European Review for Medical and Pharmacological Sciences 2023; 27: 11734-11745

Cloning and periplasmic soluble expression of hepatitis B surface antigen gene in *Escherichia coli*

M. KHAN[1], I. KHAN[2], I. TAUSEEF[1], K.S. HALEEM[1], T.A. SHAH[3], S. NAZ[4], N. NAZIR[4], T. AZIZ[5], H.A. THAMER[6]

[1]Department of Microbiology, Hazara University, Garden Campus, Mansehra, Pakistan
[2]Department of Microbiology, Abbottabad University of Science & Technology, Havelian, Pakistan
[3]National Institute for Biotechnology and Genetic Engineering, Faisalabad, Punjab, Pakistan
[4]Department of Biochemistry, University of Malakand, Chakdara, Pakistan
[5]Department of Agriculture, University of Ioannina, Arta, Greece
[6]Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia

Abstract. – OBJECTIVE: The objective of this study was to clone and express the hepatitis B surface antigen gene (HBsAg) in *Escherichia coli (E. coli)*, thereby aiming to develop potential local therapeutics for combating Hepatitis B virus (HBV) infection in the Pakistani community by producing HBsAg in *E. coli*.

MATERIALS AND METHODS: Blood serum samples were collected from hepatitis B-infected patients, and their genomic DNA was extracted. Real-time and nested polymerase chain reaction (PCR) was performed to amplify the HBsAg gene. The gene of interest was cloned into the pET20b expression vector and transformed into *E. coli* BL21 (DE3) using Isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. The gene's precise size was confirmed with gene-specific external and internal primers (681 bp and 400 bp, respectively).

RESULTS: The HBsAg gene was successfully sequenced and submitted to GenBank, exhibiting 98% homology with targeted HBV sequences worldwide. The expression of HBsAg protein was confirmed through silver staining, Coomassie staining, western blot, and dot blot analysis.

CONCLUSIONS: The expressed protein clones are now available for further development as a local recombinant DNA vaccine to prevent hepatitis B viral infection in the local community.

Key Words:
E. coli, HBsAg, Cloning, *pET20b-HBsAg* expression.

Introduction

Hepatitis B virus (HBV) is a non-cytopathic DNA virus that belongs to the Hepadnaviridae family, recognized for its hepatotoxic effects. It is responsible for causing chronic liver infections worldwide, leading to severe health consequences such as hepatocellular carcinoma and liver cirrhosis[1]. The impact of HBV infection is substantial, affecting one-third of the world's population, approximately two billion people. Among these, 400 million individuals suffer from chronic infection, and an estimated 10-30 million new cases are reported annually[2]. In 2015, the World Health Organization (WHO) estimated that 257 million people had developed chronic HBV infection, resulting in 1.34 million deaths attributed to liver cirrhosis and hepatocellular carcinoma[3]. The epidemic caused by HBV predominantly affects the African and Western Pacific regions[4].

In Pakistan, HBV infection remains a significant public health concern, with an estimated incidence of nine million infections and a high prevalence rate[5]. This prevalence can be attributed to factors such as poor hygiene practices, inadequate healthcare facilities, and limited awareness regarding disease transmission[6]. HBV exhibits genetic diversity, with nine serotypes and ten genotypes (A-J) distributed across different geographical regions[7]. Studies[8] conducted in Pakistan have reported a predominance of genotype "D," accounting for 63.71% of cases.

The hepatitis B surface antigen (*HBsAg*) gene encodes the outer surface protein of HBV, which plays a crucial role in binding to and entering liver cells[9]. Detection of this gene serves as a clinical marker for acute or chronic viral infection and helps determine the prevalence of HBV infection[10]. The immune response in HBV infection

Corresponding Author: Tariq Aziz, MD; e-mail: iwockd@gmail.com

targets virus-specific proteins for viral clearance, but HBV S mutations evade immune detection. This evasion can lead to chronic liver disease and potentially progress to liver failure and carcinoma[11]. Various expression hosts, including yeasts, bacteria, plant cells, insects, mammalian cells, and *baculovirus*, are commonly used for *HBsAg* gene expression[12]. However, *Escherichia coli* (*E. coli*) and methylotrophic yeast, such as *Pichia pastoris*, are frequently utilized. Among these, *E. coli* is the widely preferred host due to its ability to produce high copy numbers of the gene of interest[13,14].

Efforts have been made to express the *HBsAg* gene in *E. coli* as a potential strategy for developing oral, plasma-based, or recombinant vaccines against hepatitis B. The recombinant proteins produced in *E. coli* can serve as soluble and functional products for research purposes and protein production in various industries[15,16]. The first-generation vaccines for hepatitis B, derived from plasma, were made available in 1982[17]. The second-generation hepatitis B vaccines were developed in 1986 through the transfection of HBV DNA into yeast. They demonstrated efficacy and safety in preventing HBV infection[18].

By incorporating (Pre-S1/Pre-S2/S epitopes) sequences through cloning in mammalian cells, highly immunogenic vaccines have been developed, eliciting a robust immune response against hepatitis B at lower doses compared to yeast-derived vaccines[19,20]. Clinical trials of HBV vaccines have demonstrated that when co-administered with oral lamivudine treatment, they can effectively inhibit HBV replication in patients with ongoing HBV infection and promote anti-HB seroconversion[19]. These findings underscore the substantial potential of these vaccines in contributing to the management and control of hepatitis B infection.

The primary objective of this project was to clone and express the hepatitis B virus *HBsAg* gene in *E. coli.* The ultimate aim was to produce *HBsAg* in *E. coli* at a local level for treatment of HBV infection, with a primary focus on the Pakistani community.

Materials and Methods

Study Area and Duration

This study was conducted at the Molecular Diagnostic Laboratory of the Centre for Applied Molecular Biology (CAMB), University of the Punjab, Lahore, Pakistan, over a period of five months from September 10th, 2018, to February 14th, 2019.

Samples Collection and Genomic DNA Extraction

For the present study, 5 ml of blood serum samples were collected from HBV-infected Pakistani patients under aseptic conditions using sterile disposable syringes. The blood samples were transferred to clot activator tubes that were appropriately labeled. Initially, they were centrifuged at 8,000 rpm for 13 min using a centrifuge (Model: Vision/VS-35SMTi, Korea, Rotor: Bio-Hazard Safety Rotor - Duralumin) to obtain serum (10 µL). The patients were provided verbal instructions about the sample collection procedure. Genomic DNA extraction was carried out from each serum sample of the infected patient using a "Bio spin Blood Genomic DNA Miniprep Kit" (Bioer Technology Co., Germany), following the manufacturer's instructions. A 200 µL lysis buffer, 30 µL proteinase-K, and 200 µL samples were mixed in an Eppendorf tube. The mixtures were vortexed and incubated at 56°C for 15 minutes. After incubation, the mixtures were given a short spin and 250 µL ethanol was added. The mixtures were given again a short spin and shifted to column tubes. For washinf purpose, 500 µL washing buffers (W1 and W2) were added to each column tube. Thereafter, 500 µL ethanol was applied to each mixture followed by a 1-minute centrifugation at a rate of 8,000 rpm. The supernatant was removed, and dry column tubes were finally centrifuged for 4 minutes at 13,000 rpm using a centrifuge (Model: Vision/VS-35SMTi, Korea, Rotor: Bio-Hazard Safety Rotor - Duralumin). The column tubes were transferred into fresh Eppendorf tubes and 50 µL of elution buffer was added for DNA elution. The resultant pure DNA samples were collected in final tubes further examined using real-time polymerase chain reaction (PCR).

