Sequence Analysis of PreS2 Region of Hepatitis B Virus Genotype D Isolates

Amania Anwar[1], **Sheeba Murad**[1*], **Hajra Sadia**[1]

[1]Atta- ur- Rahman School of Applied Biosciences,
National University of Sciences and Technology, H-12, Islamabad, Pakistan, Telephone: +92-51-9085-6139.
***Corresponding Author:** s.mall@asab.nust.edu.pk, sheebamall@yahoo.com

ABSTRACT

Hepatitis B virus (HBV) is a well known agent of liver diseases. HBV disease burden varies across the globe with regions from low to high endemicity. Pakistan lies in the intermediate endemic zone, with high rate of mortality due to liver disease, cirrhosis and hepatocellular carcinoma. There is a wide range of heterogeneity in relation to HBV genotypes and sub-genotypes and in their patterns of pathogenesis, virulence and response to antiviral therapy. A large number of HBV genomic variations are associated with clinical outcomes such as hepatocellular carcinoma and liver cirrhosis. Thus, the present study aims to analyze PreS2 gene sequences from HBV isolates and their phylogeny. To investigate this, a study was conducted on twenty one HBV chronically infected individuals, serum samples were subjected to PCR with specific primers for PreS2 region of HBV genotype D and then sequenced. Point mutations: A39V, P41H and L42I were found in cell permeability domain of PreS2 protein. However, MHC class I and II epitopes were conserved in all sequences. Phylogenetic analysis was carried out by comparing the nucleotide sequence with 22 reference sequences of HBV sub-genotype D retrieved from the GeneBank. Phylogenetic analysis showed that two of our isolates, ASAB1 (2266) and ASAB3 (PIMS 7) shared cluster 1 with China D1, Pakistan D1, Iran D1 and Turkey D1. Meanwhile, ASAB2 (HF2) was grouped in cluster 2 with Lebanese D2 and Brazil D2.

Key words: Hepatitis B virus; Phylogenetic analysis; PreS2 region; Genotype D; Pakistan

1. INTRODUCTION

Hepatitis B virus (HBV) infection is a threat to the public health worldwide. Incidences of HBV infections are increasing at an alarming rate due to unavailability of effective vaccines and improved antiviral therapies for low income population. According to the current estimates of World Health Organization (WHO) 400 million people are chronically infected worldwide (Locarnini, 2002). In Pakistan, there are estimated 7-9 million carriers. Recently, a study reported 28.87% and 22% in HBV positive patients suffering from liver cirrhosis and hepatocellular carcinoma respectively Ali et al. (2011).

On the basis of genetic variability of (i.e.> 8%), HBV can be classified into eight genotypes including genotypes A to H Okamoto et al. (1988). All genotypes exhibit distinct geographical distributions, depicting ethnographic patterns of disease transmission Kramvis et al. (2005). Further, these eight genotypes have been grouped into 49 sub-genotypes on the basis of nucleotide variability of 4% to 8% Schaefer et al. (2005). In Pakistan, Genotype D (63.71%) is the most prevalent genotype. Moreover, sub-genotypes D1 and D3 have been reported to be prevalent in Pakistani population Baig et al. (2009). In addition, presence of D2 has also been reported Ahmed et al. (2009). However, Genotype C prevails with 7.55% in local population and is considered as an emerging genotype Ali et al. (2009).

HBV is a member of Hepadnaviridae family Pungpapong et al. (2007). The virus genome is double stranded circular virus of 3.2 kilobase pairs, which is organized in four overlapping open reading frames ORF S, C, P and X. ORF S encodes surface protein. ORF C encodes either HBV e antigen or viral capsid protein that has the ability of self assembly into the viral capsid. ORF P and ORF X encode large polymerase protein and 16.5 kDa HBV X protein respectively. ORF S is divided into three in frame AUG start codons which encodes three different surface proteins (Schadler and Hildt, 2009). Large surface protein (LHBs) encompasses PreS1 (108-119aa), PreS2 (55aa) and Surface (226aa) domains. Middle protein (MHBs) includes PreS2 and surface domain. Moreover, Small protein (S) consists of only Surface domain.

PreS2 domain consists of human serum albumin binding site (aa3-16), N-glycosylation site (aa 4), transactivator domain, cell permeability domain (aa 41-52) Lin et al. (2012) and several T and B cell epitopes Barnaba et al. (1989). Numerous mutations have been reported in PreS2 gene mutations which add complication in the diagnosis and treatment of HBV infected individuals (Weber, 2006; Gao et al., 2007; Su et al., 2007). In addition, PreS2 mutations are also associated with clinical outcomes. Point mutations; L36P, A39V, L42I, P41H, P52R were detected in Occult HBV isolated from patients with Hepatocellular Carcinoma Pollicino et al. (2007).

