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Production of hepatitis B surface antigen (HBsAg) from *Escherichia coli* for development of diagnostic kit

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Abstract
Hepatitis B virus (HBV) infection is a worldwide health problem, which can lead to severe liver disease mainly hepatocellular carcinoma and cirrhosis. The present investigation lays emphasis on expression of *HBsAg* gene in *E. coli.* The confirmed recombinant pET28a+*HBsAg* clone was transformed into *E. coli* strain BL21 (DE3) *PlysS* cells. The positive clones were used for protein expression studies and induction parameters *viz.,* IPTG concentration, temperature, induction time and pH of the medium were standardized to produce optimum HBsAg protein yield. The highest protein expression was recorded in clone 2 when it was subjected to 1.5 µM IPTG (384 µg mL^{-1}), pH at 8 (378 µg mL^{-1}) and induction time for 8 h (360 µg mL^{-1}) with 35 °C induction temperature. The cells pelleted was lysed by sonication and protein purification was done using fused *His-tag* via Ni-NTA agarose-based method. The purified protein from clone 2 was subjected for 12 % SDS-PAGE analysis. The presence of ~27 kDa protein band confirmed the expression of HBsAg protein in *E. coli* which was further confirmed through western blot.

Keywords: HBsAg, *E.coli*, Western Blot, IPTG, SDS-PAGE

Introduction
Hepatitis B is one of the major global health problems and potentially life threatening liver infection caused by the hepatitis B virus. This infection causes chronic liver disease and can lead to cirrhosis and hepatocellular carcinoma, resulting in over a million deaths worldwide each year. Globally two billion people are infected with HBV, amidst which 400 million people are chronically infected. Every year 10-30 million new cases of infection are registered. As per World Health Organization (WHO) datasheet, more than 780,000 people die every year due to acute or chronic consequences of hepatitis B (WHO, 2014) [25]. In India there are about 40 million Hepatitis B infected patients, ranking second only to China, and most people with chronic Hepatitis B or C are unaware of their infection, putting them at serious risk of developing cirrhosis or liver cancer which are life threatening (The Economic Times, 2014) [20]. Hepatitis B infection can be prevented effectively upon the development of efficient diagnostic reagent and preventive vaccine.

Immunization with hepatitis B vaccine is the most effective means of preventing HBV infection and its consequences (Gitlin, 1997) [8]. This requires large amount of HBsAg. Presently the recombinant antigen is produced in yeast (Valenzuela, Medina, Rutter, Ammerer, & Hall, 1982) [23] and mammalian cells (Chinese hamster ovary cells) replacing human serum-derived formulations (Sitrin, Wampler, & Ellis, 1993) [18].

Diagnosis of person with HBV infection is the most critical step before taking the person to effective treatment strategies (Chou, Easterbrook & Hellard, 2017) [5]. The conventional antigen of diagnostic reagent for immunoassay of HBsAb is mainly obtained from the plasma of high titer HBV carriers. With the popularization of HBV vaccination, it is difficult to find such infected individuals to obtain the sera containing high titer of HBsAg. Variety of cell systems are able to express recombinant HBsAg suitable for immunization but the glycosylation of the recombinant HBsAg occurring in 146 Asn within the sequence of 'a' common epitope, affects the immunore activity of the product, resulting in the hiding of the epitope. *E. coli* cell system lacks the glycosylation process. Hence *E. coli* expressed HBsAg can be a potential substitute.

E. coli is one of the most attractive bacterial hosts for the production of recombinant proteins (Peternel & Komel, 2011) [15]. Compared with other established and emerging expression systems, *E. coli* offers several advantages, including growth on inexpensive carbon sources,

rapid biomass accumulation, amenability to high cell-density fermentations, simple process scale-up and high protein yields (Assenberg, Wan, Geisse & Mayr, 2013) [1]. Because of its long history as a model system, *E.coli* genetics is very well characterized and many tools have been developed for chromosome engineering and to facilitate gene cloning and expression (Baneyx & Mujacic, 2004) [2].

