



Serodiagnosis of Hepatitis B Virus Using PreS Antigen from Pakistani Isolate SBS001

Saima Iftikhar^{1*}, Farheen Aslam² and Muhammad Akhtar¹

¹*School of Biological Sciences, Quid-e-Azam Campus, University of the Punjab, Lahore, Pakistan.*

²*Lahore College for Women University, Lahore, Pakistan.*

Authors' contributions

This work was carried out in collaboration among all authors. Author SI is the principle author who, in coordination with authors MA and FA carried out all the experimental work and wrote this manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2021/v33i42A32411

Editor(s):

(1) Dr. Syed A. A. Rizvi, Nova Southeastern University, USA.

Reviewers:

(1) Jitka Forstová, Charles University, Czech Republic.

(2) Dragoljub Zivanovic, University of Nis, Serbia.

Complete Peer review History: <https://www.sdiarticle4.com/review-history/71877>

Original Research Article

Received 01 June 2021

Accepted 04 August 2021

Published 28 August 2021

ABSTRACT

Background: An estimated 325 million people worldwide live with hepatitis B and/or C and approximately, 5 million people are affected with hepatitis B. in Pakistan. This study aimed at developing PreS protein from Hepatitis B Virus Pakistani isolate (SBS001) with enhanced sensitivity to detect antibodies in serum as a diagnostic method.

Methods: Gene encoding PreS region from hepatitis B Virus was cloned and expressed in *Escherchia coli*. The recombinant protein preS-His was purified by Ni-IDA affinity chromatography. Antibodies were raised in rabbit. This protein was screened for detection of antibodies in HBV patients' sera through ELISA. This ELISA procedure was compared with commercially available Kit used for diagnosis of HBV infection.

Results: Single band purified recombinant PreS protein was obtained with high titer of antibodies raised in rabbits. This recombinant protein was used in ELISA as antigen coated on the plate. That efficiently detected antibodies present in HBV patients. It was concluded that preS-His antigen/protein A HRP-conjugate ELISA method was more sensitive than the commercial kit for detecting the antibodies present in HBV patient sera.

Conclusion: It was concluded that SBS001 PreS recombinant protein can be used in ELISA kits for detection of HBV in Pakistani population.

*Corresponding author: E-mail: saima13pu@gmail.com, Saima.sbs@pu.edu.pk;

Keywords: Hepatitis B Virus; preS domain; Ni-IDA affinity chromatography; ELISA.

1. INTRODUCTIONS

The relation of man with hepatitis B virus is very ancient. The worldwide prevalence of this virus is estimated to be around 2 Billion including 400 Million people with chronic stage of infection [1]. According to WHO in 2015, hepatitis B resulted in 887 000 deaths, mostly from complications (including cirrhosis and hepatocellular carcinoma) [2].

Hepatitis B virus is the smallest DNA virus belonging to the family of *hepadnaviridae*. The partially double stranded genome of the virus is approximately 3.2 kb [3]. The surface gene, codes for three proteins L (Large), M (medium) and S (major or small) protein. These proteins are the products of the same gene with translation occurring from different methionine residues. The surface gene can be studied through its full-length translation product (L protein) or in its various parts (pre S1, pre S2, S). PreS1 and preS2 regions of DNA are collectively called preS and *in vitro* translated peptides corresponding to these DNA sequences are called preS protein. T-cell inducing immune response to preS antigen has shown that immunity can be induced using preS antigen in mice resistant to vaccine comprising PreS-protein [4]. PreS antigen induces the formation of neutralizing antibodies, which prevents endocytosis, attachment and perhaps membrane penetration of HBV into the hepatocytes. The preS1 domain of the large surface protein containing 21-47 amino acid sequence is important for attachment to hepatocytes. The preS2 domain has four reputed functions. It has a protein kinase binding sites and also a permeabilization site, which helps in the transfer of HBV particles into the cytosole of hepatocytes. It also has a proteolysis sensitive site as well as a 5-16 amino acid sequence to block human serum albumen receptor binding site. Furthermore, amino acid sequence 5-32 derived peptide vaccine can protect chimpanzees against HBV infection [5]. It is also suggested that HBV may attach to important receptors through preS1 site by reaction with interleukin 6 [6]. All these facts contribute to the enhanced immunogenicity of preS in a glycosylated and non-glycosylated form. The control of HBV on global scale is limited by several factors including high expense of yeast-derived vaccine, which inhibit their use in the third world areas. That is why recombinant vaccines produced in *E. coli* are very likely to

become the next generation vaccine containing the preS1 and preS2 region of hepatitis B virus. The present study is concerned with the cloning, expression and purification of antigenic preS protein of HBV and its serodiagnoses.

