https://doi.org/10.48047/AFJBS.7.5.2025.254-262

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Expression of Recombinant Hepatitis B Surface Antigen (HBsAg) without Transmembrane Domains for Efficient Expression in *E. coli* System.

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Volume 7, Issue 5 ,May 2025

Received: 15 Mar 2025

Accepted: 05 Apr 2025

Published: 09 May2025

doi:10.48047/AFJBS.7.5.2025.254-262

Abstract: The expression of hepatitis B surface antigen (HBsAg) in Escherichia coli (E. coli) poses considerable difficulties, especially because of its four transmembrane domains. These domains are essential for the correct assembly and operation of HBsAg, which inherently produces lipoprotein micelles needing certain membrane-spanning structures. While the structures have important functions in post-translational operations, studies have shown that HBsAg can be produced without those domains or by replacing those domains with other viral sequences. By removing its transmembrane domains, this study investigates the expression of recombinant hepatitis B surface antigen (HBsAg). We synthesised and cloned the modified gene into a suitable expression vector, pET-20b (+), and then transformed it into E. coli BL21 (DE3). The aim was to create soluble HBsAg for diagnostic use and to simplify the expression of proteins with transmembrane domains. The results showed successful expression of soluble HBsAg in E. coli BL21(DE3), paving the way for potential diagnostic applications. This study provides insights into the challenges and strategies involved in producing transmembrane protein variants for various research purposes.

Keywords: HBsAg, Recombinant expression, HBV, Diagnostic.

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Keywords: HBsAg, Recombinant expression, HBV, Diagnostic.

1. **Introduction:** Hepatitis B virus (HBV) is a major global health issue, linked to various liver diseases, including acute hepatitis, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). The transmission of HBV primarily occurs through contact with infectious body fluids, such as blood and sexual secretions. While many adults can clear the infection spontaneously, a significant portion progresses to chronic infection, characterised by the persistence of hepatitis B surface antigens (HBsAg) for more than six months (Tan et al., 2015; Hsu et al., 2023). Horizontal transmission occurs through sexual contact and needle sharing, while vertical transmission happens from mother to child during childbirth, particularly in high-prevalence regions. Nelson et al. (2016) classify areas with HBsAg positivity rates exceeding 8% as high-prevalence regions. The clinical presentation of HBV infection can range from asymptomatic to severe, with acute infections potentially causing complications like fever and jaundice. Chronic infections can lead to severe outcomes such as cirrhosis and HCC. The immune response is critical in disease progression, where liver damage is often due to an immune-mediated attack on infected hepatocytes (Tan et al., 2015), Sandhu et al. (2017).

   HBsAg is the main antigen that shows that someone has been infected with HBV. It can be found in serum anywhere from 1 to 12 weeks after infection, which means that the virus is still replicating. Its presence signifies that an individual is infectious (Jackson et al., 2018), (Tang et al., 2020). Doctors use HBsAg levels to guide their treatment decisions for HBV infections, as higher levels are associated with active viral replication and an increased risk of worsening liver disease (Pattyn et al., 2020). Elevated HBsAg levels (>50,000 IU/ml) in

chronic hepatitis B (CHB) are associated with lower HBV-specific T cell responses, indicating that high levels may promote immune tolerance instead of effective viral clearance (Gupta et al., 2024), (Sandhu et al., 2017). A decline in HBsAg levels is viewed as a positive indicator toward achieving seroconversion (loss of HBsAg and development of anti-HBs antibodies), which is a therapeutic goal in managing HBV infections (Pattyn et al., 2020), (Tajiri and Shimizu, 2015). Research suggests that quantifying HBsAg can forecast clinical outcomes and guide antiviral therapy strategies (Hsu et al., 2023; Jackson et al., 2018). HBsAg plays a vital role in both vaccines and diagnostics for HBV infection. The creation of recombinant HBsAg has led to very effective vaccines that make the immune system work very well, protecting more than 95% of healthy people (Pattyn et al., 2020; Nelson et al., 2016). These vaccines use HBsAg to create virus-like particles (VLPs), which mimic the native virus without containing any infectious material, thereby ensuring safety and efficacy in preventing HBV infections (Tan et al., 2015; Jackson et al., 2018). Furthermore, HBsAg serves as a key biomarker for diagnosing HBV infection since its presence indicates active viral replication and infectiousness. However, expressing full-length HBsAg with transmembrane domains poses significant challenges related to misfolding, aggregation, and low yields.

The transmembrane domains are important for folding and secreting proteins correctly. Changes in these areas can stop HBsAg from being secreted and lead to misfolded proteins building up in the endoplasmic reticulum (ER) (Sandhu et al. 2017), (Tajiri & Shimizu, 2015). This misfolding triggers cellular stress responses, leading to reduced yields of functional HBsAg. Research has demonstrated that optimizing expression systems and addressing TMD-related mutations can enhance HBsAg production. However, achieving stable and high-yield expression remains a critical challenge in vaccine development (Gupta et al., 2024; Hsu et al., 2023; Tajiri and Shimizu, 2015). In this study, we have explored the possibility of expressing HBsAg protein without the transmembrane domains and feasibility.