Real-time PCR Analysis

The DNA samples were quantitatively and qualitatively analyzed by using real-time PCR (Qiagen; Hilden, Germany). Two mixtures were prepared: Mixtures A included (Taq DNA polymerase) and Mixture B consisted of primer (Novacyt; Camberley, United Kingdom), buffer (Invitrogen; Waltham, MA, USA), dNTPs (Fermentas; Waltham, MA, USA), and probes (Generi Biotech; Machkova 587/42, 500 11 Hradec

Králové, Czechia). These mixtures were applied to 15 µL PCR tubes. For relative quantification, four separate concentrations (S1, S2, S3, and S4) were used. The water 10 µL and negative test control (NTC) 15 µL were added into a separate tube. The thermocycling conditions involved initial denaturation at 95°C for 10 minutes, followed by 45 cycles at 95ºC for 15 seconds, 55ºC for 30 seconds, and 72ºC for 15 seconds. Following PCR completion, various titers of hepatitis B viral DNA were shown on the screen with different values. To carry the study further, the sample with a high titer was selected. The SnapGene (https:/www.snapgene.com) software was utilized for designing specific external and internal primers of the *HBsAg* gene, as described in (Table I). The length of the *HBsAg* gene-specific external primer was 681 bp, and the internal primer was 400 bp.

Nested PCR Amplification

The *HBsAg* gene was amplified using nested PCR, which involved two rounds of PCR for the desired gene amplification. In the first round, the gene-specific external primer was used for PCR amplification. The resultant product from the first-round PCR was used as a template for second-round PCR, along with the gene-specific internal primer. The PCR conditions have been described below. Finally, the bands of the gene were excised from the gel and subjected to gene extraction and purification.

First Round of PCR with Gene-Specific External Primers

In the first round of PCR, the different reagents were mixed, such as 2 µL reverse external primer, 2 µL forward external primer, 10 µL PCR master mix, and 6 µL DNA sample. The total volume obtained was 20 µL. This reaction was further processed by PCR conditions such as:

- Initial temperature at 94°C for 5 min.
- Temperature of denaturation at 94°C for 30 sec.
- Temperature of annealing at 56°C for 30 sec.
- Temperature of elongation at 72°C for 40 sec.
- Temperature of final elongation at 72°C for 7 min.
- Temperature of Incubation at 4°C.
- Total cycles = 35.

Second-Round PCR for Gene Confirmation with Gene Specific Internal Primers

The various reagents were mixed in the second round of PCR, such as 2 µL reverse external primer, 2 µL forward external primer, 10 µL PCR master mix, and 6 µL DNA sample. The final volume obtained was 20 µL. The reaction was further processed by applying the PCR conditions such as:

- Initial temperature at 94°C for 5 min.
- Temperature of denaturation at 94°C for 30 sec.
- Temperature of annealing at 65°C for 30 sec.
- Temperature of elongation at 72°C for 40 sec.
- Temperature of final elongation at 72°C for 7 min.
- Temperature of incubation at 4°C.
- Total Cycles = 35.

TA Cloning and Transformation

TA cloning was performed using the A Cloning™ Kit and PCR™ 2.1 (Catalogue No. K2020-20), (Sigma-Aldrich; St. Louis, MI, USA) as per the manufacturer's instructions. The *HBsAg* gene was ligated into a PCRII vector (Invitrogen; Waltham, MA, USA) for gene amplification and then transformed into *E. coli* TOP10 F′. The transformation was confirmed through PCR analysis, restriction digestion, and gel electrophoresis (HE33 mini horizontal submarine unit; company, Amersham Biosciences, (Amersham United Kingdom), utilizing ampicillin resistance. Positive colonies were selected, cultured, preserved, and systematically subjected to plasmid extraction.

Plasmid Extraction and Sequencing

Table I. *HBsAg* gene-specific forward external primer (681 bp) and forward internal primer (400 bp) for amplification purposes.

Desired gene (HBsAg)	Primer sequence (5′-3′)	GC%	Tm	Amplicon size (bp)
External Reverse Primer	5′-TAA GGA TCC AAT GTA TAC CCA AAG ACA AAA-3′	33.33%	65.3	681
External Forward Primer	5′-CAT ATG ATG GAG AAC ATC ACA TCA -3′	37.5%	60.1	681
Internal Reverse Primer	5′-TAA GGA TCC CCC CCA ATA CCA CAT CAT CCA TAT-3′	45.45%	72.7	400
Internal Forward Primer	5′-CAT ATG CAT ATG ATG TGT CTG CGG CGT TTT-3′	43.33%	69.4	400

The TA-*HBsAg* plasmid purification was performed using the Gene Jet™ Plasmid Miniprep Kit (catalogue No. k0502), (Invitrogen; Waltham, MA, USA). The purification process involved treating the *HBsAg* gene with 250 µL of lysis buffer, 250 µL of re-suspension buffer, 350 µL of neutralization buffer, and a final 50 µL elution buffer. The PCR product of the desired gene was subjected to sequencing. The obtained chromatogram of the sequenced *HBsAg* gene, aligned with gene-specific forward primers of Pakistani isolates, was compared to existing sequences using the BLAST analysis network service provided by National Center for Biotechnology Information (NCBI). To construct the pET20b-*HBsAg* gene, both the *HBsAg* gene and pET20b plasmid (Novagen's, Pretoria, South Africa) were cleaved using the BamH1 and Nco1 restriction enzymes. Subsequently, the ligase enzyme was employed to ligate the expression plasmid (pET20b) with the desired gene. The resulting pET20b-*HBsAg* gene construct was transformed into competent cells of *E. coli* TOP10 F'. The confirmation of successful transformation was achieved through PCR analysis and restriction digestion (Figure 1).

Periplasmic Soluble Expression

The *HBsAg* gene cassette was cut through Nco1 and BamH1 restriction enzymes from the pET28b expression plasmid, and the pET20b plasmid was also digested with the same restriction enzymes.

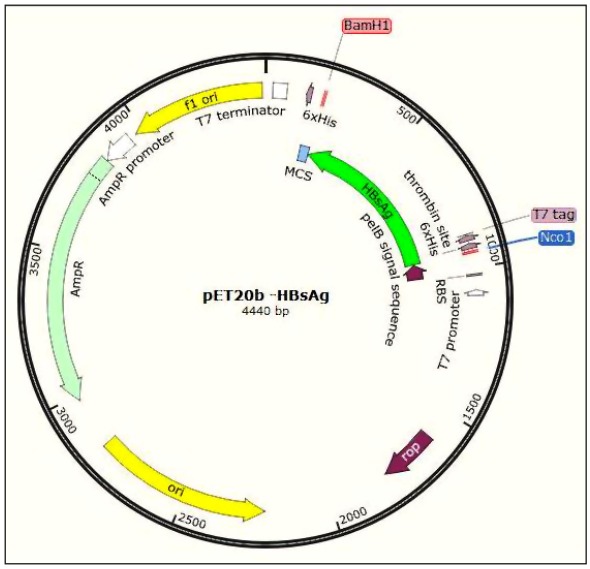

Figure 1. Circular map of Recombinant Plasmid (pET20b-HBsAg) generated *via* snap gene (https:/www.snapgene.com).

