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HCVNS3 Gene of Hepatitis C Virus Expression in *E.coli* Strain BL21 (DE) 3C+ For Diagnostic Role of HCV by Detecting the Anti HCV

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Abstract: This study aimed at understanding the antigen antibody reaction of Hepatitis C Virus for developing a kit for HCV diagnosis. For that, the plasmid DNA containing HCVNS3 gene were isolated from *E.coli* strain DH5α and transferred to expression host, *E.coli* strain BL21 (DE) 3C+ and recombinant HCVNS3 protein of interest was expressed and it was purified by using metal affinity column chromatography. Protein expression was confirmed by western blotting, ELISA and spot test. The collected purified protein samples were subjected to SDS-PAGE analysis with protein marker. The desired protein band was found to be of 37 kDa. In spot test the card showed red colored spot on test indicates that our target protein was successfully bound and it is specific for the serum antibodies present in the serum sample. The band found on the membrane was treated with positive HCV at 37 kDa position. The recombinant HCVNS3 protein was specifically interacted with the antibodies present in the serum sample. Recombinant HCVNS3 protein was refolded and purified by metal affinity chromatography and protein band (37 kDa) was confirmed in the native phage. These result suggested that recombinant protein can be extended as a diagnostic reagent for detecting HCV disease.

Key words: Hepatitis C virus · *E.coli* · BL21 (DE) 3C+ · HCVNS3 · ELISA · SDS-PAGE · Western blotting

INTRODUCTION

Hepatitis is classified into five types, Hepatitis A, Hepatitis B, Hepatitis C, Hepatitis D, Hepatitis E. Hepatitis C virus particles consist of a core of genetic material (RNA virus of the family *Flaviviridae*) surrounded by an icosahedral protective layer of protein and encased in a lipid (fatty) envelope of cellular origin. Two viral envelope glycoproteins, E1, E2, are embedded in the lipid envelope [1]. Hepatitis C virus (HCV) infection is the most public chronic blood borne infection in United States [2]. Approximately been reported in the natural history of hepatitis C virus 3% of world population is infected with hepatitis C virus [3, 4]. The risk of vertical transmission is 6% and 25% in mothers observed only to be HCV positive and in those who are HCV/HIV positive respectively [4].Chronic liver disease is the tenth leading cause of death among public in United States [5]. Current estimates of medical and work-loss costs of HCV-related acute and chronic liver disease are >$600 million annually (CDC, unpublished data) and HCV-associated end-stage liver disease is the most frequent indication for liver transplantation among adults. Because most HCV-infected persons are aged 30–49 years, the number of deaths attributable to HCV-related chronic liver disease could increase substantially during the next 10–20 years as this group of infected person reaches ages at which complications from chronic liver disease typically occur. HCV is transmitted primarily through large or repeated direct percutaneous exposures to blood [6]. There are multiple factors and regulatory mechanism operating to modulate the immune responses during hepatitis infection [7].

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Diagnostic of the HCV disease is a major problem. Recombinant proteins and synthetic peptides containing immunodominant epitopes were used as antigens in immunodiagnostic assays, leading to the development of commercially available screening and supplemental assays for anti-HCV immunoglobulin G (IgG) [8,9]. A rapid anti-HCV IgG assay was approved by the Food and Drug Administration (FDA) for clinical use in the United States [10]. These assays, however, cannot identify whether an antibody-positive person has active HCV infection, since anti-HCV IgG may be detectable in persons who have resolved infection and are no longer viremic. Nucleic acid testing (NAT) for the detection of HCV RNA remains the gold standard for diagnosing active HCV infections. However, the laboratory setup for performing NAT methods is expensive. Because of these NAT is not routinely performed in many clinical laboratories. Availability of a serologic assay not based on NAT but indicative of active infection should further facilitate identification of HCV-infected patients and enable referral to care. Chronic hepatitis C virus (HCV) infection which is often a silent disease has resulted in a global epidemic and diagnosis of hepatitis C virus often requires more confirmation with molecular techniques such as the polymerase chain reaction for detection of HCV RNA and protein expression [11,12]. In the present study, the recombinant protein HCVNS3 was expressed using *E.coli* strain BL 21 DE3 C+ and HCVNS3 recombinant protein can be used for diagnosis of HCV by detecting the anti HCV. Then the protein was refolded for improving the solubility and stability.

MATERIALS AND METHODS

HCVNS3 cloned in plasmid DNA of *E.coli* strain DH5α and expression host of *E.coli* strains BL21DE3 C+, BL21DE3, BL23DE3 obtained from Bhat biotech Bangalore, India was used in the present study. Plasmid DNA was isolated from *E.coli* strain DH5α containing HCVNS3 gene by alkaline lysis method [13]. Purity of plasmid DNA was checked using agarose gel electrophoresis. Competent cells of three different expression hosts such as *E.coli* strain BL21DE3 C+, BL21DE3, BL23DE3 were prepared and competency of the cells have been made up by addition of transfer buffer and stored at -80°C [14,15].