There is insufficient data regarding the mutations in the PreS2 region in local HBV genotype D infected individuals. Therefore; this study aimed to isolate PreS2 ORF gene and analyze the PreS2 sequence for mutations. In addition, a phylogenetic analysis was conducted to determine origin of PreS2 origin of HBV genotype D isolates.

2. MATERIALS AND METHODS

Blood Sample collection and Viral DNA extraction

A total of 21 blood samples of HBV positive patients were collected from patients residing in Islamabad during January to April, 2012 at Atta-ur-Rahman School of Applied Biosciences's Diagnostics Lab. Consent of the patients was taken and they were informed about the research study. Out of 21 samples only three samples corresponded with the inclusion criteria. Accordingly, samples having HBV infections other then genotype D and either Human immunodeficiency virus or Hepatitis C virus infections were excluded from the study; demography of the samples is shown in Tabel 1. Total of 3ml of freshly drawn blood was taken in ethylene diamine tetraacetic acid (EDTA) vacutainer tubes and centrifuged at 8000 rpm for 3 minutes to separate the serum. Genomic DNA was purified from the serum by using Nucleospin kit (Germany) according to the manufacturer's protocol. Primers were designed by retrieving the already published sequences of HBV PreS2 gene of genotype D from National Centre for Biotechnology Information (NCBI) (Available at http://www.ncbi.nlm.nih.gov/). The PreS2 gene was amplified by the following primers: TGTAGGCCCACTCACAGCA (forward) and GTAACACGGCAGGGGTCCT (reverse).

Table 1. Demography of patients

Accession no.	Sex	Age	HBsAg	HBeAg
ASAB 1(2266)	M	41	POSITIVE	POSITIVE
ASAB 2(HF2)	F	18	POSITIVE	POSITIVE
ASAB3(PIMS7)	M	33	POSITIVE	POSITIVE

PCR Amplification of HBV PreS2 gene and Sequencing

Polymerase Chain Reaction (PCR) was carried out to amplify PreS2 gene using the extracted Genomic DNA. The reaction mixture was prepared in 0.2 ml tubes (Axygen, California, USA) by adding 12.5 µL (800 ng) sample DNA template along with 2.5 µL each of gene specific forward and reverse primer (2 pmol), 5 µL of 2 mM dNTPs ,5 µL of 25 mM MgCl2 , 1.25 µL of 1.5 units of Taq polymerase (1U/µL) (Fermentas, USA) along with 5 µL of 10X Taq Polymerase Buffer and nuclease free water (16.25 µL) making a total reaction volume of 50 µL. PCR mixture was vortexed and then placed in Thermal Cycler (Swift™ MaxPro Thermal Cycler, Esco, Singapore), under the following PCR conditions: 94° C for 2 min followed by 35 cycles of 94° C for 1min, 58.5° C for 45 s, 72° C for 60 s and a final extension at 72° C for 10 min. Reactions was held at 4° C. Amplified PCR products were analyzed by electrophoresis on 1 % agarose gel. DNA fragments were purified from agarose gel by using Silica Bead DNA Gel Extraction Kit (Fermentas, USA). Purified DNA products were sent to Macrogen Korea for sequencing along with 10µl of forward HB2250P3F and HB200P3R reverse primer.

Sequence and Phylogenetic Analysis

PreS2 isolates were sequenced from each patient by using both sense and antisense primers. These sequences were aligned in CLC workbench software (CLC Inc, Aarhus, Denmark) to draw a consensus sequence for each isolate and then consensus sequences were submitted to Genebank under accession number KF482899, KF482900, KF470787. Sub-genotype D sequences were retrieved from NCBI (http://www.ncbi.nlm.nih.gov) : AY741798-IRAN D1, AY161159-INDIA D1, AY796032-TURKEY D1, AB583680.1-PAKISTAN D1, FJ562309.1-CHINA D1, FJ386590.1-CHINA D1, JN642163.1- LEBANON D2, EU594382.1-RUSSIA D2, JF815677.1-BRAZIL D2 JN642163.1- LEBANON D2, EU594382.1-RUSSIA D2, JF815677.1-BRAZIL D2, EI00615-EAST INDIA D3, X85254-ITALY D3,AB583679.1-PAKISTAN D3, AY373430-INDIA D3, JF815648.1- BRAZIL D4, GQ922005.1-CANADA D4, GQ922003.1-CANADA D4, GQ205386.1-INDIA D5, GQ205387.1-INDIA D5, JF815661.1-BRAZIL D6, JF815606.1-BRAZIL D6, FJ904444.1-TUNUSIA D7, FJ904440.1-CHINA D7, shown in Table 2. Consensus sequences were generated for sub-genotype D1 to D7. Then subsequent consensus and our PreS2 isolates sequences were aligned using CLC workbench 6.5.3 (www.clcbio.com). Further CLC tool was used to translate nucleotide sequence into protein sequence. B and T cell epitopes, along with other protein domains were analyzed. Phylogenetic analysis of our three PreS2 isolates with PreS2 sequences of sub genotype D was performed by CLC workbench software.