The cassette from the pet28b plasmid was subcloned into the pet20b expression vector. To achieve periplasmic soluble expression of the desired gene, the recombinant plasmid pET20b-*HBsAg* was introduced into the BL21 (DE3) expression host and induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG). After gene expression, the presence of the *HBsAg* protein was confirmed using various analytical techniques, including SDS-PAGE (Hoefer, Holliston, MA, USA), SE250/SE260 mighty small II, model No. PS3000-230V), silver staining kit (Thermofisher Scientific; Waltham, MA, USA), Coomassie staining kit (Thermofisher Scientific), dot blot, and western blot (Hoefer Scientific Instruments Semi-Phor, San Francisco, CA, USA) analysis.

The osmotic shock approach was applied for the extraction of periplasmic soluble protein. In the cold hypertonic buffer, the bacterial pellets were resuspended. The ice incubation was applied to the suspension mixture and shaken for up to 45 minutes. Then, centrifugation was applied to the mixture to isolate the supernatant periplasmic protein and the cells. Finally, the pellet was placed in the cold hypotonic buffer. The mixture on ice was shaken and then centrifuged at 4°C for 10 minutes. The supernatant was collected to form the periplasmic soluble protein. The extracted periplasmic soluble protein was purified by applying the NI-NTA affinity column[21]. Ni-NTA is an affinity chromatography matrix for purifying recombinant proteins carrying a His tag.

Results

Nested PCR Analysis Confirmation

The *HBsAg* gene was amplified from serum samples of hepatitis B-infected Pakistani patients using real-time PCR and nested PCR (1st and 2nd rounds). The size of the gene was confirmed using specific primers (681 bp and 400 bp), as depicted in (Figure 2a-b). Following amplification, quantitative and qualitative measurements of the gene were obtained, and a sample with high titer DNA (72,049,654 copies per ml) was selected from multiple samples for gene cloning and expression.

HBsAg Gene's Sequencing and Blast Analysis

The *HBsAg* gene was successfully sequenced and submitted to GenBank and received accession number BankIt2732749 HBV OR424356. Blast

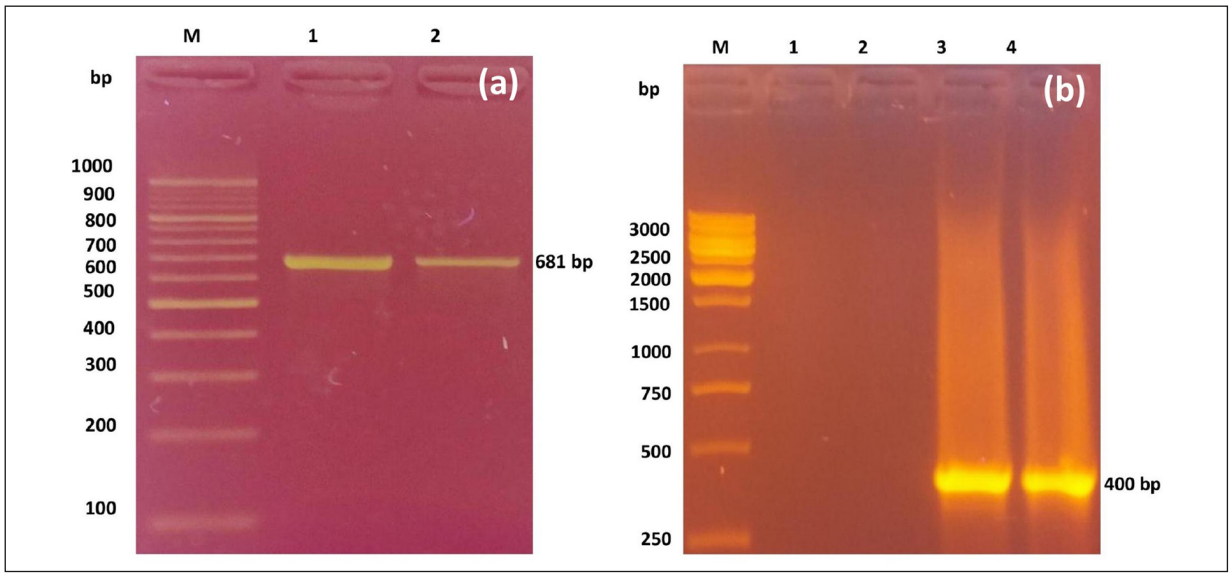

Figure 2. a, Lane M: 100 bp DNA marker, Lane 1, and Lane 2: HBsAg gene of 681 bp with gene-specific external primer. **b**, Lane M: 1 kb DNA marker, Lane 1, and Lane 2: negative control, Lane 2 and Lane 3: HBsAg gene of 400 bp with gene-specific internal primer.

analysis comparing it with the large surface protein of hepatitis B virus isolate IR-54 revealed a remarkable 98% homology in the targeted sequence (**Supplementary Data**). Translation of the sequencing result yielded an amino acid sequence with 98% homology to reported sequences worldwide. The sequencing chromatogram aligned with gene-specific forward primers exhibited similarity to the S protein of the hepatitis B virus.

Sequencing of HBV S Gene

The amino acid sequence of the amplified HBV S gene (Figure 3):

SLDSWWTSLNFLGGSPVCLGQN-
SQSPTSNHSPTSCPPICPGYRWM-
CLRRFIIFLFILLLCLIFLLVLLDY-
QGMLPVCPLIPGSSTTSTGPCRTCT-
TLAQGTSMFPSCCCSKPSDGNCTCIP-
IPSSWAFGKFLWEWASARFSWLSLL-
VPFVQWFAGLSPTVWLSVIWM-
MWYWGPSLYNILSPFIPLLPIFFCL-
WVYI

Recombinant Plasmid (pET20b-HBsAg) Expression

To achieve periplasmic soluble expression, the high titer DNA of 72,049,654 copies per ml was cloned into the expression vector pET20b. The transformed *E. coli* BL21 (DE3) expression host was induced with IPTG. Confirmation of precise gene size was conducted using a gene-specific external primer (681 bp) and a gene-specific internal primer (400 bp) (Figure 4a-b).

Confirmation of HBsAg Protein Expression

The precise size of the expressed *HBsAg* protein (28 kDa) was confirmed using techniques such as silver staining, Coomassie staining, and SDS-PAGE (Figure 5a-b).

Dot-Blot and Western-Blot Analysis

The dot-blot analysis confirmed the presence of *HBsAg* protein bands by utilizing specific primary and secondary antibodies. The expressed *HBsAg* protein appeared as dots on the nitrocellulose membrane. Furthermore, Western blot analysis confirmed the protein's exact size to be 28 kDa (Figure 6a-b).

