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Highly Immunogenic Recombinant HBV Core Protein from Pakistani Isolates: A Potential Candidate Vaccine for Hepatitis B in Pakistan

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ABSTRACT

Hepatitis B can be initially diagnosed by hepatitis B core antibody - one of the earliest proteins of immune system detected after viral infection starts. Present study was aimed at the cloning, expression, refolding, purification and immunogenicity analysis of HBV core antigen protein of a local genotype D isolate from Pakistani population. The viral protein was expressed in BL21 (DE3) strain of *E. coli* using pET22 b (+) expression plasmid vector. Recombinant protein appeared as inclusion bodies constituting about 40 to 50% of total *E. coli* proteins with a molecular weight of 21kDa. The misfolded inclusion bodies were solubilized, refolded *in vitro* and purified by anion exchange chromatography using DEAE-Sephadex. The immunogenicity of folded and purified antigen was determined and verified by antigen-antibody interaction reactions and ELISA. The recombinant antigen exhibited strong reaction against HBV positive and no reaction against HBV-negative sera. It gave a good response to the ELISA and indicated a great immune response in rabbit. The subject protein with considerably high immune response offers a useful marker for the early diagnosis of HBV and a candidate subject for vaccine development research.

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Authors' Contribution
AR and ARS conceived and designed the experimental work and wrote the article. MSN helped in part of experimental work. JAK and KM helped in interpretation of results. ARS supervised the work and finalized the article.

Key words
Hepatitis B virus, Core antigen, Gene expression, Immunogenicity, Recombinant protein, HBsAg, HBcAg

INTRODUCTION

Hepatitis B viral infections are leading cause of liver cirrhosis or hepatocellular carcinoma (Lok, 2002). The virus belongs to family hepadnaviridae and its genome consists of 3.2 kb double stranded circular DNA molecule (Ganem and Schnieder, 2001). The genome consists of four partially overlapping open reading frames encoding primarily the viral DNA polymerase, core, surface and X proteins which play important role in the viral pathogenicity (Mizokami *et al.*, 1997). Hepatitis B virus (HBV) has ten distinct genotypes with above 8% nucleotide sequence divergence and many subgenotypes having sequence divergence between 4% to 8% (McMahon, 2009; Mulyanto *et al.*, 2010). Specific genotypes and their recombinants are found in different geographical regions and populations (Owiredu *et al.*, 2001; Wang *et al.*, 2005; Lok and McMahon, 2009; Zhou *et al.*, 2011). Similarly, there is also a broad variation in the prevalence of hepatitis B in different areas of the world (Medley *et al.*, 2001).

According to recent estimates, about 7% of the world population (350 to 400 million people) is infected with HBV, resulting in 1-2 million deaths per year (Qasmi *et al.*, 2000; Lavanchy, 2004; Dienstag, 2008). The prevalence of hepatitis B in Pakistani population is up to 2.5% (Qureshi *et al.*, 2010). The most prevalent HBV genotype is D (63.8%) followed by genotype A (10.0%), genotype C (7.7%) and genotype B (5.8%), while untypable and mixed genotypes are 2.7% and 10%, respectively (Idrees *et al.*, 2004; Noorali *et al.*, 2008; Ahmed *et al.*, 2009). The proteins of HBV collectively known as hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg) are important markers for the diagnosis of infection (Krajden *et al.*, 2005). The genetic variability of HBV often becomes a challenge for the accurate diagnosis of HBV infection, particularly with respect to the detection and quantification of antigenic proteins. It has been reported as the cause of low efficacy of vaccines (Weber, 2005; Cassidy *et al.*, 2011). Many attempts have been made for the nucleotide sequence analysis, recombinant production of HBV surface and core antigenic proteins to establish the strong immunogenic epitopes detection of local HBV genotypes and their recombinant have been prepared (Stahl and Murray, 1989; Preikschat *et al.*, 1999;

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DeSouza *et al*., 2013). We have already reported the prevalence of HBV in the general populations of northern Pakistan (Rauf *et al*., 2011, 2013) and the cloning of surface protein in *E. coli* (Abbas and Shakoori, 2005). Present study was aimed at the comparative analysis of nucleotide sequences encoding core antigen protein from local isolates of HBV obtained during our studies to determine the prevalence of HBV. It was also aimed at the production of recombinant HBcAg protein in *E. coli* and its evaluation for immunogenicity.