Materials and Methods

Bacterial strains, plasmids and growth conditions

E. coli DH5α or BL21 (DE3)

The *E. coli* strains *DH5α* and BL21 (DE3) *PlysS* were used for the cloning and expression experiments. The expression vector pET-28a (+) used in this study was purchased from Novogen, USA. The *E. coli* was grown in Luria Bertini (LB) broth at 37 °C with shaking 200rpm or on LB plates solidified with 1.5 w/v agar. As per the requirement LB broth and agar plates were supplemented with kanamycin in a final concentration of 50 mg L^{-1}. The cultures grown on LB agar plates were stored at 4 °C for short term preservation with antibiotics. For long term storage, cultures were grown until mid-log phase in low salt Luria Broth with antibiotics were preserved in 50 % Glycerol, at -80 °C. The *Hepatitis B* gene was cloned and maintained in pUC_57 cloning vector. Bacterial expression vector pET-28a (+) was selected to express the gene encoding for HBsAg protein in *E. coli*. It contains T7 promoter driven expression of recombinant proteins with the addition of a 19 amino acid N-terminal fusion tag containing a 10X His tag followed by a thrombin protease cleavage site. Two stop codons are included in the vector at the C- terminal cloning site with kanamycin resistance (*nptII*) gene as selection marker and poly linker sequence with multiple cloning sites (Fig. 1.). This vector replicates in *E. coli* through its pBR322 origin of replication. Plasmid DNA was isolated and purified using QIA prep Miniprep Kit (Qiagen, USA) according to the manufacturer protocol.

Primer designing for the cloning of HBsAg gene

DNA primers for PCR amplification of *HBsAg* gene were designed to clone the desired fragment in the *NcoI* and *NotI* sites of pET-28a (+) due to the absence of these two sites within the sequence of *HBsAg* gene. Primer *NcoI*_F (5'-*CATG*CCATGGAGAATACAACTTCAGGTTTCTTGG GT-3', *NcoI* site underlined contained the first 26 nucleotides of the *HBsAg* gene and four extra nucleotides were added upstream of the *NcoI* site to increase the efficiency of the cleavage by the restriction enzyme. Primer His::Stop_*NotI*_R (5'- *ATAAGAAT*GCGGCCGCTTATTAGTGATGATG ATGGT - 3'; *NotI* site underlined) contained 20 nucleotides of the reverse stand of the end of the HBsAg fused His tag. An additional eight nucleotide were added to increase the efficiency of the cleavage by the restriction enzyme.

Cloning of HBsAg gene into bacterial expression vector pET-28a (+)

The *HBsAg* gene in the pUC_57 vector was used as a template and was amplified with *Pfu* polymerase using a pair of gene specific primers HBsAg *NcoI* forward primer and HBsAg *Not I* reverse primer in 50 µL reaction mixture. The programme followed was; each cycle consisted of a denaturation phase of 30 sec. at 95 °C, followed by annealing at melting temperature (Tm) of primer for 30 sec. and primer extension at 72°C for 1min/kb, depending on the length of the fragment to be amplified. The unfinished extensions were allowed to completion in the final extension phase at 72 °C for 5 min. The PCR product was resolved on 1 percent agarose gel and the 714 bp *HBsAg* gene was eluted from the agarose using Qiagen gel elution kit. The purified PCR product (*HBsAg* gene) was restriction digested in 50µL reaction mixture (2µg DNA, *NcoI* and *NotI*).

The bacterial expression vector pET-28a (+) was restriction digested with *NcoI* and *NotI*. The pET-28a (+) vector carries an N-terminal 6X His tag. The *HBsAg* gene was cloned to the upstream of N-terminal coding portion of pET-28a (+) vector (Fig. 1.).

The digested PCR product was ligated into digested pET-28a (+) vector in 1:3 ratio using T4 DNA ligase enzyme. The ligated mixture was transformed into *E.coli* DH5α competent cells by electroporation as described by Sambrook & Russel (2001) [17]. The recombinant plasmid thus obtained was designated as pET-28a (+) *HBsAg*. Transformants were screened using 50 mg L^{-1} kanamycin selection.

To confirm the recombinant clones, the colony PCR was done using a pair of gene specific primers. Further, these clones were also confirmed by restriction digestion with *NcoI* and *NotI* and confirmed on 0.8 percent agarose gel.

HBsAg protein expression

The *E. coli* BL21 competent cells were transformed with confirmed recombinant clone as described by Sambrook & Russel (2001) [17] and screened using 50 mg L^{-1} kanamycin selection. The single colony was cultured by inoculating it in 10 mL LB broth containing kanamycin (50 mg L^{-1}) and incubated at 37 °C overnight. From the overnight grown culture 1mL was inoculated into 100 mL LB broth to make 1:100 dilution and incubated at 37 °C until the OD reached 0.4-0.6. At this point, different parameters were used to optimize HBsAg protein expression in *E. coli* i.e., different IPTG concentration (0.5, 1.0, 1.5, 2.0, and 2.5 µM), temperature (30 °C, 35 °C and 40 °C), time interval (8,16 and 18 h) and different pH (5, 6, 7 and 8) in the LB medium to standardize the optimum parameters for protein expression. The cells were harvested by centrifugation at 10,000 rpm for 15 min at 4 °C. From each set of experiments protein samples were isolated and purified.