2. MATERIALS AND METHODS

2.1 Cloning of pres Antigen

To amplify fragment corresponding to gene encoding preS region, primer set PSF 5' TCTACCATATGGGAGGTTGGTCTTCCAAACC 3' and PSR 5' CTAAAGCTTAATGGTGGTGGTGGTGGTGGT T 3' was used. PCR was done using with initial incubation at 94 °C for 5 min, followed by 30 cycles with denaturation at 96 °C for 30s, annealing for 30s at 50 °C and extension at 72 °C for 1 min. The final extension was done at 72 °C for 7 min. The PCR product was first cloned in pTZ57R/T by InsT/A clone PCR product cloning Kit (Fermentas, USA). After restriction, PreS was ligated into Nde1 and Hind III sites of pET 21a (+) vector. *Escherichia coli* DH5 α cells were prepared and transformed with the recombinant vector [7].

2.2 Expression and Purification of preS Antigen

E. coli BL21 codon plus (DE3)-RIL (BL⁺) bacteria were transformed with expression plasmid for preS antigen production and were grown in LB broth containing 100 μ g/ml ampicillin at 37 °C to an OD₆₀₀ of 0.5 and then induced with 0.5 mM IPTG for another six hours. The expressed protein in soluble fractions of *E. coli* cells was checked by disrupting the cells by sonication for 7 min in ice water with one minute intermittent cooling interval at 50 amplitude (BANDELIN SONOPULS HD2070). To prepare lysate under denaturing conditions, the induced cells were resuspended in 100 mM of phosphate buffer pH 8.0 containing 6 M urea, and subjected to sonication. The supernatant was applied to Ni-IDA column to purify the protein as the recombinant proteins contained 6X His-tag at their C-terminal. Fractions containing specific protein were pooled and dialysed against 50 mM sodium phosphate buffer pH 5.2 with a linear decrease of urea i.e. 4, 2, 0 M during 24 hours. Total cell protein, soluble and insoluble fractions of the cell lysate and purified protein samples

were analysed on SDS-PAGE [8]. HPLC profile of PreS protein showed 2 major peaks which were analyzed on SDS – PAGE and also on MALDI – TOF. It was observed that there is protein specie, which was attributed to the oxidization of methionine and separated from the desired protein in the HPLC separation.

2.3 MALDI – TOF Analysis and Mass Determination

2 µl of protein sample (concentration 0.5 - 1 mg/ml) was mixed with 12 µl of matrix B solution and 2 µl sample was loaded on MALDI plate (polished steel). The sample plate was allowed to dry in a desiccator for 20 - 30 min. The sample was analysed in flex control programme, using LP-Clin prot.par method with default settings; detector gain (6X), and centroid algorithm for the peak detection, in a linear, positive (LP) mode. The detection range was adjusted between 10000 - 30000 Da. Spectrum was obtained by striking 200 - 400 shots at 60 - 70 % *arb* units of laser intensity on a sample.

2.4 Immunogenicity of the Recombinant PreS Protein

Antisera were raised in rabbit against PreS protein and examined by treating against the PreS protein through ELISA. Serum samples of six Hepatitis B patients were also added in the study. Serum was collected from patients before the start of Hepatitis B Virus treatment. The control group includes serum from 3 healthy individuals. The antigenicity of this protein was also compared with a commercially available kit containing recombinant HBsAg (small surface protein) coated plates. The serological assay was performed in three ways. First, in which P-preS-His was used as an antigen and the formation of antigen-antibody complex was quantified using protein A horseradish peroxidase conjugate (protein A-HRP). The second, was the use of commercial kit in which the wells are coated with HBsAg and following incubation with the antisera the complex formation was measured using a conjugate provided with kit. The third, was a hybrid of the two preceding methods. Here, the commercial plates containing HBsAg were treated with the experimental serum and the complex then detected with protein A-HRP conjugate.