MATERIALS AND METHODS

Materials and HBV isolates

HBV screening kits were obtained from (ACON~ACON Laboratories Inc., San Diego, CA 92121, USA), PCR reagents, DNA/protein markers, restriction and ligation enzymes were obtained from Thermo Scientific (Thermo Fisher Scientific, Inc. NYSE: TMO), Ion exchangers were obtained from Calzyme Laboratories (3443 Miguelito Ct, San Luis Obispo, CA 93401, United States). DNAzol ®BD. Cat. No.DN129 was obtained from Molecular Research Centre, Inc., Cincinnati, OH, USA). All the reagents and chemicals used in the present study were of molecular biology grade. The HBV isolates were selected from PCR positive samples in the local populations.

Anti HBsAg screening

HBV screening was based on the detection of antibodies against the virus in the sera. Screening kits (ICT) were used for the detection of anti HBsAg (ACON~, ACON Laboratories Inc., San Diego, CA 92121, USA). Procedures provided by manufacturers were adopted.

PCR-based detection of HBV DNA

For the confirmation of viral DNA in the anti HBsAg positive samples, the serum obtained from venou-puncture was used for DNA isolation by DNAzol ®BD kit based method provided by the manufacturers.

PCR amplification of gene for core protein

Antigen positive samples were used for the DNA isolation and PCR amplification of gene encoding core protein. The primers with nucleotide sequences
5'–TCCAAGCTGTGCCTTGGGTG- 3' and
5' -GAAGAATAAAGCCCAGTAAA- 3'
were used in the PCR reaction. Reaction mixture included 40 pmole primers, 1.6 µM $MgCl_2$, 8 µM dNTPs, 5 U *Taq* polymerase, 1X *Taq* buffer, 10-20 ng of DNA with total volume of 20 µL. PCR-thermocycler was adjusted at 94ºC for denaturation for 5 min, followed by 30 cycles, each of 20 Sec at 94ºC (denaturation), 25 Sec at 55ºC (annealing) and 30 sec at 72ºC (extension) with final extension at 72ºC for 5 min. PCR product was checked out by agarose gel with the DNA marker (Fermentas cat. No. SM0403).

T/A cloning and sequence analysis

HBV core gene was extracted from the agarose gel using silica based DNA extraction kit (Thermo Scientific Cat. No. K0513) and ligated with pTZ57R/T plasmid vector using PCR cloning kit (Thermo Scientific Cat. No. K1213). The ligation mixture was used for the transformation of competent cells of *E.coli* (DH5α strain) which were spread on the LB agarose plates containing 100 µg of ampicillin, 120 µg of X-gal, 120 µg of IPTG per mL of medium. The plates were incubated at 37ºC overnight, greenish-blue colonies and white colonies appeared on each experimental plate. White colonies were further grown in LB broth medium with ampicillin, plasmid was isolated and subjected to restriction analysis using EcoRI and SalI. Plasmid isolated from a single colony, confirmed for the presence of core protein gene (pTZ-HBVc) was analyzed for nucleotide sequence of ligated gene using plasmid specific M-13 primers.

Expression of HBV core protein in E. coli

The primers with nucleotide sequence:
5' CTTTGGCATATGGACATTGACC 3' and
5' GAAGAATTCAGCCCAGTAAA 3'
were used for the amplification of core gene from pTZ57RT plasmid. Hence, the restriction sites for NdeI and EcoRI were introduced before the start codon and after the stop codon respectively in the confirmed gene sequence. It was ligated and cloned as pTZ-HBVc, restricted with *Eco*RI and *NdeI* and ligated in to pET22b (+) plasmid restricted with the same enzymes. Competent cells BL21codonplus RIL strains of *E. coli* were transformed with the ligation mixture above, the cells were spread on the LB-Amp plates for overnight growth. The presence of target gene the expression plasmid was confirmed by restriction analysis of recombinant plasmid pET-HBVc with *NdeI* and *Eco*RI.