3. RESULTS

Nucleotide Sequence Alignment of PreS2 gene

A total of 22 Sub-genotypes (D1-D7) of genotype D HBV PreS2 sequences were retrieved from Genebank as shown in Table 2. PreS2 sequences for every genotype D sub-genotypes were aligned and a consensus sequence was generated. Consensus sequences were then compared with our isolates: ASAB1 (2266), ASAB2 (HF2) and ASAB3 (PIMS7). Point mutations at C28T, T125C and C122A were found in PreS2 sequences as shown in Figure 1, which were further analyzed via protein alignment.

Protein Sequence Alignment of PreS2 protein

Amino acid sequences were predicted for ASAB1 (2266), ASAB2 (HF2), ASAB3 (PIMS7) and PreS2 consensus sequences using CLC Work Bench 6.5.3. Later, amino acid sequences were aligned. Mutations were found in cell permeability domain of three of our isolates as shown in Figure 2. In ASAB1 (2266) and ASAB2 (HF2) isolates, ALA at position 39 is substituted with VAL, where both are neutral and non polar in nature. Moreover, in all three isolates at position 42, LEU is replaced by another neutral and non polar amino acid ILE. However, PRO at position 41 which is neutral and non polar, is substituted by HIS a basic and polar amino acid.

Phylogenetic analysis

Phylogenetic tree was constructed by using three different PreS2 gene sequences of HBV reported in this study along with twenty two PreS2 sequences obtained from Genebank with different sub-genotypes. Hepatitis B virus sub-genotypes were grouped in individual clusters. Two of our isolates, ASAB1 (2266) and ASAB3 (PIMS 7) shared cluster 1 with China D1, Pakistan D1, Iran D1 and Turkey D1. Meanwhile, ASAB2 (HF2) was grouped in cluster 2 with Lebanese D2 and Brazil D2 as shown in Figure 3.

Table 2. Retrieved PreS2 sequences with sub-genotype of D from NCBI

SUB-GENOTYPE	NCBI ACCESSION NO-ORIGIN
D1	AY741798-IRAN, AY161159-INDIA, AY796032-TURKEY, AB583680.1-PAKISTAN, FJ562309.1-CHINA, FJ386590.1-CHINA
D2	JN642163.1- LEBANON, EU594382.1-RUSSIA, JF815677.1-BRAZIL
D3	EI00615-EAST INDIA, X85254-ITALY, AB583679.1-PAKISTAN, AY373430-INDIA
D4	JF815648.1- BRAZIL, GQ922005.1-CANADA, GQ922003.1-CANADA
D5	GQ205386.1-INDIA, GQ205387.1-INDIA
D6	JF815661.1-BRAZIL, JF815606.1-BRAZIL
D7	FJ904444.1-TUNUSIA, FJ904440.1-CHINA