HBsAg protein extraction and purification

The harvested cells were suspended in lysis buffer 1X PBS, 10mM PMSF and 1mg mL^{-1} lysozyme and incubated on ice for 15 min. The cells were lysed by sonication at 200-300 W for 15 min with alternative 10 sec sonication and 10 sec rest on ice. After sonication, cells were pelleted out at 10 000 rpm for 10 min at 4 °C. The supernatant was collected and subjected for purification using Ni-NTA agarose column. The column of Ni-NTA agarose was prepared, equilibrated and activated with 1X PBS. The column was loaded with protein sample and flow through was collected.

The column was washed with 1X PBS to remove unbound foreign or host proteins. Then the HBsAg protein was eluted from the column using different concentration of imidazole (75,100, 150 and 250 mM). The concentration of purified HBsAg protein isolated from *E. coli* BL21 cells was measured by using Bradford protein assay (Bradford, 1976).

SDS-PAGE and Western blot analysis

The purified HBsAg protein was analyzed by SDS-PAGE as per the standard procedure of Lamelli (1970) [12]. The protein

samples were prepared by denaturing the proteins by boiling for 15 min with SDS loading dye. The protein samples were run on 12 percent acrylamide gel along with standard protein marker. Further the expression of the HBsAg protein was confirmed by western blot as per the standard protocol of Towbin, Staehelin, & Gordon, 1979. [22] The protein ladder (Pure-gene, Genetix) was used as marker. The protein bands were transferred from SDS-PAGE gel to activated PVDF membrane by electro blotting. The unspecific protein binding sites were blocked with blocking buffer containing 2 percent skimmed milk and incubated at 37 °C for 1 h. Then the membrane was washed with 2 percent PBST and incubated for 1 h at 37°C. The membrane was probed consecutively with a primary antibody (mouse anti-hepatitis B surface antigen, Invitrogen, 1:1000 dilution) and secondary antibody (goat anti-mouse IgG horseradish peroxidase conjugate, Invitrogen, 1: 20,000 dilution). The binding of the antibody was visualized using goat antibody antimouse IgG conjugate with peroxidase enzyme using DAB substrate.

Statistical analysis

Each experiment was carried out with two replications by following Complete Randomized Design (CRD). Statistical analysis was carried out by applying *f test* or analysis of variance (ANOVA) using Software Windostat ver. 8.5.

Result and Discussion

Generation of recombinant clones of pET- 28a (+) HBsAg

The *E. coli* strain DH5α was transformed with the ligation mixture and the recombinants were grown on kanamycin selection media. The colony PCR resulted in the amplification of *HBsAg* gene, amplicon size ~714bp (Fig. 2.). Further, restriction digestion of recombinant clones with *NotI* and *NcoI* restriction enzymes resulted in linearisation of pET-28a (+) vector (~5.23 Kb) and the release of the gene (~714bp) (Fig. 3). This confirms the proper integration of the gene into the expression vector. Thereafter, clones were designated as recombinant clones.

Effect of IPTG concentration on recombinant HBsAg protein expression

In the present study, IPTG concentration was examined from 0.5, 1.0, 1.5, 2.0 and 2.5 µM at 30 °C, 35 °C and 40 °C for HBsAg protein expression. The clone 2 produced higher concentration of *HBsAg* protein. The clones induced with 1.5 µM IPTG concentration kept at 35 °C and 30 °C recorded the highest protein expression of 384 µg mL^{-1} and 353 µg mL^{-1} respectively and the least expression was observed in clones induced by 2.5 µM IPTG concentration (Fig 4). We found that the protein concentration decreased with increase in IPTG concentration of 1.5 µM IPTG onwards. It may be due to higher concentration of IPTG, which decreased OD_{600} of host cells after 4 h of IPTG addition as found in HBsAg polypetide fused with HCV epitopes (Gholizadeh *et al*., 2015) [7]. Elghanam, Attia, Shoeb, & Hashem, (2012) [6] reported a protein yield of 140 µg per liter of culture at 1.0 µM IPTG concentration, in contrast we found ~3.8 mg L^{-1} of culture and higher yield was may be because of partial purification of the protein sample.

Effect of induction temperatures and time on recombinant HBsAg protein expression

E. coli can grow and divide at a wide range of pressure (1-400 atm) and temperature (23-40 °C) (Kumar & Libchaber, 2013) [10]. The temperature is known to influence the expression of recombinant proteins in *E. coli.* The optimum temperature for *E. coli* growth is 37 °C but induction for recombinant protein expression are carried out at low temperature to retain the solubility, proper folding of the expressed protein. Therefore, the effect of temperature on the expression of *HBsAg* protein was studied in the present study. We found that among different induction temperatures used on an average irrespective of IPTG concentrations induction at 35°C resulted in protein yield of 360 µg mL^{-1} (Fig 5) and there was significant decrease in protein yield to 239 µg mL^{-1} and 126 µg mL^{-1} at both 30 °C and 40 °C respectively in clone 2, similar results were found in other clones (Data not shown) The decrease in HBsAg protein yield at 40 °C was may be due to relatively higher temperature.