Discussion

The global impact of HBV infection remains a formidable challenge, persisting despite the availability of effective treatments and vaccines. This ongoing battle has mobilized a community of relentless researchers and scientists, united in their commitment to conquer this pervasive global threat[22]. By recognizing the collective efforts and dedication of these individuals, we can draw inspiration from their tireless pursuit of solutions to overcome HBV and safeguard global health. In

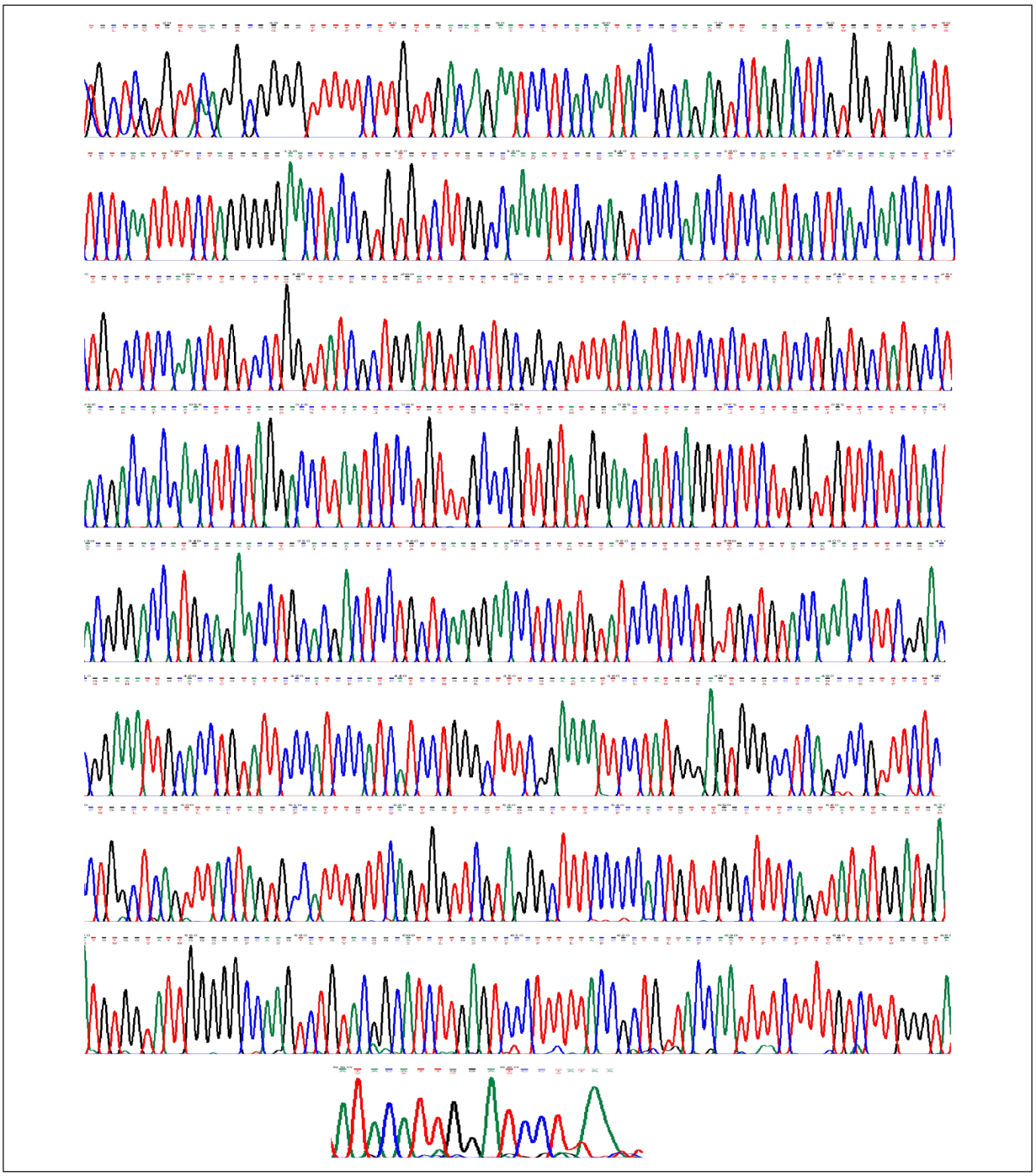

Figure 3. Chromatogram peaks of the hepatitis B virus showed 98% homology with the targeted sequences along with reported sequence ID: AHJ59092.1 on the NCBI database.

the present study, *E. coli* BL21 (DE3) is the most preferred candidate for the efficient expression of *HBsAg* protein that is widely used in association with pET expression vectors. For global vaccine production to combat HBV infection, a prokaryotic expression machinery, specifically E. coli, is considered as the most accepted host[23]. According to the previous research[24], *E. coli* has been considered as the suitable expression system to regulate the high yield expression of *HBsAg* protein in a short duration and with low costs.

In previous research[25-27], many expression vec-

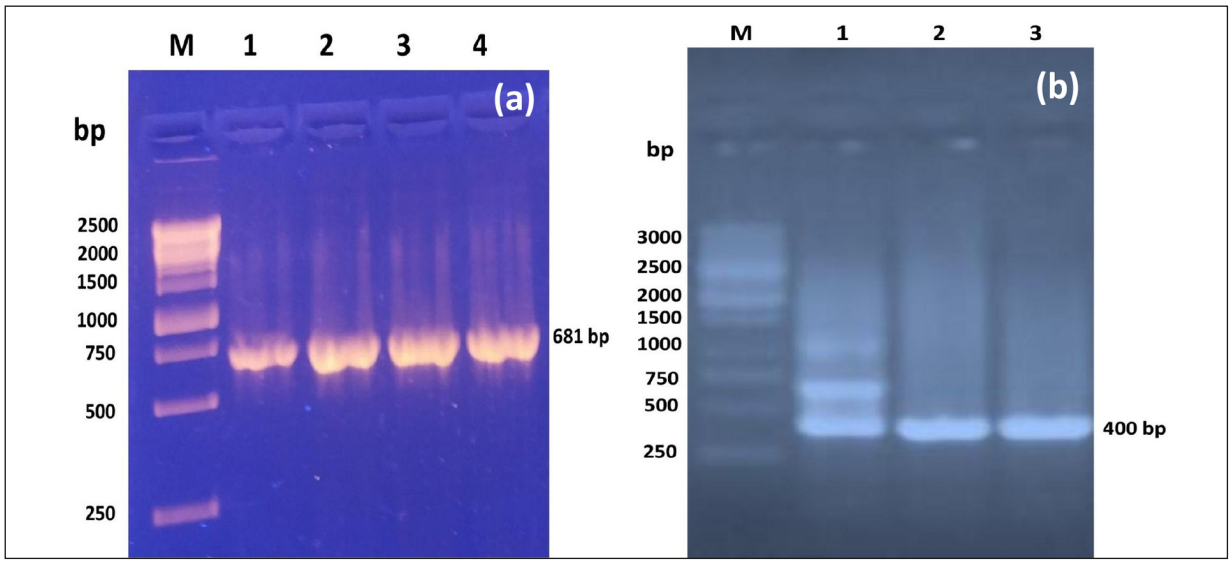

Figure 4. a, Lane M: 1 kb DNA ladder, Lane 1 to 4: HBsAg (681 bp) length in pET20b transformed into *E. coli* BL21 (DE3) with gene-specific external primer. **b**, Lane M: 1 kb DNA marker, Lane 1 to 3: HBsAg (400 bp) in pET20b plasmid transformed into E. coli BL21 (DE3) with gene-specific internal primer.