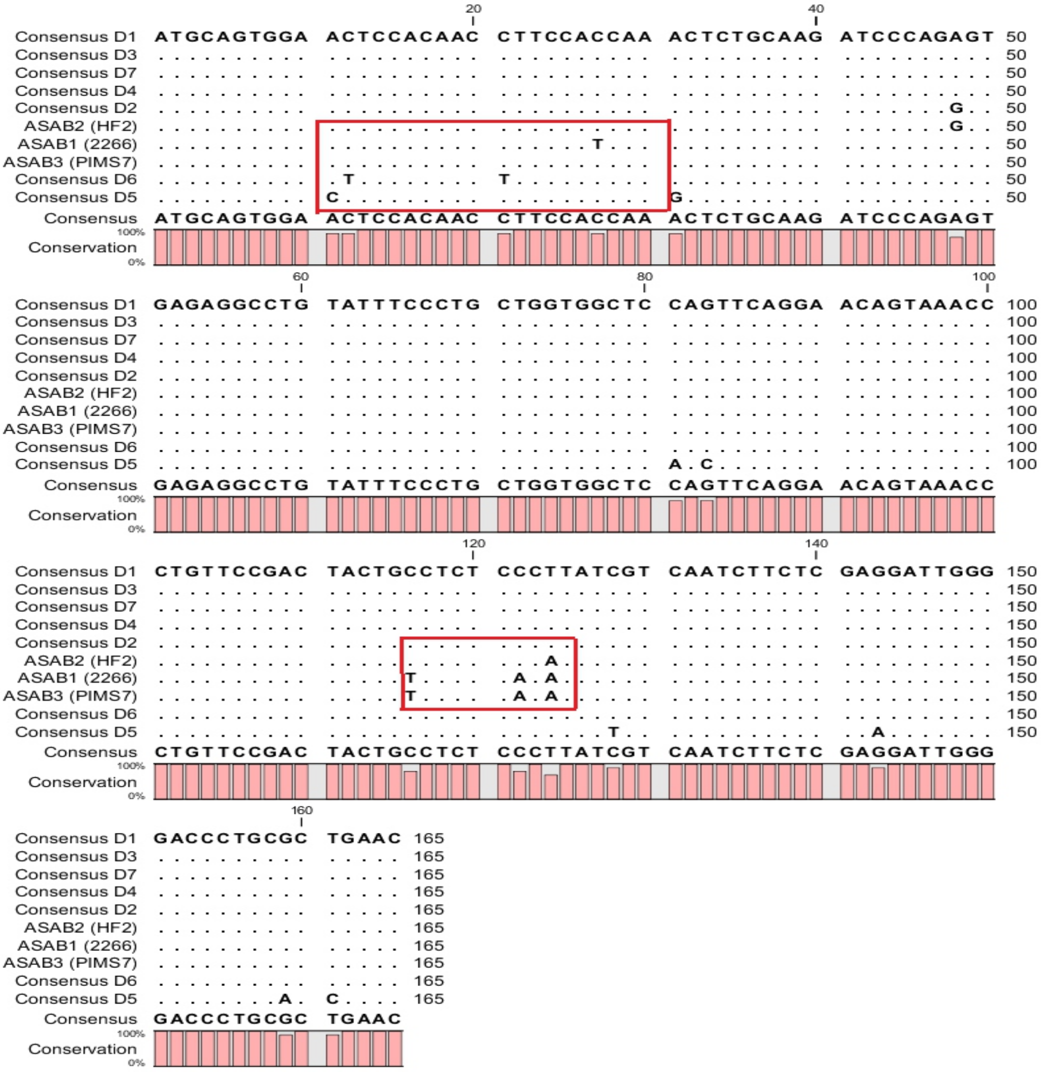

Figure 1. Multiple sequence alignment (MSC) of HBV PreS2 gene sequences of sub-genotype D strains with ASAB1 (2266), ASAB2 (HF2) and ASAB3 (PIMS7) isolates by means of CLC workbench 6.5.3 (http://www.clcbio.com).

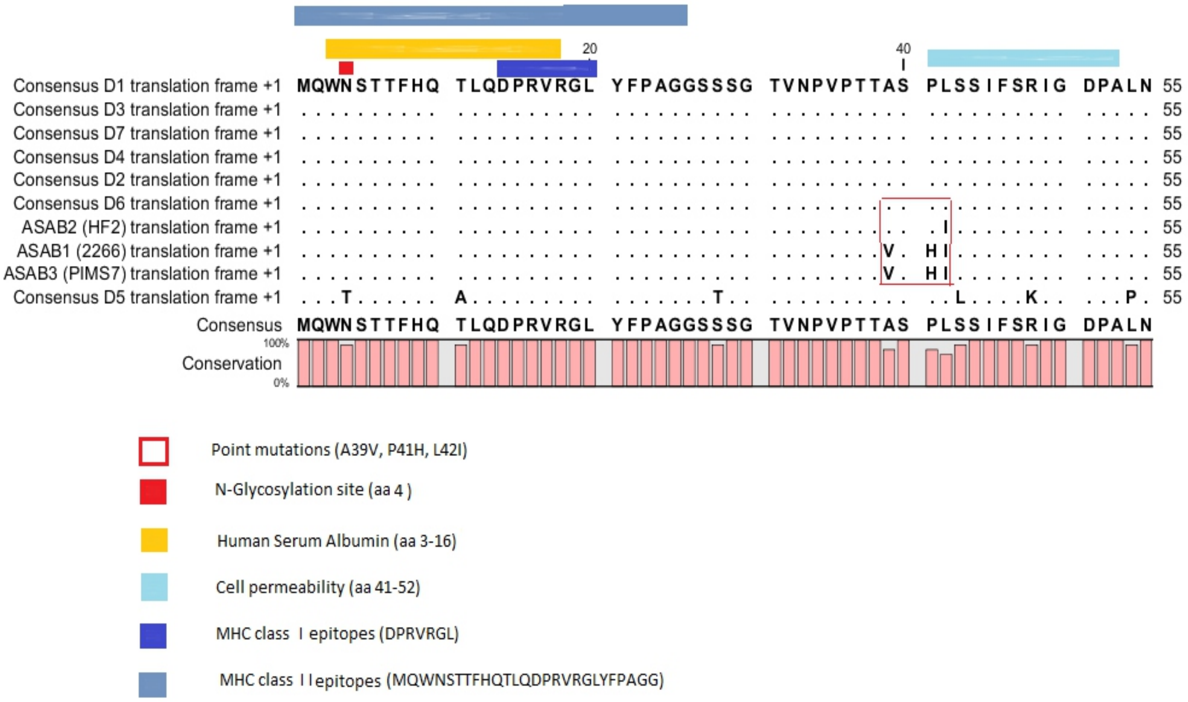

Figure 2. Protein sequence alignment of 3 of our isolates with other protein consensus sequences of sub-genotype D mentioned in Table 2. The conserved bases are shown as dots whereas the mutated residues are marked with the single letter code of that amino acid. Colored squares represent respective domains in the PreS proteins (Lin et al., 2012) .Used software CLC workbench 6.5.3 (http://www.clcbio.com).