Lower IPTG concentration, lower temperature with longer induction time leads to the slower expression rate of the protein which allows better folding of the protein but reduces yield. The higher IPTG concentration with shorter induction time at low temperature may allow properly folded protein expression which leads to increased yields of protein (Tolia & Joshua- Tor, 2006) [21]. In the present study we found maximum HBsAg protein expression of 360 µg mL^{-1} when induction was done for 8 h and decreased with the increase in time this may be due to change in pH of growth medium because of longer incubation time after induction. Sivashanmugam *et al*. (2009) [19] found that, pH of the expression medium drops from the starting pH 7.2-6.01 after 36 h resulting in plasmid loss and significant reduction of the protein yield.

Effect of different media pH and temperature on recombinant HBsAg protein expression

Medium pH is one of the crucial factors for the expression of HBsAg protein that was studied. Protein expression was done at different pH (5, 6, 7 and 8) of LB media and induction at different temperature. The maximum HBsAg protein expression of 212 µg $mL^{-1}$was recorded at media pH 8 followed by 135 µg mL^{-1} at pH 7, when induction was done at 30 °C. In similar way when induction was done at 35 °C with different pH of media the maximum HBsAg protein expression of 378 µg mL^{-1} was recorded followed by 220 µg mL^{-1} at pH 7. When induction was done at 40 °C with different IPTG concentrations there was no significant HBsAg protein production (Fig. 6). It was found that the HBsAg protein expression was less at acidic pH 5 and 6 and highest in medium with pH 8 which was 378 µg mL^{-1} and 212 µg mL^{-1} at 35 °C and 30 °C respectively in clone 8. Similar results were reported by Wang *et al*. (2014) [24] where they expressed GFP, GST, CYP (*cytochrome P450 monooxygenase*) in *E. coli* and reported that alkaline pH (7.5-8.5) did not inhibit protein expression. The BL21 cells inoculated in the medium with pH 5 reported very slow growth rate. This may be due to decrease in pH in the medium which may cause stress to the bacterial cells, resulting in plasmid loss and reduction in the protein yield. The manipulation of pH has an impact on protein solubility, yield and physiochemical characteristics. Alkaline pH is shown to increase the solubility of the protein and has larger buffering capability (Sivashanmugam *et al*., 2009) [19].

Purification of histidine tag fused recombinant HBsAg protein

The Histidine Tag fused HBsAg protein expressed in *E. coli*

BL21 cells was extracted and purified by metal affinity chromatography. The HBsAg protein samples were passed through Ni-NTA agarose column. Different imidazole concentrations (75, 100, 150 and 250 mM) were used to elute the HBsAg protein from the column. The optimum elution of HBsAg protein from the Ni-NTA agarose column was obtained when 75 mM imidazole was used. Yu *et al*. (2010) [26] expressed *HBv polymerase* enzyme as a fusion protein with 6x His tag which helped in the purification of *HBv* enzyme protein and maximum purified protein was recovered when they used 50 and 75 mM imidazol buffer. Similarly, Elghanam, Attia, Shoeb, & Hashem, (2012) [6] expressed HBsAg (s) fusion protein with Glutathione-s-transferase tag (GST) and purified by using glutathion sepharose beads slurry. The expression and subsequent purification of recombinant proteins are widely employed in biochemical studies.

A powerful purification method involves the use of peptide affinity tags, which are fused to the protein of interest and used to expedite protein purification via affinity chromatography (Bornhorst & Falke, 2000) [3]. Hence, immobilized metal-affinity chromatography (IMAC) can be used to purify recombinant proteins containing a short affinity tag consisting of polyhistidine residues, theoredoxin residues etc., and the Ni NTA passed protein could keep its function.

Molecular characterization of recombinant HBsAg protein

SDS-PAGE analysis of recombinant HBsAg protein

Expressed HBsAg was confirmed by SDS-PAGE analysis along with protein ladder, which revealed that the protein size of ~27 kDa protein compared to reported size of 24 kDa HBsAg (Fig 7). The increase in size was may be due to 10x His tag fused to the protein. Pumpen *et al*. (1984) [16] reported the 24 kDa HBsAg protein, since the expressed protein didn't carry 10x His tag. Whereas, Elghanam, Attia, Shoeb, & Hashem, (2012) [6] reported higher protein size of ~52 kDa. High molecular size of the protein was due to GST tag fused to the HBsAg.