tors such as pRc/CMV, pGBKT7, pNI2, and pS-G5Flag have been used for the cloning of the hepatitis B surface antigen gene. To analyze the expression of the *HBsAg* gene, we have constructed an expression system of the pET20b expression vector. This vector not only prefers the expression process but also facilitates the periplasmic soluble expression of the *HBsAg* protein, which

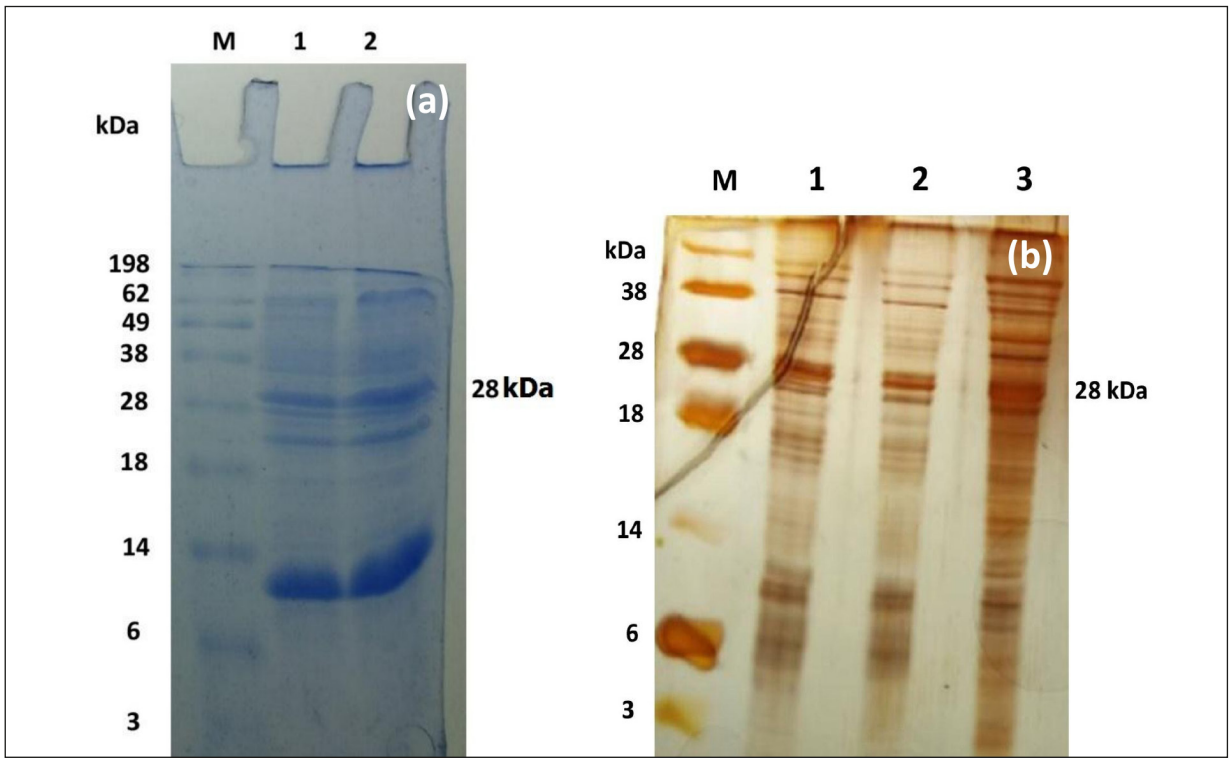

Figure 5. a, Lane M: Protein marker, Lane 1 and 2: Inclusion bodies of HBsAg protein of size 28 kDa. **b**, Lane M: Protein marker, Lane 1 to 3: inclusion bodies of HBsAg protein of 28 kDa size.

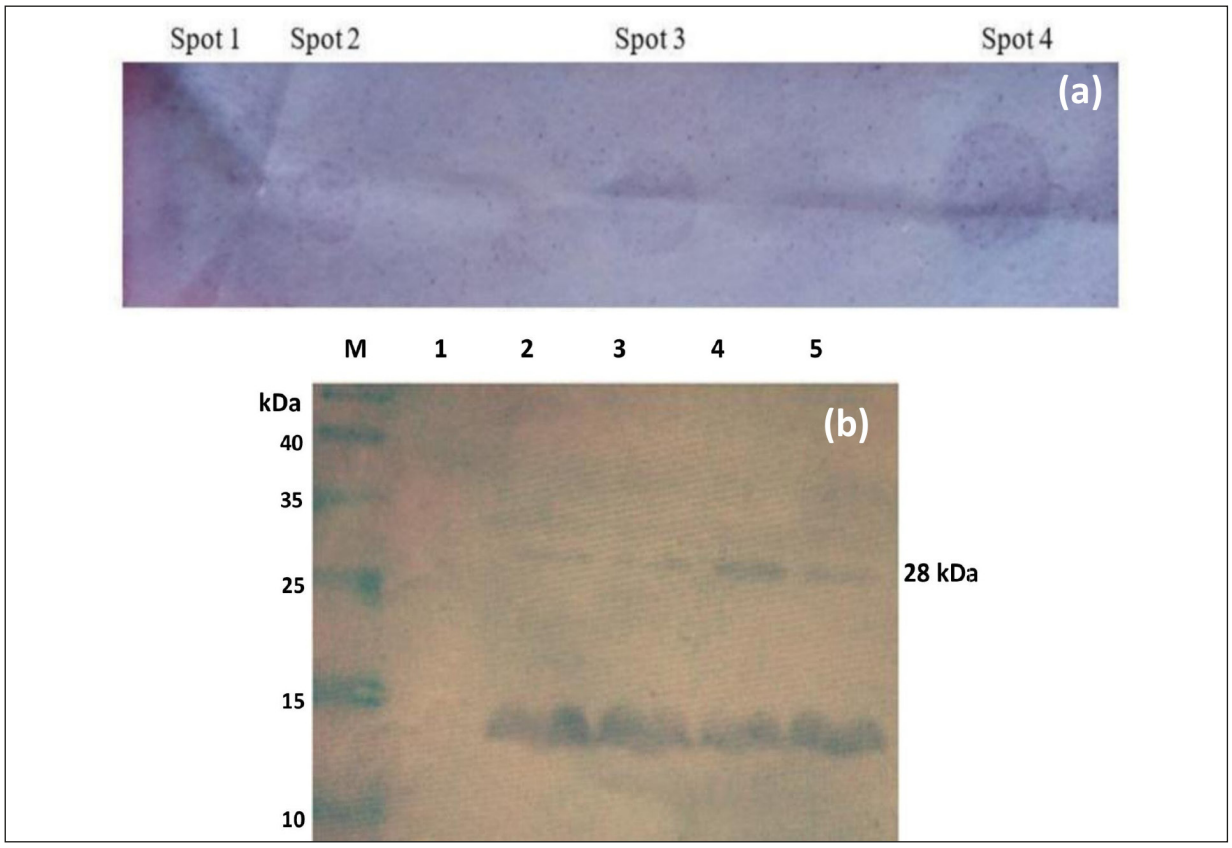

Figure 6. a, Represents Dot-Blot Analysis: spot 1: negative control, spot 2: sample 10 µl, spot 3: sample 15 µl, spot 4: sample 20 µl. **b**, Represents Western blot analysis cell lysate: M: protein marker (catalogue No. 26616, Thermo fisher pre-stained), lane 1 to 5: HBsAg gene protein of 28 kDa length.

can be easily extracted from the cell for the purpose of protein purification. Periplasmic soluble expression of recombinant proteins has many advantages over cytoplasmic expression: it provides a good medium for protein folding and disulfide bond formation. In the periplasmic space, there is less proteolytic activity. The protein expressed in the periplasmic space contains the amino-terminal as the original one. These benefits make the periplasmic expression of recombinant proteins most desirable for vaccine development.