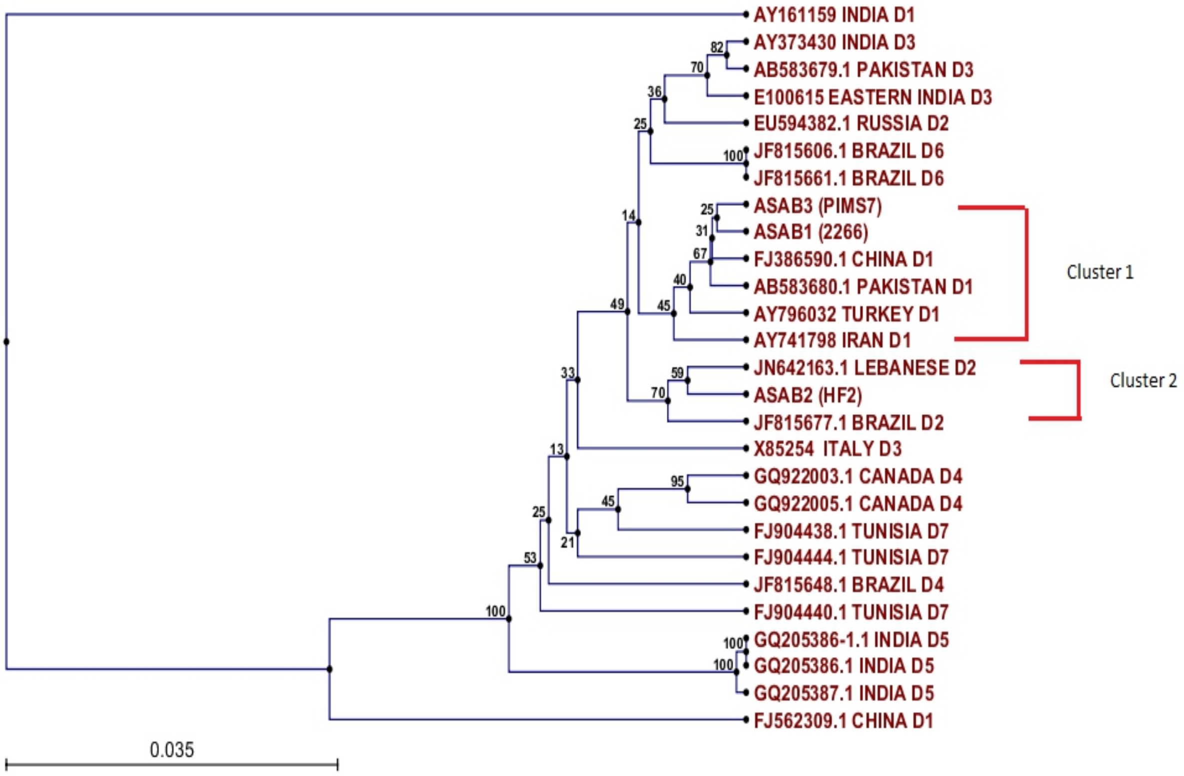

Figure 3. Phylogenetic tree of HBV PreS2 gene sequences. Tree was constructed by UPGMA algorithm. Bootstrap values are mentioned at the nodes. Tree shows a phylogenetic relationship between three of our isolates and sub-genotypes from the rest of the world. References sequences are labeled by Genebank accession number, Country name and by their respective sub-genotypes.

4. DISCUSSION

In Pakistan, the prevalence of HBV infections has increased beyond the reported findings. There are estimated 7-9 million carriers with carrier rate of 3-5%. In Pakistan, HBV infected population is distributed among general population including healthy blood donors, military recruits, prisoners and healthcare persons Ali et al. (2008). There are 20 to 30% HBV infected patients with cirrhosis and hepatocellular carcinoma Ali et al. (2011). Thus, HBV is a real challenge for scientist community.

HBV mutants have substantial impact to trigger pathogenesis and cause severe form of liver disease Mendy et al. (2008). Deletion mutations spanning 42-54 bp within Pre-S2 N-terminal, Pre-S2 internal deletions: 1-15 PreS2, 1-26 PreS2, 12-20 PreS2, 44-53 PreS2 have been reported (Gao et al., 2007; Su et al., 2007). Where, PreS2 internal deletions lead to the loss of M protein-glycosylation site (Asn-4 glycan site) in PreS2 protein, which impairs virion secretion (Margaret and Reinhild, 1998). Further, deletions in PreS2 region alter T and B cell epitopes giving PreS2 mutants selective advantage over wild type viruses Chisari et al. (1995). Studies report, HBV PreS2 deletion mutants confer an inefficient immune response, hence PreS2 deletion mutants persists in infected individual Barnaba et al. (1989). HBV Isolates from Ground glass Hepatocytes showed deletions at 5 terminus of PreS2 region (nt 2-55) and point mutation within the start codon (ATG to ATA) of middle surface protein (Fan, 2000). Recent studies found, prevalence of 18.8% Pre-S2 deletions in the HCC group and 5.8% PreS2 deletions in non-HCC group. In addition, PreS2 deletions were more prevalent in HCC patients aged < 50 years as compared with older HCC patients Yeung et al. (2011). Thus, keeping in view this scenario, study was conducted to determine PreS2 variants. PreS2 gene was successfully isolated and sequenced from three genotype D patients suffering from HBV chronic infections. Moreover, protein sequence derived from nucleotide sequences were analyzed for mutations.