Western blot analysis of purified recombinant HBsAg protein

The purified protein was further confirmed by western blot analysis. The purified and crude HBsAg protein developed colour after incubation with primary antibody followed by secondary antibody conjugated to horse radish peroxidase enzyme and finally with DAB substrate (Fig 8). It confirmed the presence of HBsAg protein. Similarly, Kumar (2015) [11] Jayanth *et al.* (2016) [9] and Madhusudhan *et al.* (2017) [13] confirmed the expression of HBsAg protein, GAD 65 protein and rabies glycoproteins of rabies virus using western blot analysis respectively.

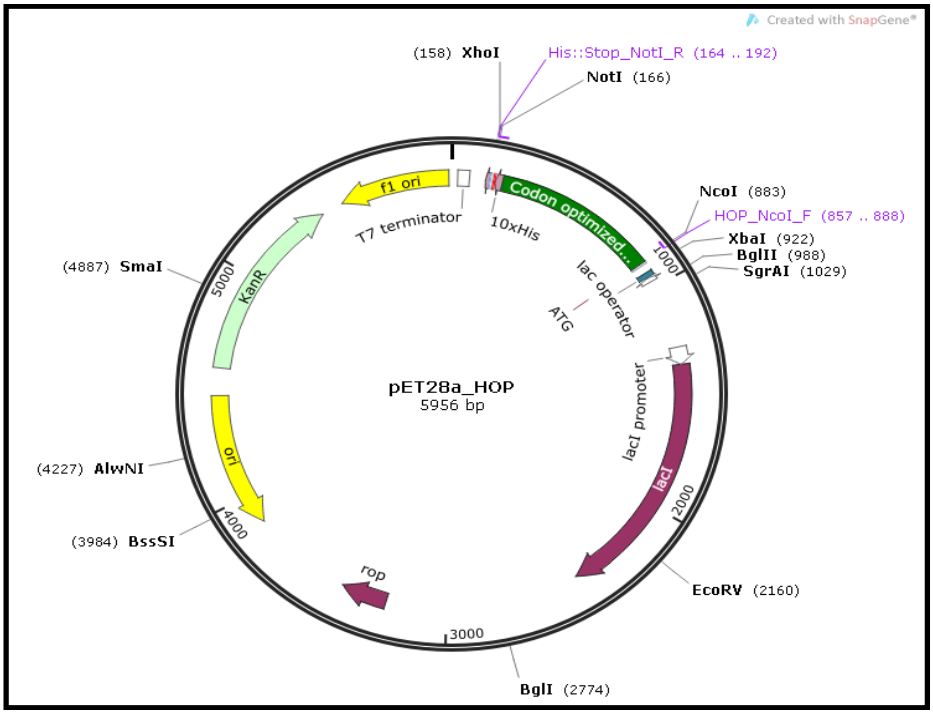

Fig 1: Construct map of pET28a (+) *HBsAg*

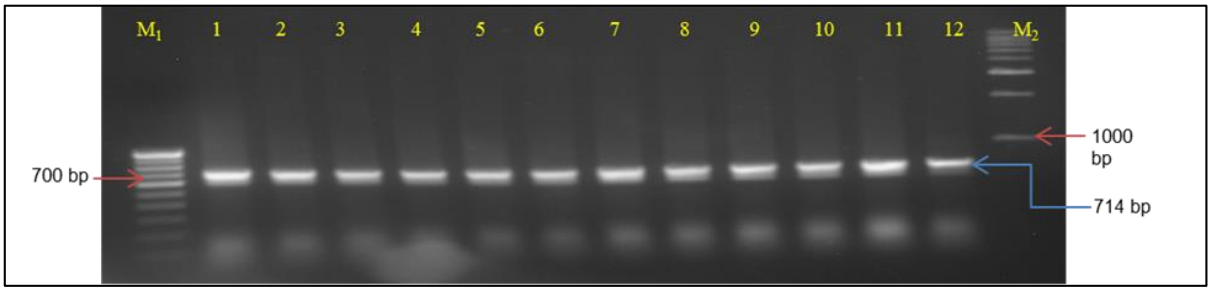

Fig 2: PCR confirmation of recombinant HBsAg *E. coli* DH5α clones with gene specific primers M_1: 100 bp DNA ladder, Lane 1: pHB118 vector (positive control), Lane 2- 12: PCR amplicon of recombinant *HBsAg* clones, M_2: 1000 bp DNA ladder