The plasmid DNA containing HCVNS3 gene was transferred into different expression hosts of *E.coli* strains and transferred colonies were selected and cultured in LB medium containing required antibiotics. The protein induction was achieved by adding (IPTG) Isopropyl β D thio-galactoside for 2 hours and then pellet was harvested. Pellets were homogenized by the breaking up the cell wall using cracking buffer. The protein was separated from the cell debris by centrifugation and stored at 2-8°C. Extracted proteins were observed through SDS PAGE. Protein was purified by using metal (NI-NTA; Nickel Nitritotriacetic acid) affinity column chromatography [16]. Ten fractions of protein sample were eluted by adding elution buffer and stored at 2-8°C. Then the protein was estimated Bradford's method and the O.D was measured at 595 nm [17]. BSA (0.5mg/ml) was used as a standard. Purified proteins were observed through SDS PAGE [18].

For Confirmation of Protein Expression: Confirmation of protein expression was ended by spot assay, western blotting and indirect ELISA methods. In spot assay extracted protein (antigen) was allowed to interact with specific serum antibody. Proteins resolved by SDS-PAGE were electrophoretically transferred onto a nitrocellulose membrane for western blot analysis [19]. The protein which blotted on the nitrocellulose membrane was incubated with HCV positive and negative serum antibodies for two hours. After two hours, the membrane was washed several times and subsequently treated with PRO-A conjugated with horseradish peroxidase (HRP) for one hour at room temperature. The protein was detected by adding corresponding substrates. In the ELISA test the cut-off value is calculated by multiplying the average absorbance value of the positive control by 0.1 and adding the average absorbance value of the negative control.

Protein Refolding: The *in vitro* refolding of solubilized recombinant HCVNS3 was carried out by pulsatile renaturation in order to increase overall yield of the recombinant HCVNS3 protein. The refolded recombinant protein was subjected to dialysis to remove the β-mercaptoethanol, sucrose, glycerol and urea whose concentration decreased due to the dialysis. Refolded protein was purified metal affinity chromatography by refolding buffer and refolded protein band was confirmed in the native phage [20].

RESULTS

Plasmid DNA containing HCVNS3 gene was isolated from the DH5α, in agarose gel electrophoresis two or more bands of DNA were observed (Fig.1) under UV transilluminator. The plasmid DNA containing HCVNS3 gene was transferred into different expression hosts of

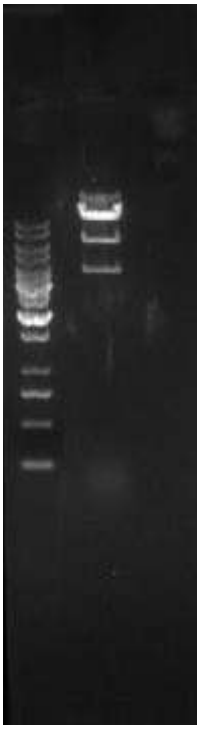

Fig. 1: Isolated Plasmid DNA by agarose gel electrophoresis

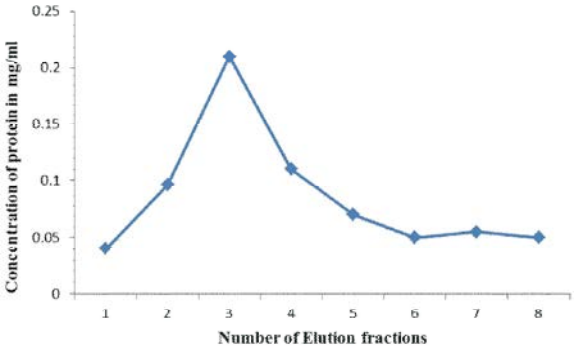

Fig. 2: Protein estimation of HCV NS3 recombinant protein by Bradfoard's method

E.coli strains and the colonies were observed on the plate which indicated successful transformation of the plasmid DNA containing HCVNS3 gene. The protein induction was successfully achieved by adding (IPTG) Isopropyl β D thio-galactoside. Protein extracted from the expression host of the *E.coli* strains and proteins were observed through SDS PAGE. Among the three strains used, BL23DE3 expressed more, hence, showed highly intense band. BL23DE3 was selected for large scale production of HCV NS3. Purified protein showed among 10 fractions, the amount of desired protein was found to be higher in 2nd, 3rd, 4th and 5th fractions. The 3rd fraction was exhibiting highest protein content with

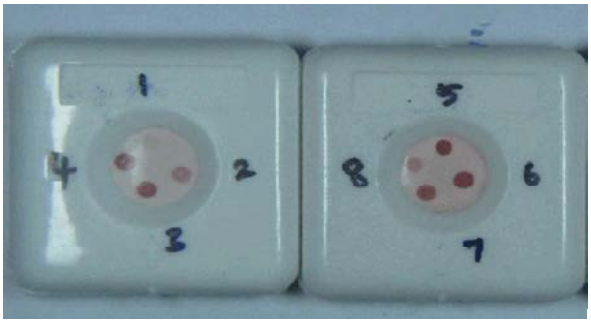

Fig. 3: The elutes analysed by spot test showed in this picture, presence of HCVNS3 in fractions 2, 3,4,5,6 and 7

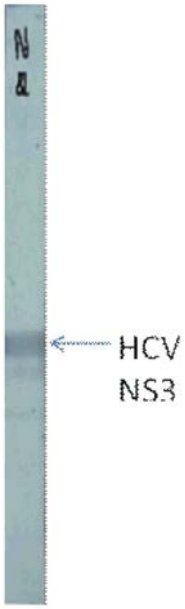

Fig. 4: Purified recombinant protein was confirmed by western blottingL1-Purified recombinant HCVNS3 fraction

0.42mg/ml. (Fig.2). Purified protein samples through affinity chromatography were subjected to SDS-PAGE analysis with protein marker conformed the desired protein band was found to be 37kDa.