The expressed protein was confirmed by performing SDS-PAGE, which revealed a 28 kDa size protein compared to the reported size of 25 kDa (Figure 6b). This increase in the protein's size may be due to the fusion of 10X His tag to the protein[28]. The purified *HBsAg* protein was further confirmed *via* dot blot and western blot analysis.

The presence of drug resistance mutations (DRMs), vaccine escape mutations, and diagnostic escape mutations in the hepatitis B virus (HBV) can hinder the effectiveness of current treatment and prevention strategies, causing delays in achieving desired outcomes[29]. Mutations in *HBsAg* that occur naturally during infection primarily affect the first loop (amino acids 107 to 138). The second hydrophilic loop (aa 139-147) is the primary target for neutralizing antibodies induced by vaccines and immunoglobulins[30]. HBV variants with a polymerase gene mutation may evolve under drug selective pressure with associated concomitant mutations in the antigenic region of the overlapping S gene, which is capable of altering the immune reactivity of the S antigen[31]. HBV mutations occur due to its distinctive replication strategy, which involves a non-proofreading reverse transcriptase, resulting in higher mutability compared to other DNA viruses. The mutation rate in HBV ranges from 1.4 to 3.2 × 10−5 base substitutions/site/year[32].

Currently there is no proper treatment available for chronic hepatitis B viral infection. Therefore, an alternative therapy is urgently required by using new approach. In this present investigation,

HBsAg protein has been expressed and cloned in order to develop an effective vaccine that will use as a complete therapy for this infection. It is clearly investigated that the antibody humoral response plays a vital role in the clearance and prevention of the spreading of hepatitis B infection. On the other hand, the cellular immune response plays a key role in the eradication of infected hepatocytes[33].

The hepatitis B surface antigen gene of 681 bp size has been cloned and expressed in different vectors and expression hosts. The *HBsAg* gene of 681 bp was cloned and expressed by using the pGEMEX expression vector and JM109 (DE3) expression host[34]. On the other hand, this gene of 681 bp size has also been cloned and expressed by using a pABII expression vector and transgenic banana as expression host[35]. One other research[36] shows that the same *HBsAg* of 681 bp has been cloned and expressed for the purpose of vaccine development by using a pBI121 expression vector and Solanum lycopersicum expression host. The expression system which has been used in the present study may show a remarkable result as compared to other. The immune responses mounted within the liver during HBV infection exemplify the remarkable adaptability and efficiency of our immune system[37].

This study represents a significant breakthrough in hepatitis B research, achieved through careful and rigorous experimentation. The *HBsAg* gene, a key player in HBV infection, was not only amplified but also successfully cloned and expressed in a soluble form. This significant milestone holds tremendous promise for advancing our understanding of the virus and potentially revolutionizing therapeutic approaches[38]. The precision of this scientific endeavor was further demonstrated by confirming the exact size of the *HBsAg* gene using gene-specific external and internal primers, yielding an awe-inspiring 681 bp and 400 bp, respectively (Figure 2a-b). In a comparable study[39-40], real-time and nested PCR techniques were utilized to amplify HBV DNA. This approach highlights the use of molecular biology methods to detect and quantify the presence of HBV in samples.

In this study, the *HBsAg* gene underwent successful sequencing and was subsequently submitted to GenBank. The obtained *HBsAg* gene sequence was then compared to the hepatitis B virus isolate IR-54 large surface protein, resulting in an impressive 98% homology with the targeted sequence. To further decipher the genetic information, the sequencing results were translated using online Expasy tools, revealing an amino acid sequence that exhibited a striking 98% homology with previously reported sequences from diverse regions worldwide. These findings shed light on the genetic characteristics of the *HBsAg* gene and contribute to the broader understanding of hepatitis B virus variants, facilitating future research and potentially aiding in the development of targeted interventions against the virus[41-43].

To achieve efficient periplasmic soluble expression, the desired gene, containing high titer DNA at a remarkable concentration of 72,049,654 copies per ml, was cloned into the expression vector pET20b. Through transformation into the *E. coli* strain BL21 (DE3) expression host *via* IPTG induction, successful expression was achieved. The accurate size of the gene was confirmed using gene-specific external and internal primers, resulting in a length of 681 bp and 400 bp, respectively (refer to Figure 4a-b). This study aligns with previous research efforts[44-45], where the *HBsAg* gene was cloned and expressed in *E. coli*, demonstrating its potential as a cost-effective and highly efficient approach for *HBsAg* production. Previous studies[44] utilizing *Saccharomyces cerevisiae* or *E. coli* as hosts for cloning and expressing the HBsAg gene reported low expression levels. In contrast, our findings reveal a substantial expression of the *HBsAg* protein, suggesting its potential utility in local therapeutics for the treatment of HBV infection. These results present a promising step forward in the field of HBV research and offer new possibilities for developing improved therapeutic strategies against the virus.

The successful confirmation of the precise 28 kDa size of the expressed *HBsAg* protein was accomplished using multiple techniques. Silver staining, Coomassie staining, and Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) were employed, yielding conclusive evidence (Figure 5a-b). Silver staining is a sensitive technique that detects proteins on gels by impregnating them with silver ions, forming visible bands or spots. It is used in 2D gel electrophoresis for high-resolution protein detection[46]. On the other hand, Coomassie staining uses Coomassie Brilliant Blue dye to bind proteins, creating blue bands/spots on gels. It is sensitive, works with different protein gels, and enables quick protein visualization/quantification in proteomics and analysis experiments[47].

Furthermore, in the current study, dot-blot

analysis was conducted, involving the binding of the *HBsAg* protein with specific primary and secondary antibodies. This analysis revealed the presence of *HBsAg* protein as distinct dots on a nitrocellulose membrane. Additionally, the Western blot analysis further verified the exact size of the HBsAg protein to be 28 kDa (Figure 6a-b). These comprehensive analyses provide robust evidence for the successful expression and accurate determination of the size of the *HBsAg* protein.

The present study confirms the readiness of expressed protein clones, specifically the hepatitis B surface antigen (*HBsAg*), for an experimental investigation using a mouse model. The aim is to explore the intricate interaction between the *HBsAg* gene and the immune system, shedding light on the pivotal role played by various immune system components in combating hepatitis B infection within the local context of Pakistan. To conduct these experiments, previously established transgenic mouse models have been utilized, enabling researchers to delve into viral entry mechanisms and the molecular intricacies of replication. It's important to note that while mouse models offer valuable insights, limitations in the comparison of mouse and human immune systems restrict their broader application[48-51]. Nonetheless, the experimental findings from this study hold great potential in advancing our understanding of the immune response to hepatitis B infection, which may pave the way for the development of novel therapeutic strategies and interventions.