For ELISA PreS antigen (2 µg/ well) in 50 mM carbonate buffer (pH 9.6: 200 ul) was coated on

Maxisorp microtiter plates (Nunc, Denmark) and incubated at 4 °C overnight. The wells were washed three times with PBS-Tween (200 µl). Serial dilutions (200 µl) in PBS-Tween were prepared in duplicate across the plate for both the test and control sera harvested from a pre immunized rabbit. Initial dilution was hundred fold. The sera were incubated at 37 °C for one hour in an incubator. The unbound sera washed from the wells with PBS-Tween (3x5 min) and goat anti-rabbit IgG HRP-conjugate second antibody, (0.01 µg in 200 µl of PBS-Tween), was incubated at 37 °C for an hour and the unbound proteins removed with PBS-Tween (3x5 min). Binding was visualized by the oxidation of 0.1 mg/ml of Tetramethylbenzidine (TMB) in 150 mM citrate-phosphate buffer (200 µl, pH 5.0) containing 0.01% (v/v) hydrogen peroxide. The color was allowed to develop in the dark for 5 min. The reaction was stopped with 12.5 % (v/v) H₂SO₄ (50 µl). The absorbance was measured at 450 nm.

2.5 Analysis of Human Sera Using Commercial kit

ELISA was performed using commercial ELISA kit, LH IRMA KIT IMMUNOTECH (A Beckman Coulter Company). In this method, polystyrene micro well strips were coated with HBsAg (small surface protein). In case of presence of anti-HBs in the sample the precoated and conjugated antigen was bounded to the two variable domains of the antibody and during incubation the immune complex formed is captured on the solid phase.

3. RESULTS

3.1 Cloning, Expression and Purification

PreS protein with His6 tag attached at C-terminus was successfully cloned in pET 21a (+) vector and expressed in *E. Coli* (Figs. 1 & 2). Level of protein expressed, as determined by Dolphin Gene Tool software, was 30 % of the total cell proteins (Table 1). The soluble proteins after dissolving in 8 M urea were loaded onto the Ni-Sepharose column. The protein obtained with elution buffer (0.1 M phosphate buffer having 6 M urea pH 5.2) was purified 80-90 %, as analyzed by SDS-PAGE (Fig. 3 (A) & (B)). Total of 58 OD₂₈₀ units of purified protein (in 70ml elution buffer) were recovered from one-liter culture. The combined eluted fractions were dialyzed against 20 mM phosphate buffer pH 5.2 with decreasing

the urea concentration linearly from 6 to 0 M during a 24 period.

3.2 Detection of Antibodies Raised in Rabbit

Polyclonal antibodies were produced by immunizing rabbits with 200 µg of preS antigen in complete Freund's (sp) adjuvant and booster dose given every 3-week, with 100 µg of the same antigen in incomplete Freund's adjuvant. Blood was collected after seven weeks of

immunization and processed to obtain the serum. To check the antigenicity of the collected serum ELISA assay was performed using 2 µg of recombinant preS protein incubated with serially diluted rabbit antisera taken after 7 weeks post initial immunization. The result of the titration of antibody binding activity in antiserum samples showed high titer after 7 weeks. This titer was much higher in rabbit #2 than in rabbit #1 (Fig. 4). The antiserum without antigen treatment was used as control and showed no reactivity.

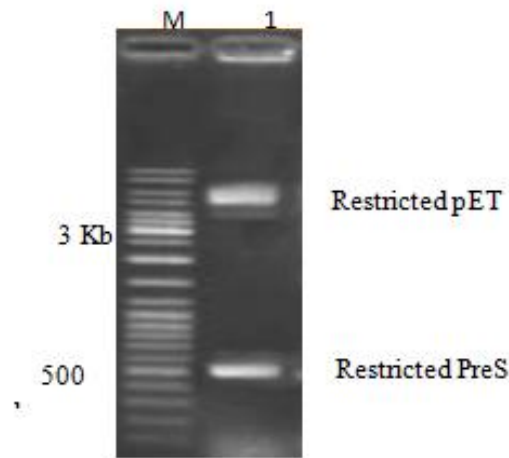


Fig. 1. Restriction analysis *preS/pET* with *NdeI* and *HindIII*. Lane M, 1kb DNA Molecular weight marker; Lane 1: Restricted *preS/pET*

