionine | Methionine |
| 134 | Tyrosine | Tyrosine | Tyrosine | Tyrosine | Tyrosine | Tyrosine | Tyrosine | Tyrosine |
| 135 | Proline | Proline | Proline | Proline | Proline | Proline | Proline | Proline |
| 136 | Serine | Serine | Serine | Serine | Serine | Serine | Serine | Serine |
| 137 | Cysteine | Cysteine | Cysteine | Cysteine | Arginine | Cysteine | Cysteine | Cysteine |

**Table-2:** Mutations in MHR Region from amino acid sequence 118-137.

escape variants or for patients who have been therapeutically administered monoclonal anti-HBs.[7] This hypothesis could not be tested in this study as none of eleven patients showed G145R change and moreover none of these eleven patients gave history of receiving HBV vaccine. It is important that the changes in residues observed in the subjects with presence of both HBsAg and anti-HBs antibodies are not only at the characteristic positions but also throughout loops 2 to 4 of the antigenic loops. However, it is difficult to predict the structural and biochemical effects of these amino acid substitutions.

To summarize, several residue changes within the MHR that are found in patients carrying both HBsAg and anti-HBs antibodies need to be further characterized immunologically, it seems quite compelling that these changes may alter antibody recognition of the S protein. Little is known about the ramifications of such alterations on T-cell epitope recognition.

This coexistence of both HBsAg and anti HBs antibodies in patients can have several deleterious consequences though this profile is not associated with change in HBsAg quantification, differences of HBsAg quantification noticed depended on HBV genotype, and type of antibody (monoclonal or polyclonal) and the targeted epitope used in the assays.[13]

The accumulation of HBV carrying immune escape mutations in CHB patients with fairly high viral loads raises the issue of transmission of such variant virus as they may not be fully neutralized by vaccine-induced antibodies, leading to widespread vaccination failure. In light of our data, chronic carriers possessing both HBsAg and anti-HBs antibodies should be considered potential reservoirs of immune escape variants. Further epidemiological studies are required to scientifically assess the probable threat of such chronic carriers in areas of high endemicity because in this setting, ambiguity about the response of patients to conventional therapies.[14]

There are studies which suggest that these patients may be more prone to advance liver diseases like hepatocellular carcinoma so there can be unknown factors other than immune suppression which may also be responsible for this coexistence of HBsAg and anti HBs antibodies in patients of chronic hepatitis B infection.[15] This profile may not always be associated with chronic active hepatitis as mentioned in Western literature. There is no clinical or biochemical parameter present which can flag these patients, hence high degree of suspicion is necessary to diagnose the condition. Most common accumulation of mutations (5 out of 7 samples) seen in this study was at position 127. Other common sites of mutation were at positions 128 and 118 (4 out of 7 samples).

Finally when screening a patient for the presence of vaccine induced antibodies, it may be useful to propose at least the detection of HBsAg as both markers may be present simultaneously and such an infected person can go undetected

## CONCLUSION

Although our findings are limited by a relatively younger population, accumulation of residue changes within the MHR, including the highly conformational and cysteine-rich “a” determinant can be a possible mechanism for coexistence of HBsAg and anti HBs antibodies in patients of chronic hepatitis B infection. The accumulation of HBV carrying immune escape variants in patients of CHB infection with relatively high viral loads raises the problem of transmission of such variants as they may not be fully neutralized by vaccine-induced antibodies, leading to wide spread vaccination failure. There is no clinical or biochemical parameter which could be used to flag these patients from other CHB patients hence these patients were indistinguishable clinically. Therefore, when screening a patient for the presence of vaccine induced antibodies, it is useful to propose detection of HBs Ag since both markers may be present simultaneously and a person with hepatitis B infection can thus go undetected.

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