Point mutations ALA to VAL, LEU to ILE and PRO to HIS were found, these have been previously reported in occult HBV isolates from patients with hepatocellular carcinoma Pollicino et al. (2007). These mutations were present in cell permeability domain which is important for translocation of viral protein and nucleic acids Hildt et al. (2002). Several MHC epitopes are reported in the entire HBV envelope protein Barnaba et al. (1989). Moreover, MHC class I and MHC class II epitopes were conserved in all sequences. Humoral and cellular immune response is directed against HBV envelope protein which leads to viral clearance, and neutralizing activity of anti-HBs antibodies. So far, only small protein (S) has been extensively used for the preparation of the vaccines and production of antibodies for therapeutic purposes. However recently, Madalinski et al. (2004) demonstrated that the third-generation preS1/preS2/S vaccine containing PreS2 major histocompatibilty epitopes showed more rapid onset and pronounced antibody response as compared to the S-gene-derived vaccine in healthy children and newborns (Madalinski et al., 2004; Madalinski et al., 2001). Thus, third generation vaccines can be used with further validation whether PreS2 MHC epitopes are conserved in local HBV variants.

Moreover, recently several researches demonstrated that type of chronic outcome; prevalence of mutations and the severity of virulence vary for genotypes and sub-genotypes. Thus, for better disease management and prognosis, it is imperative to determine their global distribution. Several studies report distribution of HBV sub genotypes around the globe. Where, Genotype A is further categorized in sub genotypes A1 predominant in Asia and Africa, A2 in Northern Europe and America (Bowyer et al., 1997; Kramvis et al., 2002) and A3 (Mulders et al., 2004; Kurbanov et al., 2005) in Central Africa. Sub-genotypes B1 of genotype B dominates in Japan, whereas B2 in China and Vietnam (Sugauchi et al., 2004; Norder et al., 2004). Genotype C with C1 as sub-genotype is common in South-East Asia and Bangladesh, C2 in Japan, Korea, and China (Chan et al., 2005; Sugauchi et al., 2004), C3 in Oceania and C4 in Australia (Sugauchi et al., 2003; Norder et al., 2004). In addition, geographical distribution of sub-genotypes D designated as D1–D7 are found to be in India, Iran, and Indonesia.

In this study, a phylogenetic analysis was conducted for three of our isolates. ASAB1 (2266) and ASAB3 (PIMS7) were grouped in cluster 1 and shared common ancestors with other members of the clusters, hence belong to sub genotype D1. While, ASAB2 (HF2) showed sequence homology with sub genotype D2 in cluster 2.

5. CONCLUSION

In conclusion, mutations were detected in PreS2 cell permeable motif. However, the implication of mutations in this region needs to be further elucidated by in silico protein modeling. Phylogenetic study demonstrated two sequences from sub-genotype D1 and one from sub-genotype D2. By further increasing number of PreS2 sequence analysis of HBV infected patients a considerable statement can be made about different mutations associated with sub-genotype.

6. ACKNOWLEDGEMENTS

We thank Dr Hajra Sadia for providing us valuable suggestions during the course of the study. This work was supported by Pak-US Science and Technology cooperative program entitled “Hepatitis B Virus-Associated Hepatocellular Carcinoma in Pakistan”.

REFERENCES

Ahmed, C. S., Wang, Z. H., Bin, Z., Chen, J. J., Kamal, M., Hou, J. L., 2009. Hepatitis B Virus Genotypes, Subgenotypes, Precore, And Basal Core Promoter Mutations In The Two Largest Provinces Of Pakistan. J Gastroenterol Hepatol., 4:569-73.

Ali, M., Idrees, M., Ali, L., Hussain, A., Ur Rehman, I., Saleem, S., Afzal, S., Butt, S., 2011. Hepatitis B Virus In Pakistan: A Systematic Review Of Prevalence, Risk Factors, Awareness Status And Genotypes. Virol J., 8:102.

Ali, S. A., Donahue, R. M. J., Qureshi, H., Vermund, S. H., 2009. Hepatitis B And Hepatitis C In Pakistan: Prevalence And Risk Factors. Int J Infect Dis., 13: 9-19.