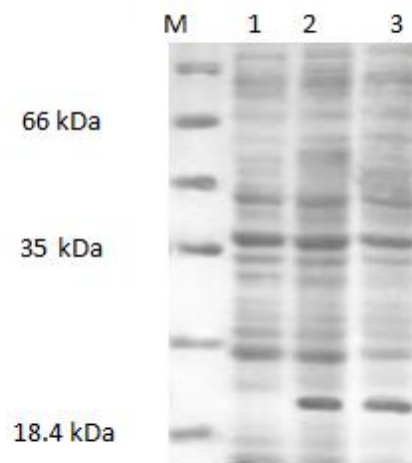


Fig. 2. SDS-PAGE analysis of the proteins of *E.coli* BL⁺ harboring *preS* antigen. Lane M, Molecular weight marker; 1, pET induced without *preS* antigen; 2, Total cell lysate containing *preS* antigen; 3, supernatant of above

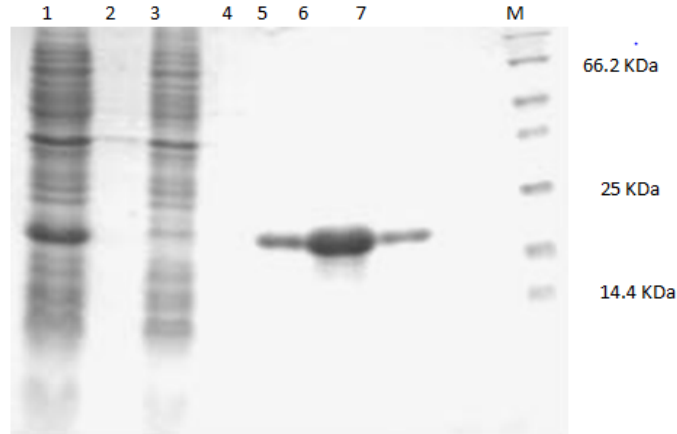


Fig. 3 (A). SDS-PAGE analysis of expressed *PreS* antigen purified by using Ni- column. Lane M, Protein molecular weight marker: 1, Induced supernatant after sonication harboring *preS* antigen in *E.coli* BL⁺: 2, *inclusions* bodies: 3, flow through after loading sonicated supernatant: 4, 5, 6, and 7, protein eluted with phosphate buffer (pH 5.2)

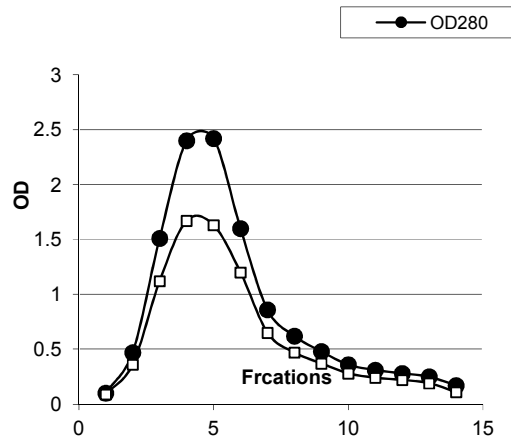


Fig. 3 (B). Data is plotted from different fractions of eluted *PreS* protein

3.3 Immunogenicity against Human Serum Samples

Anti-*preS* antibodies start to form during the early stages of HBV infection and play an important role in the elimination of virus from the infected host. Its presence in the serum indicates subsequent recovery during acute infection. Therefore, immunogenicity assay assists in the detection of anti-*preS* response in the sera from HBV infected patients. This experiment was planned for the evaluation of *preS* in the diagnosis and management of patients suspected of infection with HBV virus. Six fresh serum samples of HBV infected patients were

collected from National Genetics Laboratory Pakistan. Samples were stored at -20°C until required for assay.

3.4 Detection of Antibodies Against *preS* Antigen in the Serum from HBV Infected Patients

Three types of ELISA methods were used. First, in which P-*preS*-His was used as an antigen and the formation of antigen-antibody complex was quantified using protein A-horse radish peroxidase conjugate (protein A-HRP). The second, was the use of commercial kit in which the wells are coated with HBsAg and following incubation with

the antisera the complex formation is measured using a conjugate provided with the kit. The third, was a hybrid of the two preceding methods. Here, the commercial plates containing HBsAg are reacted with the experimental serum and the complex then detected with protein A-HRP conjugate. Samples from six HBV positive patients were analysed using these three methods (Fig. 5 (a-f)).