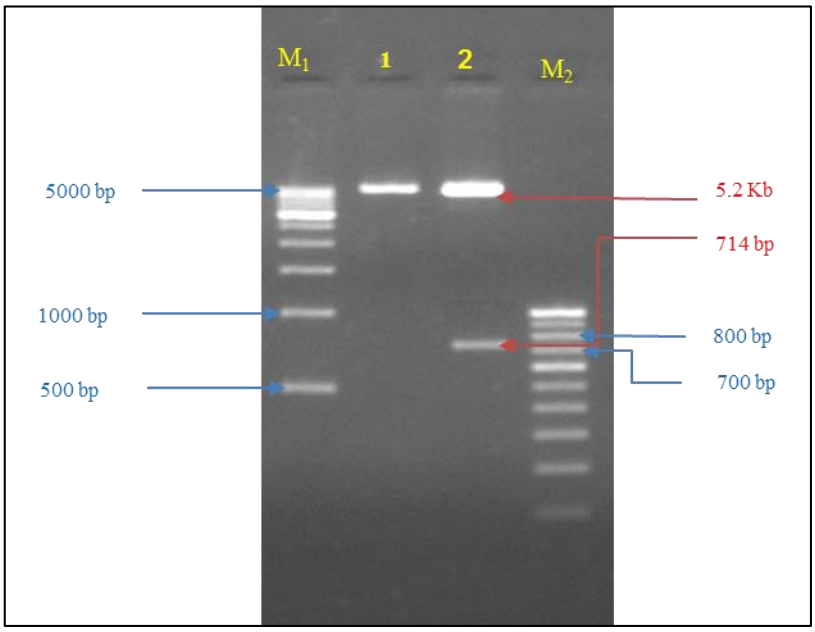

Fig 3: Agarose gel electrophoresis for validation of pET28a+*HBsAg* clone via restriction enzyme analysis M_1: 500 bp DNA Ladder, Lane 1: Restriction digested plasmid DNA vector pET (+) 28a with *Nco I*, Lane 2: Restriction digested recombinant construct pET28a+*HBsAg Nco I* and *Not* M_2: 100 bp DNA Ladder

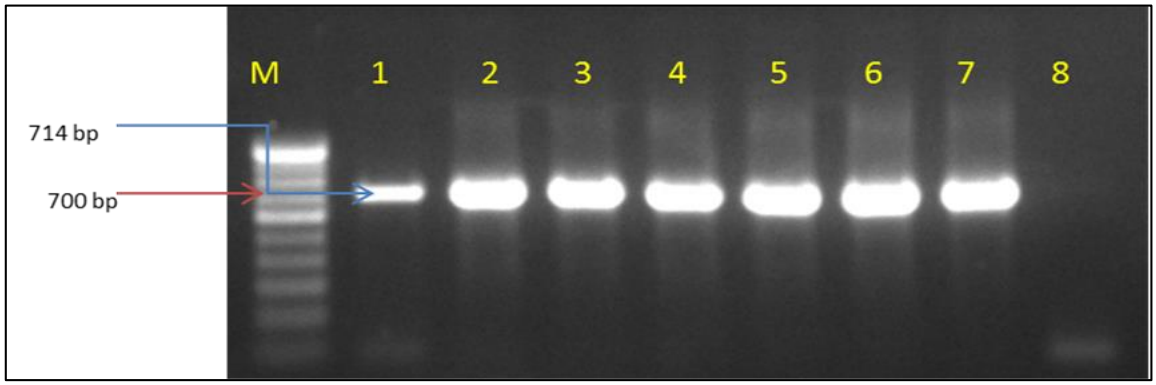

Fig 4: PCR confirmation of recombinant pET28a+*HBsAg* clones with gene specific primers M: 100 bp DNA ladder, Lane 1: pUC57 vector (positive control), Lane 2-7: PCR amplicon of recombinant pET28a+*HBsAg* clones, Lane 8: Negative control