Expression, refolding and purification

Recombinant HBV core protein was produced on large scale in LB–broth containing 100 µg of ampicillin per milliliter of medium and 0.5 mM final concentration of IPTG. The bacterial cell pellet was sonicated and extract was analyzed on SDS-PAGE for the presence of protein. All the recombinant protein was found as misfolded inclusion bodies which were solubilized in a buffer containing 8M urea, 50mM Glycine, 10mM EDTA, 50mM Tris-HCl pH 8.0 and 2mM DTT to make a solution having an OD of 5.0 at 280nm. Small fractions of clear solubilized inclusion bodies were added to the refolding sink solution

containing 100mM Tris-HCl pH 9.0, 10mM ETDA, 0.5mM cystine, 2M urea and 5mM cysteine in such a way that the final absorbance of sink solution at 280 nm was increased up to 0.05 after the addition of each fraction. Final concentration of sink with 0.5 absorbance values was achieved in 10 h with constant stirring at 4ºC. This mixture was placed on stirrer overnight in a closed beaker covered with aluminum foil followed by overnight dialysis against 20 mM Tris-HCl buffer pH 7.5. The folded inclusion bodies were purified by FPLC based resource Q column equilibrated with 5 column volumes of dialysis buffer. Clear dialyzed sample after centrifugation at 25,000 rpm (23,600x *g*) for 30 min was loaded on to the column. Unbound proteins were washed with 2 column volumes of equilibration buffer and bound proteins were eluted with 0 to 500 mM linear NaCl gradient in 5 column volumes. Fractions of 1ml were collected and analyzed on SDS-PAGE.

Immunogenicity of recombinant protein

Purified recombinant protein was quantified by Bradford method (Bradford, 1976), 100 µg of protein was mixed in 100µl of phosphate buffer saline (PBS) pH 7.4 and equal volume of Freund's Complete Adjuvant was added. The mixture was emulsified by vortexing for 3 h. The immunogen was administered to two male, 12 months old rabbits, *Oryctolagus cuniculus*. The immunogen (200 µL) was injected subcutaneously at the back of the rabbit at four different sites with 50 µL at each site using 1 ml insulin syringe. Pre-immune serum of the animal was obtained before injecting the first dose of immunogen at 0 week and stored for control purpose. First booster dose was given after 4 weeks of the first dose. The quantity of the dose was kept half of the first. Second booster was given after 3 weeks of the first booster. After two weeks of second booster administration final bleed was taken and serum was isolated and stored. Antigen-antibody precipitation test was performed using purified HBV core protein and antisera of the immunized rabbits. Three different concentrations of the reagents were used in the test. Volume of the antisera was kept constant (50 µL) while the volume of antigen (recombinant protein) and saline was varied as 0 µL, 100 µL, 150 µL of saline solution and 200 µL (200 µg), 100 µL (100µg) and 50 µL (50 µg) of antigen per reaction. The reaction mixture was incubated at 37ºC for 30 min and then incubated at 4ºC overnight. The overnight-incubated mixture of antigen and antibody was checked out for precipitation.

Enzyme-linked immunosorbent assay (ELISA) of rabbit antisera

ELISA of rabbit antisera was carried out to determine the immunogenicity of the HBV core antigen. The wells of microtiter plate were coated with recombinant protein (2.0 µg) in 200 µL of 50mM carbonate buffer (pH 9.5), incubated overnight at 4ºC and washed three times with PBS containing 0.05% Tween. The microtiter plate was incubated at 37ºC for 1 h in humid environment, washed four times with PBS-Tween buffer. The secondary antibody *i.e*. anti IgG of rabbit coupled with HRP, prepared in PBS was added to each well and the plate was incubated at 37ºC for 1 h again. The wells were washed four times with PBS-Tween buffer using 200 µL per wash. The plate was allowed to dry for 20 min, then 200 µL of TMB substrate solution containing hydrogen peroxide was added to each well and incubated at 37ºC for 5 min. The color reaction was stopped by adding 50 µL of stop solution (12.5% v/v sulphuric acid solution). The absorbance was measured at 450nm by ELISA reader.