Expression protein HCVNS3 was successfully conformed by spot assay, western blotting and Indirect ELISA methods. The spot assay card showed red colored spot on test indicated that our target protein was successfully bound and it is specific for the particular antibody (HCV Ab). The card, which was added with negative serum sample, develops red colored spot at the control and do not develop color at the test, giving the negative result because of absence of specific HCV antibody and this conformed HCVNS3 protein expression (Fig.3). In western blotting the band found on the

membrane was treated with positive HCV serum sample at 37 kDa position (Fig.4). No bands were found on the membrane incubated with negative serum sample. This result indicated HCVNS3 protein was successfully expressed. In ELISA analysis calculation showed the cut-off value 0.165. The positive control average absorbance 1.011 and negative control of average absorbance 0.0642. The sample with absorbance value less than the cut off value were consider non-reactive and were considered negative for antibody to HCV. This result also supported to HCVNS3 protein expression and conformed to detection of HCV antibody in the infected persons. Expressed protein was successfully refolded and protein band (37 kDa) conformed in the native PAGE.

DISCUSSION

In this study the protein genome was cloned in to PET plasmid vector and gene was expressed successfully. The prokaryotic expression strains that efficiently expressed recombinant of HCV gene and protein were obtained successfully [21]. This is in conformity with previous reports of HCV that core gene sequence was cloned to the plasmid vector pET-32a and gene was expressed [22]. The human MBL-CLR gene was cloned pET32a and gene was expressed [23]. HCVNS3 gene was transformed into *E.coli*BL21 (DE) 3C+ strain and HCVNS3 protein was obtained. In earlier study Cai *et al*. [24] reported the human MBL-CLR gene was transformed into other *E.coli* BL21 (DE3) strains. Expressed protein was purified by Immobilized Metal Affinity Chromatography (IMAC) and identified by spot assay, western blot and indirect enzyme-linked immunosorbent assay (ELISA). In western blotting the protein was detected by adding corresponding Substrates. The band found on the membrane was treated with positive HCV serum sample at 37kDa position. In indirect ELISA analysis calculation showed the cut-off value 0.165. The positive control average absorbance 1.011 and negative control of average absorbance 0.0642. This is in conformity with previous report [24] wherein the expressed product was purified by Immobilized Metal Affinity Chromatography (IMAC) and western blot and indirect (ELISA) using the antibody from BALB/c mice immunized with the recombinant human MBL protein. The spot assay card showed red colored spot on test indicates that our target protein was successfully bound and it is specific for the particular antibody (HCV Ab) [25].

For expression of the gene of interest to deliver T7 RNA polymerase to the cells by either including expression in of the polymerase using the inducer IPTG (Isopropyl β-D-1-thiogalactopyranoside) then T7 promoters and transcribe the gene of interest. After induction the protein was extracted by mechanical homogenization. In induction, PMSF play a pivotal role because it acts as a protease inhibitor. In present study our target protein did not degraded because of PMSF. It is confirmed by different immunological method such as spot test, western blotting and ELISA. In previous studies others have done the development of anti HCV assay using the NS3 genes from genotypes commonly found in Thailand which were amplified and cloned into a bacterial expression system. These recombinant NS3 proteins were immunogenic and reacted with plasma samples of Thai patients infected with various HCV genotypes. The NS3 proteins from the genotypes could react with 3 plasma samples from HCV infected blood donors which could not bind to the NS3 protein in the commercial HCV immunoblotting kit using antigen from HCV genotype [26]. Another studies showing that HCV NS4B protein regulates STAT3, MMP-2 and Bcl-2 in the human liver cell line Huh7. STAT3 is essential for NS4B-induced gene expression, as knockdown of STAT3 prevents NS4B from activating MMP-2 and Bcl-2 [27].

Nonstructural 3 protein (NS3) of hepatitis c virus is one of the antigens commonly used in diagnostic assays for antibody to hepatitis c virus however immune response to the NS3 protein from one genotype may not cross react with that from other genotypes. We have revealed a novel mechanism in which we produced diagnostic kit in *in vitro* for anti HCV disease. The present study supports the recombinant protein used to diagnose HCV disease against the appropriate HCV antigens from HCV affected patients successfully it should be derived from the virus genotypes and our target protein which was developed in this study it will be acting as a specific antibody for HCV positive patients. The present study is simple and reliable method for diagnosis of HCV disease. In conclusion, our results revealed that the HCVNS3 recombinant protein can be used for diagnosis of HCV by detecting the anti HCV.

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