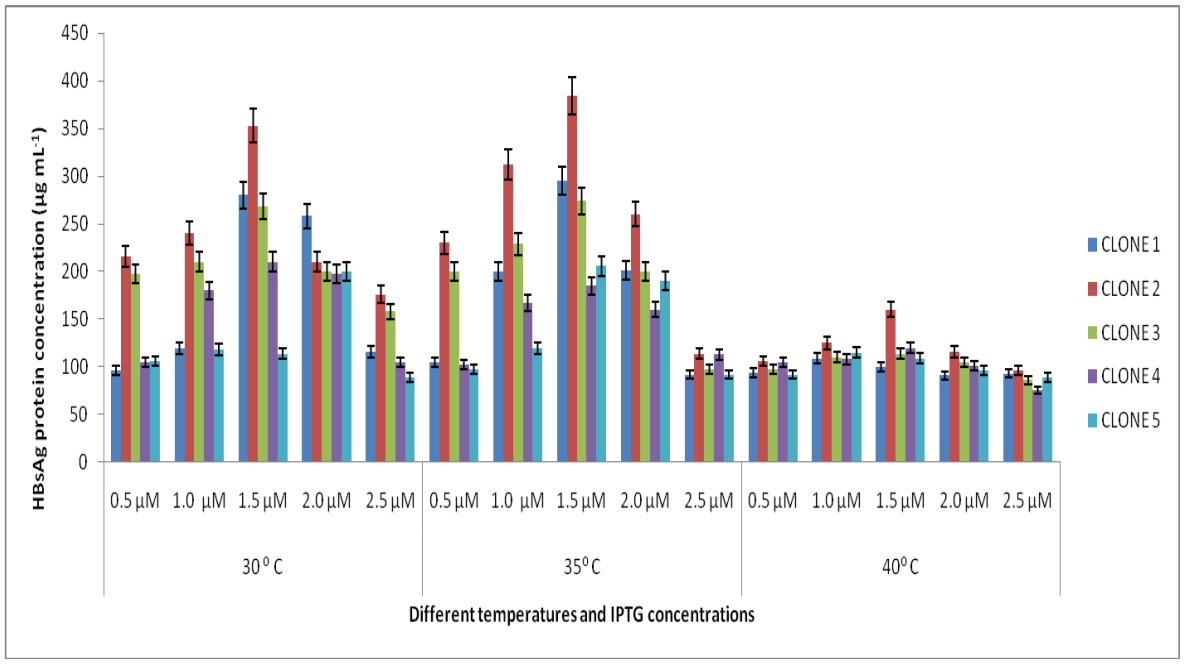

Fig 5: Effect of different temperatures and IPTG concentrations on HBsAg protein expression in *E. coli*

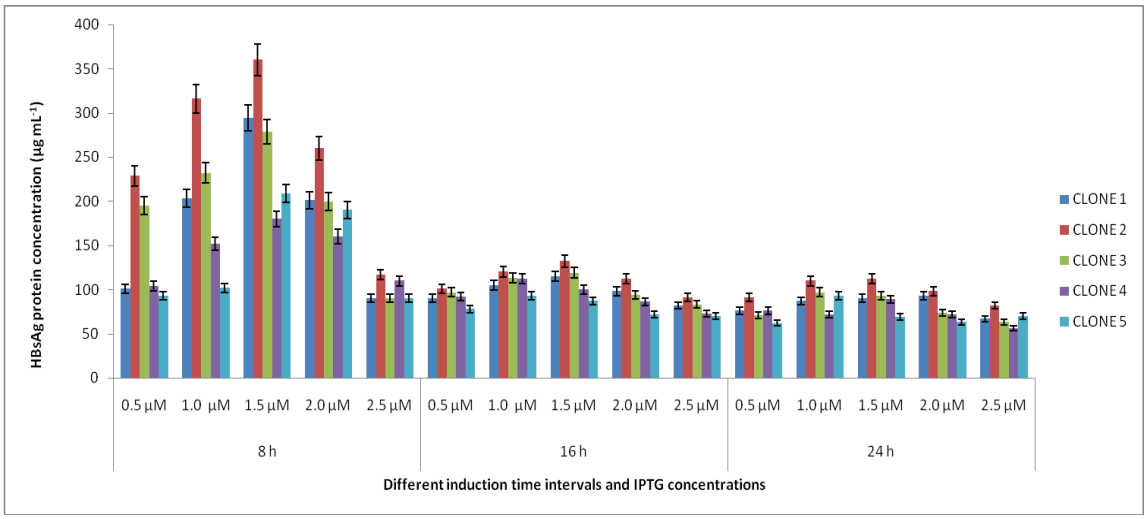

Fig 6: Effect of different induction time and IPTG concentrations on HBsAg protein expression in *E. coli* at 35 °C