Baig, S., Siddiqui, A., Chakravarty, R. And Moatter, T., 2009. Hepatitis B Virus Subgenotypes D1 And D3 Are Prevalent In Pakistan. Bmc Research Notes., 2:1.

Barnaba, V., Franco, A., Alberti, A., Balsano, A., Benvenuto, R., Balsano, F., 1989. Recognition Of Hepatitis B Virus Envelope Proteins By Liver- Infiltrating T Lymphocytes In Chronic Hbv Infection. The Journal Of Immunology., 143: 2650-2655.

Bowyer, S. M., Staden, L.v., Kew, M. C., Sim, J. G., 1997. A Unique Segment Of The Hepatitis B Virus Group A Genotype Identified In Isolates From South Africa. J Gen Virol., 78: 1719-1729.

Chan, P. C., Chen, H. L., Kong, M. S., Huang, F. C., Lee, H. C., Lin, C. C., Liu, C. C., Lee, I. H., Wu, T. C., Wu, S. F., Ni, Y. H., Hsu. H. Y., Chan, M. H., 2005. Factors Affecting The Mortality Of Pediatric Fulminant Hepatic Failure In Relation To Hepatitis B Virus Infection. J Gastroenterol Hepatol., 20: 1223-27.

Chisari, F. V., Ferrari, C., 1995. Hepatitis B Virus Immunopathology. Springer Semin Immunopathol., 17:261–81.

Chu, C. J., Keeffe, E. B., Han, S. H., Perrillo, R. P., Min, A. D., Soldevila, P. C., 2003. Prevalence Of Hbv Precore/core Promoter Variants In The United States. Hepatology., 38: 619-628.

Fan, Y. F., Lu, C. C., Chang, Y. C., 2000. Identification Of A Pre-s2 Mutant In Hepatocytes Expressing A Novel Marginal Pattern Of Surface Antigen In Advanced Diseases Of Chronic Hepatitis B Virus Infection. J Gastroenterol Hepatol., 15: 519–28.

Gao, Z. Y., Li, T., Wang, L. Du., Li, Y. J., Jie, Li. Lu., M, F., Zhuang, H., 2007. Mutations In Pres Genes Of Genotype C Hepatitis B Virus In Patients With Chronic Hepatitis B And Hepatocellular Carcinoma. J Gastroenterol., 42: 761–768.

Hildt, E., Munz, B., Saher, G., Reifenberg, K., Hofschneider, P. H., 2002. The Pres2 Activator Mhbs (t) Of Hepatitis B Virus Activates C-raf-1/erk2 Signaling In Transgenic Mice. Embo J., 15: 525-35.

Huang, X., Qin, Y., Zhang, P., Tang, G., Shi, Q., Xu, J., Qi, F., Shen, Q., 2010. Pres Deletion Mutations Of Hepatitis B Virus In Chronically Infected Patients With Simultaneous Seropositivity For Hepatitis-b Surface Antigen And Anti-hbs Antibodies. J Med Virol., 82: 23–31.

Kramvis, A., Kew, M. C., 2005. Relationship Of Genotypes Of Hepatitis B Virus To Mutations, Disease Progression And Response To Antiviral Therapy. J Viral Hepatitis., 12: 456–464.

Kramvis, A., Weitzmann, L., Owiredu, W. K., Kew, Mc., 2002. Analysis Of The Complete Genome Of Subgroup A' Hepatitis B Virus Isolates From South Africa. J Gen Virol., 83: 835-839

Kurbanov, F., Tanaka, Y., Fujiwara, K., Sugauchi, F., Mbany, D., Zekeng, L., Ndembi, N., Ngansop, C., Kaptue, L., Miura, T., Ido, E., Hayami, M., Ichimura, H., Mizokami, M. A., 2005. New Subtype (subgenotype) Ac (a3) Of Hepatitis B Virus And Recombination Between Genotypes A And E In Cameroon. J Gen Virol., 86: 2047-56.

Lin, C. M, Wang, G. M., Jow, G. M., Chen, B. F., 2012. Functional Analysis Of Hepatitis B Virus Pre-s Deletion Variants Associated With Hepatocellular Carcinoma. Journal Of Biomedical Science, 19:17.

Locarnini, S. A., 2002. Clinical Relevance Of Viral Dynamics And Genotypes In Hepatitis B Virus. Journal Of Gastroenterology & Hepatology., 17 Suppl 3: 322–s328.

Madalinski, K., Sylvan, S. P., Hellstrom, U., Mikolajewicz, J., Dzierzanowsk, F. K., 2004. Presence Of Anti-pres1, Anti-pres2, And Anti-hbs Antibodies In Newborns Immunized With Bio-hep-b Vaccine. Med Sci Monit., 10: 110-7.

Madalinski, K., Sylvan, S.P., Hellstrom, U., Mikolajewicz, J., Zemdrzuska, S. E., Piontek, E., 2001. Antibody responses to preS components after immunization of children with low doses of Bio HepB. Vaccine., 20: 1133-40.