These results from six sera clearly show that the method using preS as the antigen was the most sensitive. The next was the kit method, which gave substantially better results than the one in which antibodies against HBsAg were detected with protein A-HRP conjugate. Thus, we conclude that preS engineered in this study, can be used for the development of diagnostic kit.

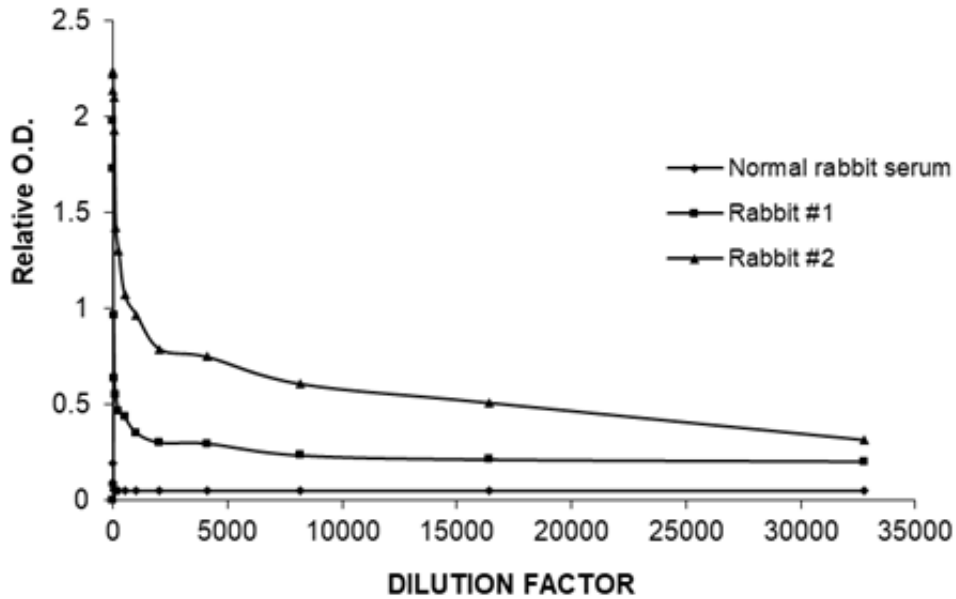
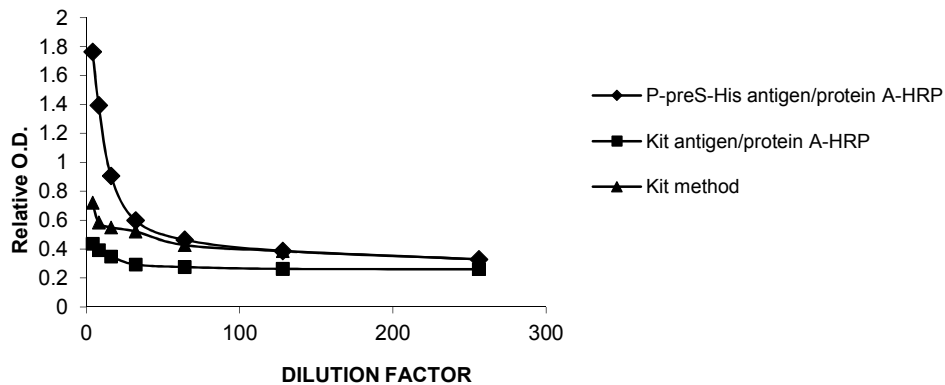
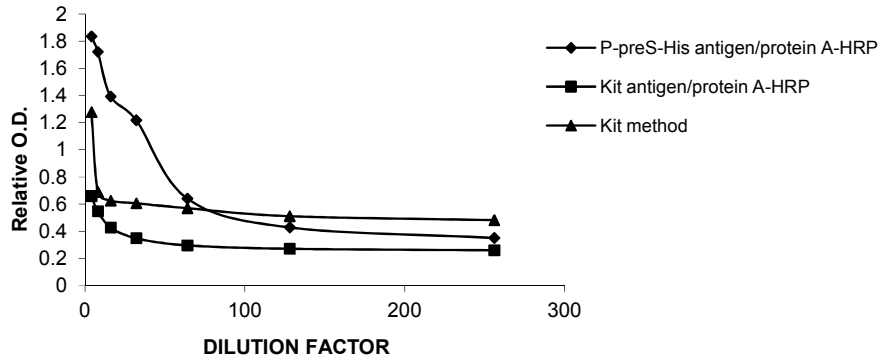