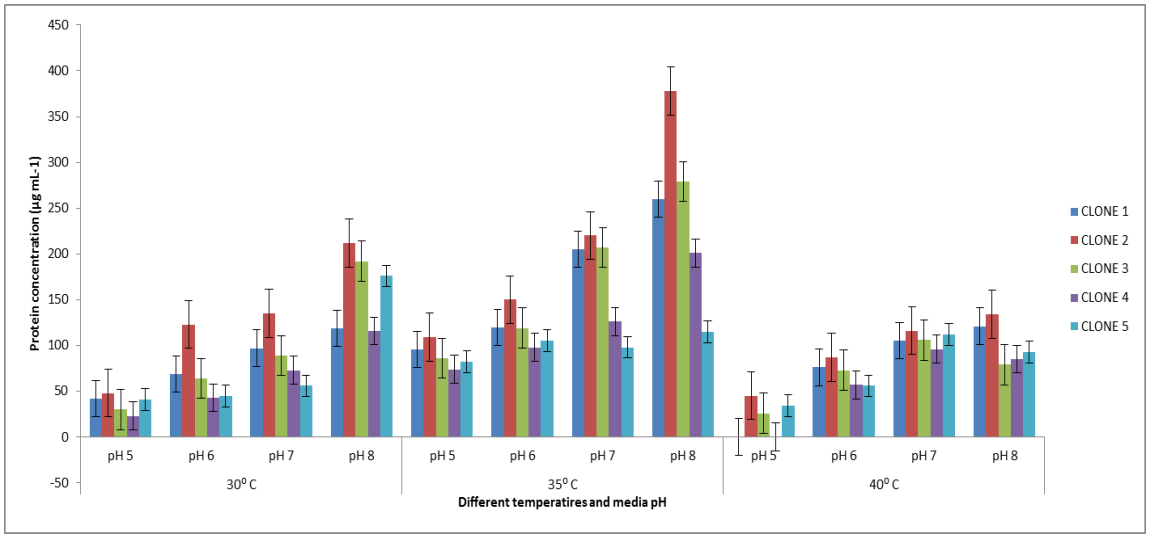

Fig 7: Effect of different temperatures and media pH on HBsAg protein expression in *E. coli* at 1.5 µM IPTG concentration

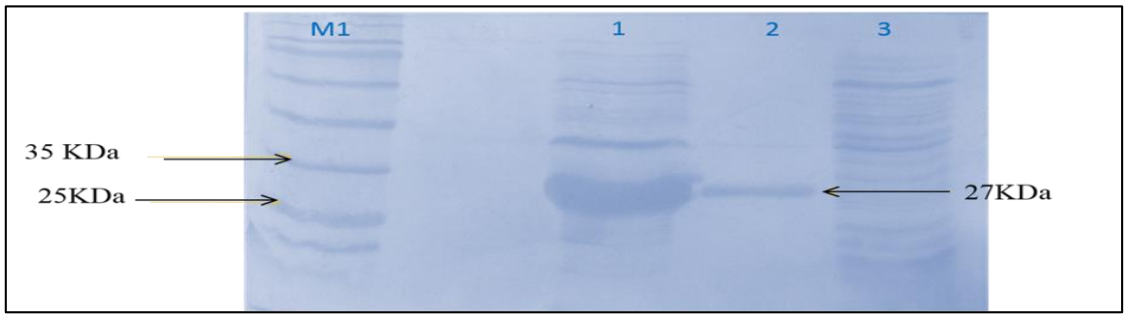

Fig 8: SDS-PAGE analysis of expressed HBsAg recombinant protein. M1: Protein molecular marker, Lane1: Induced total cellular protein, Lane 2: Column Purified Protein (Fused Protein 27kDa), Lane 3: Uninduced total cellular protein

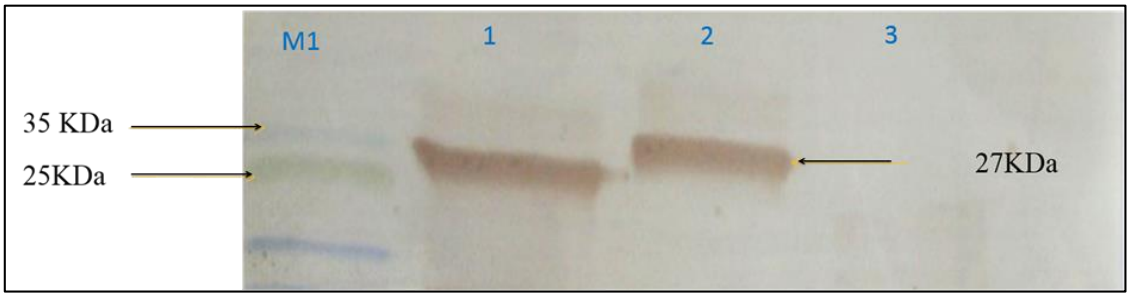

Fig 9: Western blot analysis of expressed HBsAg recombinant protein. M1: Protein Molecular marker, Lane1: Commercial Vaccine, Lane 2: Column Purified, Protein (Fused Protein 27kDa), Lane 3: Uninduced total cellular protein

Conclusion

In conclusion, the different parameters were standardized for the expression of HBsAg protein in *E. coli*. Fused recombinant HBsAg protein expressed in *E. coli* along with simple low cost purification protocol can be aimed at developing a less expensive diagnostic kit for screening of Hepatitis B.

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