RESULTS

HBV surface antigen positive samples were verified for the presence of viral DNA by nested PCR. A 630 bp fragment containing an open reading frame of 552bp of core protein gene encoding 183 amino acid protein was amplified from the samples positive for viral DNA. The DNA fragment was ligated into pTZ57RT plasmid vector the *E. coli* cells successfully transformed with this plasmid were confirmed by plasmid isolation and restriction analysis with *Eco*RI and *Sal*I. The nucleotide sequence of HBV core gene was determined for local isolates using M13 primers which had highest homology with genotype D reported in the literature. The restriction site for NdeI enzyme was added before the start codon of confirmed gene sequence and complete open reading frame was ligated into pET22b (+) expression vector

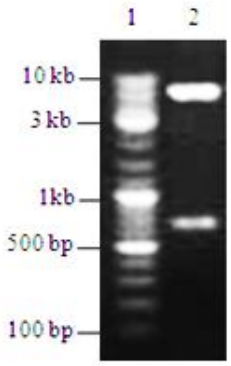

Fig. 1. Agarose gel electrophoresis of double restricted recombinant plasmid (pET22 b +). Lane **1,** 10kb DNA marker (Fermentas Cat# SM0334); Lane **2,** double restricted plasmid pET22 b (+).

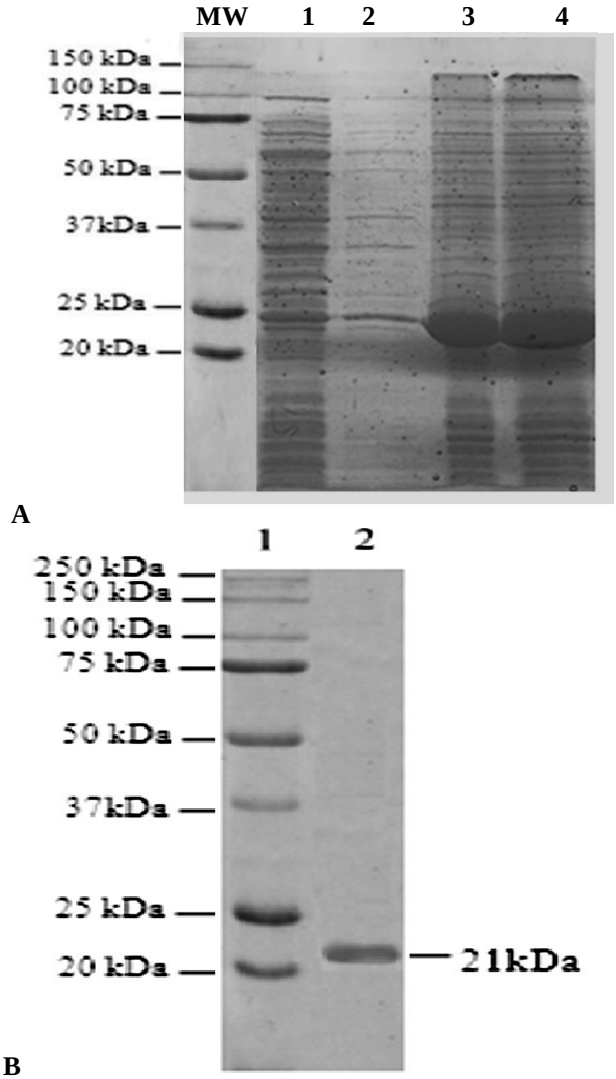

Fig.2. SDS-PAGE pattern of HBV core protein expressed in *E.coli* cells. **(A)**. Lane 1, Un-induced cell lysate. Lane 2, washing of inclusion bodies; Lanes 3 and 4, inclusion bodies. **(B)** Lane 1, protein markers, 2, purified recombinant protein with molecular weight of about 21 kDa.

using NdeI and EcoRI restriction sites for both the gene and the vector. The transformed BL21 (DE3) cells transformed with the ligation mixture were confirmed by plasmid isolation and restriction analysis. The plasmid and gene were shown as separate bands while subjected to agarose gel electrophoresis at 5.5 kb and 630bp molecular weight bands (Fig. 1). The expression of recombinant protein was induced in the presence of 0.7 mM IPTG in LB broth medium containing 100µg of ampicillin per mL of medium. All the recombinant protein appeared as misfolded inclusion bodies on SDS PAGE (Fig. 2A). The inclusion bodies were solubilized in urea solution, folded *in vitro* and purified. Purified protein was found as a single band on SDS-PAGE with a molecular weight of about 21 kDa (Fig. 2B).