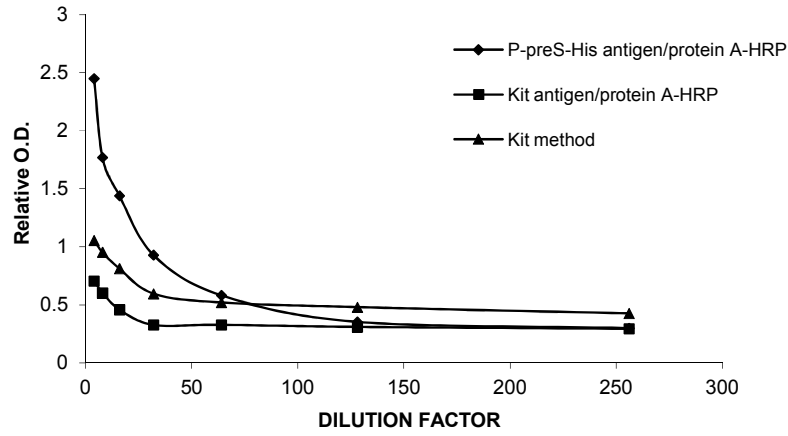
Fig. 4. Measuring the immunogenicity in rabbits injected with PreS antigen. Serum was obtained 7 weeks after the first injection and graph was plotted between the different dilutions of rabbit antisera and their corresponding absorbance measured at 450 nm



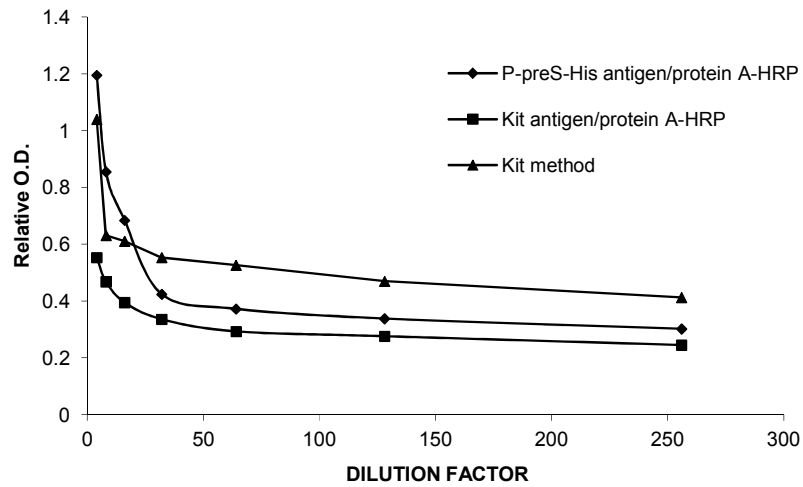
(a)



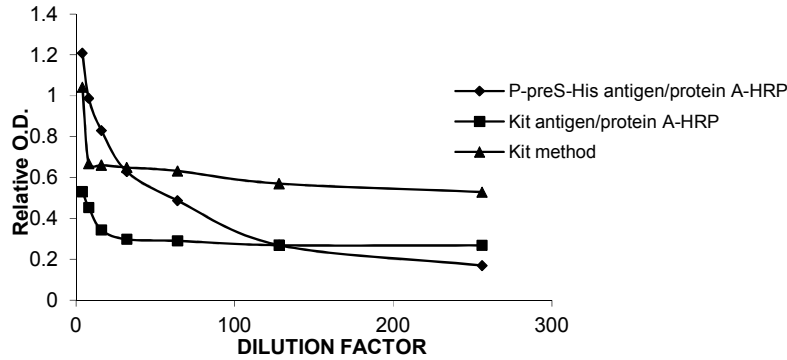
(b)