Margaret, W., Reinhild, P., 1998. Role For Calnexin, N-linked Glycosylation In The Assembly And Secretion Of Hepatitis B Virus Middle Envelope Protein Particles. J Virol., 72: 778–782.

Mendy, M., D'mello, F., Kanellos, T., Oliver, S., Whittle, H., Howard, C. R., 2008. Envelope Protein Variability Among Hbv-infected Asymptomatic Carriers And Immunized Children With Breakthrough Infections. J Med Virol., 80 : 1537-1546.

Mulders, M. N., Venard, V., Njayyou, M., Edorh, A. P., Bola, A. O., Kehinde, M. O., Tamfum, J. J., Nebie, Y. K., Maiga, I., Ammerlaan, W., Fack, F., Omilabu, S. A., Le, Faou. A., Muller, C P., 2004. Low Genetic Diversity Despite Hyperendemicity Of Hepatitis B Virus Genotype E Throughout West Africa. J Infect Dis., 190: 400-08.

Norder, H., Courouce, A.m., Coursaget, P., Echevarria, J.m., Lee, S.d., Mushahwa, I.k., Robertson, B.h., Locarnini, S., Magnius, Lo., 2004. Genetic Diversity Of Hepatitis B Virus Strains Derived Worldwide: Genotypes, Subgenotypes, And Hbsag Subtypes. Intervirology., 47: 289-309.

Okamoto, H., Tsuda, F., Sakugawa, H., Sastrosoewignjo, 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:

Okamoto, H., Tsuda, F., Sakugawa, H., Sastrosoewignjo, 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.

Pollicino, T., Raffa, G., Costantino, L., Lisa, A., Campello, C., Squadrito, G., Levrero, M., Raimondo, G., 2007. Molecular And Functional Analysis Of Occult Hepatitis B Virus Isolates From Patients With Hepatocellular Carcinoma. Hepatology., 45: 277-285.

Pruitt, K.d., Brown, G.r., Hiatt S.m., Thibaud,n. F., Astashyn,a., Ermolaeva, O.,farell C.m., Hart, J, Landrum, M.j., Mcgravey, K. M., Murphy, M. R., O'leary, N.a., Pujar, S., Rajput, B., Rangwala, S.h., Riddick, L.d., Shkeda, A., Sun,h., Tamez, P., Tully, R.e., Wallin,c., Webb,d., Weber,j., Wu, W., Dicuccio, M., Kitts, P., Maglott, D.r., Murphy, T.d., Ostell, J.m., Refseq: An Update On Mammalian Reference Sequences. Nucleic Acids Res. 2013

Pungpapong, S., Kim, W. R., Poterucha, J. J., 2007. Natural History Of Hepatitis B Virus Infection: An Update For Clinicians. Mayo Clin Proc., 82 : 967-975.

Schadler, S., Hildt, E., 2009. Hbv Life Cycle: Entry And Morphogenesis. Viruses., 1,185-209.

Schaefer, S., 2005. Hepatitis B Virus: Significance Of Genotypes. J Viral Hepat., 12: 111-124.

Su, I. J., Wang, H. C., Wu, H. C., Huang, W. Y., 2008. Ground Glass Hepatocytes Contain Pre-s Mutants And Represent Preneoplastic Lesions In Chronic Hepatitis B Virus Infection. J Gastroenterol Hepatol., 23:1169–1174.

Sugauchi, F., Kumada, H., Sakugawa, H., Komatsu, M., Niitsuma, H., Watanabe, H., Akahane, Y., Tokita, H., Kato, T., Tanaka, Y., Orito, E., Ueda, R., Miyakawa, Y., Mizokami, M., 2004. Two Subtypes Of Genotype B (ba And Bj) Of Hepatitis B Virus In Japan. Clin Infect Dis., 38: 1222-28.

Sugauchi, F., Ohno, T., Orito, E., Sakugawa, H., Ichida, T., Komatsu, M., Kuramitsu, T., Ueda, R., Miyakawa, Y., Mizokami, M., 2003. Influence Of Hepatitis B Virus Genotypes On The Development Of Pres Deletions And Advanced Liver Disease. J Med Virol., 70: 537–544.

Sumi, H., Yokosuka, O., Seki, N., Arari, M., Imazeki, F., Kurihara, T., 2003. Influence Of Hepatitis B Virus Genotypes On The Progression Of Chronic Type B Liver Disease. Hepatology., 37: 19-26.

Weber, B., 2006. Diagnostic Impact Of The Genetic Variability Of The Hepatitis B Virus Surface Antigen Gene. J Med Virol., 78 Suppl 1: 59–65.

Yeung, P., Wong, D. K. H., Lai, C. L., Fung, J., Seto, W. K., Yuen, M. F., 2011. Association Of Hepatitis B Virus Pre-s Deletions With The Development Of Hepatocellular Carcinoma In Chronic Hepatitis B. J Infect Dis., 203: 646–654.