Antibodies against the HBV core protein were raised in two male rabbits which were confirmed by antibody-antigen precipitation test and Enzyme Linked Immunosorbent Assay (ELISA) test. It was found that maximum titer of antibodies was achieved after the second booster dose of immunogen in both the rabbits. The serum of both rabbits exhibited similar response with absorbance at 450 nm after first and second booster which was 0.6 to 1.2 for rabbit one and 0.71 to 1.3 for rabbit two respectively (Figs. 3, 4).

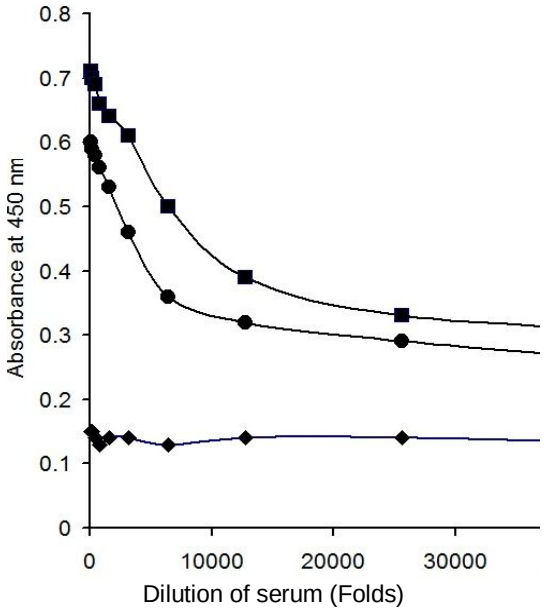

Fig. 3. Antibody titer in the serum of rabbits after the first booster dose of immunogen. Antiserum of rabbit1 (●) exhibits maximum absorbance (0.6) with 100 folds dilution of serum and minimum (0.25) at 51200 folds dilution. Rabbit 2 (■) shows maximum absorbance (0.71) at 100 folds dilution and minimum (0.30) at 51200. Pre immune serum (control) (♦) shows no significant difference in absorbance with varied dilution.

DISCUSSION

Present study describes the expression, purification and immunogenic analysis of a gene encoding HBV core protein in type D genotype of local isolates. HBV gene encoding the small fragment of core protein was analyzed for nucleotide sequences and expressed in BL21 (DE3) cells of *E. coli*. Gene was over-expressed in the presence of 0.7mM IPTG in LB medium. The expression level of

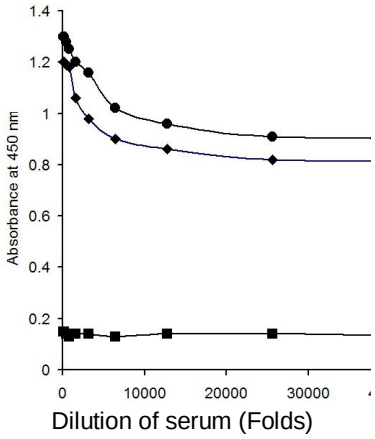

Fig.4. Antibody titer in the antisera of rabbits after the second booster dose of immunogen. Antiserum of rabbit1 (♦) exhibits maximum absorbance (1.2) with 100 folds dilution of serum and minimum (0.81) at 51200 folds dilution. Rabbit 2 (●) shows maximum absorbance (1.3) and minimum (0.90) at 100 and 51200 folds dilutions, respectively. Pre immune serum (control) (■) shows no significant difference in absorbance with varied dilution.

protein in the present study was higher than reports available in the literature about the expression of viral genes in *E. coli* (Borisova *et al*., 1989; Azizi *et al*., 2000). Refolding and purification results are similar to those described by Preikschat *et al*. (1999). Antibodies against the recombinant HBV core protein were raised in the rabbits and confirmed by reaction of antigen versus antiserum. Precipitates were observed within the tubes which indicated the antibodies in the serum of experimental rabbits against the antigen. The quantitative test for the polyclonal antibodies in the antisera of rabbits was performed by ELISA. However, the specificity of the antibody could not be determined and therefore its veracity is an open question for further investigations. The ELISA results also highlighted the fact that the HBVcAg is highly immunogenic and may be a candidate of future vaccine studies for hepatitis B.

CONCLUSIONS

The recombinant HBV core protein has shown a high level of immune response in animal model. It can be a potential candidate for the vaccine development against local HBV strains in Pakistani population.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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