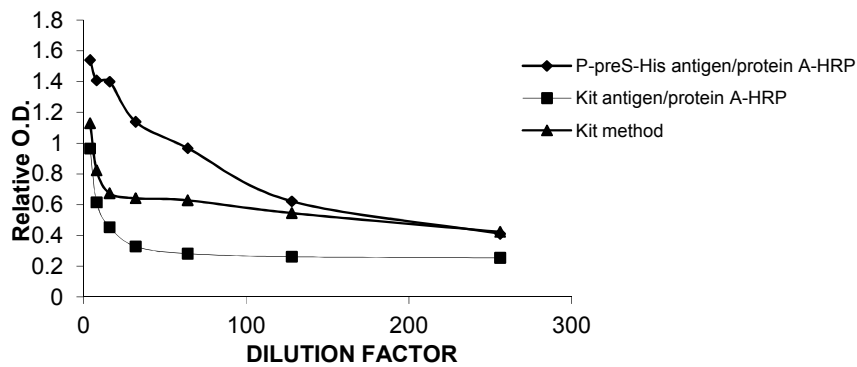
(c)



(d)



(e)



(f)

Fig. 5. Comparison of three ELISA methods to detect antibody in sera of patients #1-6 (graph a-f). In preS antigen/protein A-HRP, pres antigen was used while in other two methods commercially available HBsAg coated strips were used

Table 1. Purification of P-preS-His (preS domain of Pakistani isolates)

Culture (In liter)	Wet cell weight (g)	Supernatant after sonication (ml)	Total preS protein (OD units)	Total protein eluted (OD)	Dialysis		Total ODs and % Yield
					Super-natan (OD)	Pellet (OD)	
One liter	1.5	20	60	56	50	6	48 74%

4. DISCUSSION

The preS domain of hepatitis B virus possesses important biological functions, which are necessary for its infection. This region possesses many properties including intracellular retention of HBsAg, specific binding of HBV to hepatocytes and induction of virus neutralizing antibodies [9]. However, the detailed study of all these properties is complicated due to the difficulty of isolating these proteins. So, we took into consideration another approach, that is, the synthesis of preS domain. There are several indications suggesting that the preS exists as

independent domain retaining its native structural [10].

PreS domain has previously been expressed in many hosts, like yeast [11], plants [12] and eukaryotic cells [13], but it is most easy to express this region in bacterial system due to ease of purification and high yield. To simplify the purification method and avoid the degradation of recombinant protein, 6X His tag was added to the carboxyl end of the preS polypeptide. Using this procedure, preS domain of Pakistani isolate (SBS001) was expressed and purified using Ni-IDA column. The yield of the proteins was

increased due to minimum time taken for purification and 35-40 mg of highly pure proteins were obtained from 1 L of culture media. Neither the addition of 6X His tag at the carboxyl end of the proteins, nor the purification protocol, including denaturation step from 6 M urea modified the conformation of the protein. The addition of 6X His tag prevented the proteolysis of the preS2 region. So all the desirable properties including increased stability of recombinant proteins, isolation by simple method and high yield make the present approach a useful tool for the clinical application of the preS domain of HBV. Antibodies were raised against preS domain in rabbit to study the immunogenicity of this recombinant protein. Comparison of this recombinant protein with commercially available antigen (HBsAg) used in diagnostic kits was also done. It is inferred that preS1 (residue number 21-47) and preS2 (residue number 120-145) are involved in receptor recognition and are important in immunization [14] Although antibody response to S region protects human from HBV infection, it was suggested that incorporation of preS domain in the HBV vaccine can significantly improve its effectiveness. The persistence of preS antigen and absence of its antibodies are the indications of development of a chronic course of disease [15]. Therefore, the production of recombinant preS with proven immunogenicity would help in the detection of anti preS response in sera from vaccine recipient and HBV infected patients. To establish a useful method for the detection of anti-preS antibodies, it was important to obtain a preS antigen with good immune-reactivity. Immune-reactivity of purified preS was checked by raising antibodies in rabbit. For qualitative analysis of preS, an ELISA method using rabbit antisera and goat anti-rabbit IgG HRP-conjugate was used that showed high antibody titer corresponding to high immune-reactivity of this recombinant protein. In our experiment, antibodies started to rise and reached its maximum after 7th week.

In further study indirect ELISA was performed using 2 µg of recombinant P-preS-His incubated with serially diluted sera of HBV patients and using protein A-HRP conjugate to capture preS-antibody complex. To extend the study, this ELISA method was compared with the commercially available kit method and results showed that the method that used preS as antigen was much more sensitive method in detecting antibodies in HBV patients than the former procedure. This may be due to the

presence of preS antibodies early in the course of infection which play an important role in the elimination of virus from the infected patients [16].