Study Limitations

The effectiveness and immune response of recombinant HBV vaccines may be affected by the limited post-translational modification in *E. coli*. To assess the immunogenicity and efficacy of the desired vaccine, an *in vivo* study is necessary. It is important to acknowledge that using *E. coli* has limitations due to its lack of glycosylation machinery, which can impact the antigenicity of the HBV vaccine. Additionally, optimizing the purification process is crucial to ensure the complete removal of endotoxins, as their presence in the final vaccine product can be harmful.

Future Objectives

This study will provide our group with valuable insights for conducting additional molecular investigations on the immune responses triggered by the *HBsAg* protein. Furthermore, conducting animal trials with the expressed *HBsAg* protein will enhance our understanding of how various components of the immune system combat hepatitis B infection. Furthermore, this expression will facilitate the development of alternative immunotherapy approaches to effectively combat hepatitis B.

Conclusions

In conclusion, this study successfully extracted, cloned, sequenced, and expressed the *HBsAg* gene in *E. coli*. The high level of expression and purification of the *HBsAg* protein has significant implications for improving local recombinant DNA vaccines against hepatitis B. Furthermore, this expression system enables comprehensive molecular studies on the interaction between *HBsAg* and the immune system, providing insights into the immune response against hepatitis B infection. These findings pave the way for the development of alternative immunotherapy strategies for the treatment of hepatitis B infection.

Conflict of Interest

The authors declare that they have no conflict of interests.

Ethics Approval

Not applicable. No humans or animals are directly involved, and approval by a bioethical committee was not required.

Informed Consent

Not applicable.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Acknowledgements

The authors greatly acknowledge and express their gratitude to the Researchers Supporting Project number (RSP-D2023R568), King Saud University, Riyadh, Saudi Arabia.

Authors' Contribution

Conceptualization: Kashif Syed Haleem; methodology: Isfahan Tauseef; software: Ibrar Khan; validation: Thamer H Abkari; formal analysis: Matiuallah Khan; investigation: Tawaf Ali Shah; data curation: Sumaira Naz. Writing - original draft and editing: Ibrar Khan, and Matiullah Khan; Resources: Tariq Aziz: visualization: Nausheen Nazir. Data curation: Thamer H Albekairi. Supervision and Funding acquisition: Tariq Aziz.

ORCID ID
T. Aziz: 0000-0003-0905-8076
T.A Shah: 0000-0002-5030-4729

References

1) Liu Y, Maya S, Ploss A. Animal Models of Hepatitis B Virus Infection–Success, Challenges, and Future Directions. Viruses 2021; 13: 777.
2) Hesari A, Ghasemi F, Salarinia R, Biglari H, Tabar M, Abdoli V, Mirzaeh H. . Effects of curcumin on NF-κB, AP-1, and Wnt/β-catenin signaling pathway in hepatitis B virus infection. J Cell Biochem 2018; 119: 7898-7904.
3) Arya M, Niguse S, Gerbekidan K, Hailekiros H, Berhe N, Asmelash D. Hepatitis B virus infection and associated risk factors among pregnant women attending antenatal care in health facilities of Tigray, Northern Ethiopia. J Med Virol 2018; 90: 503-509.
4) Schweitzer A, Horn J, Mikolajczyk R, Krause G, Ott J. Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. The Lancet 2015; 386: 1546-1555.
5) Mehmood S, Raza H, Abid F, Saeed N, Rehman M, Javed S, Khan M. National prevalence rate of hepatitis B and C in Pakistan and its risk factors. J Public Health 2020; 28: 751-764.
6) Ali M, Idrees M, Ali M, Hussain A, Rehman I, Saleem S, Afzal S, Butt S. Hepatitis B virus in Pakistan: a systematic review of prevalence, risk factors, awareness status and genotypes. Virol J 2011; 8: 1-9.
7) Liu Z, Zhang Y, Xu M, Li X, Zhang Z. Distribution of hepatitis B virus genotypes and subgenotypes: A meta-analysis. Medicine 2021; 100.
8) Khan A, Qazi J. Risk factors and molecular epidemiology of HBV and HCV in internally displaced persons (IDPs) of North Waziristan Agency, Pakistan. J P M A 2018; 68: 165-169.
9) Warner N, Locarnini S, Xu H. The role of hepatitis B surface antibodies in HBV infection, disease, and clearance. Future Virol 2020; 15: 293-306.
10) Alsadeq D, Taleb S, Zaied R, Fahad S, Smatti M, Rizeq B, Althani A, Yassine H, Nasrallah G. Hepatitis B virus molecular epidemiology, host-virus interaction, coinfection, and laboratory diagnosis in the MENA region: An update. Pathog 2019; 8: 63.
11) Keefli A, Tutkaoglu S, Coskun U. Risk of hepatitis B virus reactivation during immunosuppressive treatment. Eur Rev Med Pharmacol Sci 2023; 27: 2823-2831.
12) Sarkar B, Islam S, Zohora U, Ullah M. Virus like particles-A recent advancement in vaccine development. The M S K 2019; 55: 327-343.
13) Helian Y, Gai Y, Fang H, Sun Y, Zhang D. A multistrategy approach for improving the expression of E. coli phytase in Pichia pastoris. J Ind Microbiol Biotechnol 2020; 47: 1161-1172.
14) Kaur J, Kumar A, Kaur J. Strategies for optimization of heterologous protein expression in E. coli: Roadblocks and reinforcements. Int J Biol Macromol 2018; 106: 803-822.
15) Huang L, Lin H, Lyu L, Gung C, Huang C. Development of a transgenic Flammulina velutipes oral vaccine for hepatitis B. F T B 2019; 57: 105.
16) Rauf A, Nadeem M, Khan J, Muhammad K, Shakoori A. Highly Immunogenic Recombinant HBV Core Protein from Pakistani Isolates: A Potential Candidate Vaccine for Hepatitis B in Pakistan. Pakistan J Zool 2016; 48.
17) Huang Q, Lims S. New Advances in Hepatitis B Vaccination for Adults. Curr Hepatol Rep 2018; 17: 466-474.
18) Fabrizi F, Cerutti R, Dixit V, Ridreujo E. Hepatitis B virus vaccine and chronic kidney disease. The adv Nefrología (English Edition) 2021; 41: 115-122.
19) Dealmeida PR. Expression and detection of anti-HBs antibodies after hepatitis B virus infection or vaccination in the context of protective immunity. Arch Virol 2019; 164: 2645-2658.
20) Shouval D, Roggendrof H, Roggendrof M. Enhanced immune response to hepatitis B vaccination through immunization with a Pre-S1/Pre-S2/S vaccine. Med Microbiol Immunol 2015; 204: 57-68.
21) Malekian R, Jahanian-Najafabadi A, Moazen F, Ghavimi R, Mohammadi E, Akbari V. High-yield production of granulocyte-macrophage colony-stimulating factor in E. coli BL21 DE3 by an auto-induction strategy. Iran J Pharm Res 2019; 18: 469-478.
22) Koffas A, Kumar M, Gill U, Jindal A, Kennedy P, Sarin S. Chronic hepatitis B: the demise of the 'inactive carrier' phase. Hepatol Int 2021; 15: 290-300.
23) Huang CH, Chen E, Yang YY, Chen YY, Chang JJ, Chen KJ, Lu CH, Lee KD, Chen PC, Chen CC. The impact of hepatitis B virus infection and vaccination on the development of non-Hodgkin lymphoma. J Viral Hepat 2017; 24: 885-894.
24) Elghanam MS, Attia AS, Shoeb HA, Hashem AE. Expression and purification of hepatitis B surface antigen S from Escherichia coli; a new simple method. BMC Res Notes 2012; 5: 125.
25) Harlow E, Lane D. A Laboratory Manual, New York: Cold Spring Harbor Laboratory. 1988, p. 579.
26) Qin S, Tang H, Zhao LS, He F, Lin Y, Liu L, He XM. Cloning of HBsag -encoded genes in different vectors and their expression in eukaryotic cells. World J 2003; 9: 1111.
27) Lambert C, Prange R. Dual topology of hepatitis B virus large envelope protein determinants influencing post- translational pre-translocation. J Biol Chem 2001; 276: 22265-22272.