5. CONCLUSION

This study has focused on preS region of HBV in Pakistani isolate. This information can be used in diagnosis and vaccine development in Pakistan. The information obtained during this study may also lead to the development of commercial ELISA and diagnostic kits.

ACKNOWLEDGEMENTS

I am much indebted to the Dr Altaf Khan for his help during the sequencing. I also express my great gratitude to Dr Saadat and Faisal Basheer who helped me a lot in collecting the HBV samples

COMPETING INTERESTS

Authors have declared that no competing interests exist.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

REFERENCES

1. Alam MM, Zaidi SZ, Malik SA, Naeem A, Shaukat S, Sharif S. et al. Serology based disease status of Pakistani population infected with hepatitis B virus. BMC Infect. Dis. 2007;7:64. Available: <https://doi.org/10.1186/1471-2334-7-64> [Online 27 June 2007]
2. WHO. Hepatitis B: Fact sheet No. WHO. 204;2015 [updated 2015 Jul cited 2015 Feb3,2016] Available from: <http://www.who.int/mediacentre/factsheets/fs204/en/>
3. Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF. et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol. 2000;81:67- 74.
4. Milich DR, Thornton GB, Neurath AR, Kent SB, Michel ML, Tiollais P. et al. Enhanced

- immunogenicity of the pre-S region of hepatitis B surface antigen. *Science* 1985;228:1195–9
5. Itoh Y, Takai E, Ohnuma H, Kitajima K, Tsuda F, Machida A. et al. A synthetic peptide vaccine involving the product of the pre-S(2) region of hepatitis B virus DNA: Protective efficacy in chimpanzees. *Proc Natl Acad Sci.* 1986; 83:9174–78
 6. Klinkert MQ, Theilmann L, Pfaff E, Schaller H. Pre-S1 antigens and antibodies early in the course of acute hepatitis B virus infection. *J Virol.* 1986;58:522–25.
 7. Sambrook J, Russel DW. *Molecular cloning: a laboratory manual.* 3rd ed. Cold Spring Harbor Laboratory; 2001.s
 8. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1979;227: 680-5.
 9. Neurath AR, Seto B, and Strick N. Antibodies to synthetic peptides from the preS1 region of the hepatitis B virus (HBV) envelope (env) sprotein are virus-neutralizing and protective. *Vaccine.* 1989; 7:234-6.
 10. Cooper A, Paran N, and Shaul Y. The earliest steps in hepatitis B virus infection. *Biochim Biophys Acta.* 2003;13:2273-79. DOI: 10.1016/s0005-2736(03)00166-4.
 11. Lu YY, L K, Cheng J, Wang L, Liu Y, Zhang LX. Cloning and expression of the preS1 gene of hepatitis B virus in yeast cells. *Hepatobiliary Pancreat Dis Int.* 2002;1:238–42
 12. Gao Y, Ma Y, Li M, Cheng T, Li SW, Zhang J. et al. Oral immunization of animals with transgenic cherry tomatillo expressing HBsAg. *World J Gastroenterol.* 2003;9:996-1002.
 13. Qin S, Tang H, Zhao L, He F, Lin Y, Liu L. et al. Cloning of HBsAg-encoded genes in different vectors and their expression in eukaryotic cells. *World J. Gastroenterol.* 2003;9:1111–1113. DOI: 10.3748/wjg.v9.i5.1111
 14. Neurath AR, Adamowicz P, Kent SBH, Riottot MM, Strick N, Parker K. et al. Characterization of monoclonal antibodies specific for the pre-S2 region of the hepatitis B virus envelope protein. *Mol. Immunol.* 1986a;23:991-97
 15. Theilmann L, Burkardt HD, Galle PR, Gemelin K, Kommerell B, and Pfaff E. Detection of antibodies against preS1 proteins in sera from patients with hepatitis B virus infection by ELISA using a psreS fusion protein expressed in *E. coli*. *Arzneimittelforschung.* 1988;38:1856-58.
 16. Madalinski K, Sylvan SP, Hellstrom U, Mikolajewicz J, Zembrzuska-Sadkowska E, and Piontek E. Antibody responses to preS components after immunization of children with low doses of BioHepB. *Vaccine.* 2001; 20:92-97.

© 2021 Iftikhar et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:

The peer review history for this paper can be accessed here:
<https://www.sdiarticle4.com/review-history/71877>