28) Pumpen P, Kozlovskaya TM, Borisova GP, Bichko VV, Dishler A, Kalis J. Expression of hepatitis B virus surface antigen gene in Escherichia coli. Gene 1984; 30: 201-210.

29) Washizaki A, Murayama A, Murata M, Kiyohara T, Yato K, Yamada N, Kato T. Neutralization of hepatitis B virus with vaccine-escape mutations by hepatitis B vaccine with large-HBs antigen. Nat Commun 2022; 13: 5207.

30) Harris B, Holzmayer V, Quershi H, Khan M, Khan S, Salamat N, Brennan C. Hepatitis B genotypes and surface antigen mutants present in Pakistani blood donors. PloS one 2017; 12: e0178988.

31) Zoulim F, Locarnini S. Hepatitis B virus resistance to nucleos (t) ide analogues. Gastroenterology 2009; 137: 1593-1608.

32) Osasona O, Oguntoye O, Arowosaye A, Adwumi M, Happi C, Folarin O. Patterns of hepatitis b virus immune escape and pol/rt mutations across clinical cohorts of patients with genotypes a, e and occult hepatitis b infection in Nigeria: A multi-centre study. Virulence 2023; 14: 2218076.

33) Chisari FV, Isogawa M, Wieland SF. Pathogenesis of hepatitis B virus infection. Pathol Biol (Paris) 2010; 58: 258-266.

34) Mojgan B, Mahvash K, Bahram K. Cloning and Expression of Hepatitis B Surface Antigen. Hepatitis Monthly 2008; 8: 17-21.

35) Shereen F. Elkholy, Roba M. Ismail, Ahmad Bahieldin, Atef S. Sadik, Magdy A. Madkour. Expression of Hepatitis B Surface Antigen (HBsAg) gene in transgenic banana (Musa Sp.). Arab J Biotech 2009; 12: 291-302.

36) Inam S, Abbas Z, Noor S, Rehman N, Adeel Zafar S, Ramzan Khan M, Ali Kaimkhani Z, Al-Misned F, Shah M, Mahboob S, Muhammad Ali G. Isolation, cloning and transgenic expression of hepatitis B surface antigen (HBsAg) in Solanum lycopersicum L. Saudi J Biol Sci 2022; 29: 1559-1564.

37) Kubes P, Jenne C. Immune responses in the liver. Annu Rev Immunol 2018; 36: 247-277.

38) Moyo B, Bloom K, Scott T, Ely A, Arbuthnot P. Advances with using CRISPR/Cas-mediated gene editing to treat infections with hepatitis B virus and hepatitis C virus. Virus Res 2018; 244: 311-320.

39) Chavan YG, Pawar SR, Wani M, Raut AD, Misra RN. Hepatitis B virus DNA polymerase gene polymorphism-based prediction of genotypes in chronic HBV patients from Western India. Afr Health Sci 2017; 17: 762-772.

40) M Hamid, U Salar, Y Rashid, MK Azim, KM Khan, S Naz, T Aziz, M Alharbi, A Alshammari, AF. Alasmari. Determining the 3-substituted Coumarins inhibitory potential against the HslV protease of E. coli. Eur Rev Med Pharmacol Sci 2023; 27: 9169-9182.

41) Lago B, Espiritosanto M, Costa V, Marques V, Villar L, Lewisximenz L, Mello F. Genetic diversity of the hepatitis B virus subgenotypes in Brazil. Viruses 2019; 11: 860.

42) M Naveed, J Hassan, T Aziz, U Ali, IK Rana, AA Khan, H Fakhrul, M Alharbi, A Alshammari, AF Alasmari. A one-health approach to design an mRNA-based vaccine candidate against the lumpy skin disease virus as an alternative to live-attenuated vaccines. Eur Rev Med Pharmacol Sci 2023; 27: 6401-6413.

43) Naveed M, Shabbir MA, Ain NU, Javed K, Mahmood S, Aziz T, Khan AA, Nabi G, Shahzad M, Alharbi ME, M Alharbi, A Alshammari AF Chain-Engineering-Based De Novo Drug Design against MPXVgp169 Virulent Protein of Monkeypox Virus: A Molecular Modification Approach. Bioengineering 2023; 10: 11.

44) Elghanam M, Attia A, Shoeb H, Abdelgawad M. Expression and purification of hepatitis B surface antigen S from Escherichia coli; a new simple method. BMC research notes 2012; 5: 1-8.

45) Naveed M, Makhdoom SI, Ali U, Jabeen K, Aziz T, Khan AA, Jamil S, Shahzad M, Alharbi M, Alshammari A. Immunoinformatics Approach to Design Multi-Epitope-Based Vaccine against Machupo Virus Taking Viral Nucleocapsid as a Potential Candidate. Vaccines 2022; 10: 1732.

46) Esser K, Cheng X, Wettengel JM, Lucifora J, Hansen-Palmus L, Austen K, Roca Suarez AA, Heintz S, Testoni B, Nebioglu F, Pham MT, Yang S, Zernecke A, Wohlleber D, Ringelhan M, Broxtermann M, Hartmann D, Hüser N, Mergner J, Pichlmair A, Thasler WE, Heikenwalder M, Gasteiger G, Blutke A, Walch A, Knolle PA, Bartenschlager R, Protzer U. Hepatitis B Virus Targets Lipid Transport Pathways to Infect Hepatocytes. Cell Mol Gastroenterol Hepatol 2023; 16: 201-221.

47) Wei C, Li S, Hu J. A Turbo ID-based proximity labelling approach for identifying the DNA-binding proteins. S T A R PROT 2023; 4: 102139.

48) Mestas J, Hughes C. Of mice and not men: differences between mouse and human immunology. J Immunol 2004; 172: 2731-2738.

49) Rongvaux A, Takizawa H, Strowig T, Willinger T, Eynon E, Flavell R, Manz M. Human haemato-lymphoid system mice: current use and future potential for medicine. Annu Rev Immunol 2013; 31: 635-674.

50) Zhang W, Sun H, Sun R, Lian Z, Wei H, Tian Z, Chen Y. HBV immune tolerance of HBs-transgenic mice observed through parabiosis with W.T. mice. Front Immunol 2022; 13: 993246.

51) Zhou Z, Li C, Tan Z, Sun G, Peng B, Ren T, Li W. A spatiotemporally controlled recombinant cccDNA mouse model for studying HBV and developing drugs against the virus. Antivir Res 2023; 216: 105642.