2. Materials and Methods:

2.1. Gene Design, Synthesis, and Cloning into Expression Vector: The gene encoding the transmembrane-less hepatitis B surface antigen (HBsAg) was synthesised, and the gene was then cloned into the pET-20b (+) expression vector, which was ordered from Synbio Technologies. This facilitates solubility and purification of the expressed

protein. We removed the four transmembrane domains (TMDs) of the Hepatitis B surface antigen (HBsAg) from the following positions: TMD1: Amino acids 4–24, TMD2: Amino acids 80–98, TMD3: Amino acids 160–193, TMD4: Amino acids 202–222. Table 1 presents a comparison between the original HBsAg amino acid sequence and the newly synthesized sequence. The sequence was synthesised with the EcoRV and XhoI sites at the extremities, and it was cloned in the vector by Gibson assembly.

2.2. Transformation: The recombinant plasmid was transformed into *E. coli* BL21 (DE3) competent cells using heat shock method. Competent cells were thawed on ice for 10 minutes, followed by the addition of 50 ng of plasmid DNA containing the HBsAg insert. The mixture was incubated on ice for 30 minutes before a heat shock at 42° C for 10 seconds, then returned to ice for an additional 5 minutes. Subsequently, 950 µl of SOC medium was added, and the cells were incubated at 37° C with shaking at 250 rpm for 60 minutes before plating on LB agar containing 100ug/mL Ampicillin. Single colonies were picked and streaked onto new plates to achieve purity of clones.

2.3. Conditions For protein expression: A single colony from the transformation plate was inoculated into LB broth with ampicillin and grown overnight at 37° C with shaking at 200 rpm. The overnight culture was diluted to an optical density (OD600) of approximately 0.1 in fresh LB medium and incubated until it reached an OD600 of 0.6-0.8. Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM, followed by incubation at 18° C for 16 hours to promote proper expression and solubility of the transmembrane-less HBsAg. The culture samples were then subjected SDS-PAGE and western blot analysis using Anti-His tag antibodies.

2.4. Protein Purification: Cell pellets were harvested by centrifugation at 5,000x g for 20 minutes at 4° C and resuspended in lysis buffer containing Tris-HCl, NaCl, and lysozyme. The lysate was subjected to sonication to disrupt cell membranes and release soluble proteins. After centrifugation at 8,000 x g for 30 minutes, the supernatant containing the soluble HBsAg was collected. Purification was achieved using affinity chromatography with a Ni-NTA column, followed by dialysis against phosphate-buffered saline (PBS) to remove imidazole. The purity of the protein was assessed using SDS-PAGE and western blot analysis. Finally, the purified protein was checked for its reactivity using commercially available RDT (Virdict 4, Tulip diagnostics) kit.

3. **Results:** The methods employed for the design, synthesis, cloning, expression, and purification of the transmembrane-less hepatitis B surface antigen (HBsAg) yielded a successfully synthesized gene even after the removal of four transmembrane domains, resulting in a 132-amino-acid sequence cloned into the pET-20b (+) expression vector. After heat shock treatment, E. coli BL21 (DE3) cells transformed successfully, as evidenced by the presence of sufficient colonies on ampicillin plates (Fig. 1.). Protein expression was induced by adding IPTG at 18 C for 16 hours, resulting in soluble HBsAg, confirmed by SDS-PAGE and western blot analysis with anti-His tag antibodies (Fig. 2.). Subsequent cell lysis via sonication and purification using Ni-NTA affinity chromatography, we obtained highly pure HBsAg, which was then validated for purity and reactivity using SDS-PAGE and a rapid diagnostic test kit (Fig. 3.), ensuring that the expressed protein retains its functional properties for potential applications in research or diagnostics.
4. **Discussion and Conclusion**: New developments in the expression of hepatitis B surface antigen (HBsAg) have shown that it is possible to make this important protein without its transmembrane domains (TMDs). This achievement is significant as it opens new avenues for vaccine development and diagnostic applications, allowing for the generation of virus-like particles (VLPs) that can elicit robust immune responses without the complications associated with full-length HBsAg expression. Strategic mutations and modifications that enable proper folding and assembly have facilitated the successful expression of transmembrane-less HBsAg. By removing the TMDs, we have minimised issues related to misfolding and aggregation, which are common challenges when expressing full-length proteins. This approach has led to the generation of soluble forms of HBsAg that retain immunogenic properties, making them suitable for use in vaccines (Berkower et al., 2010), (Liu et al., 2024). However, while we have achieved successful expression, one of the primary challenges that remain is optimizing the protein's yield. Current production levels are not yet sufficient to meet the demands for large-scale vaccine manufacturing or diagnostic applications. The low yield can be attributed to several factors, including the inherent instability of the protein in solution and potential inefficiencies in the expression system used (Berkower et al., 2010), (Alpsoy et al., 2022).

   In conclusion, we have successfully expressed HBsAg without transmembrane domains; further optimization is crucial to improving protein yields. Addressing these challenges will be essential for advancing the practical applications of this technology in vaccine

development and diagnostic tools for hepatitis B virus infection. As for other protein of interest with multiple transmembrane domains, which makes the expression of the protein a difficult task, this method can be applied to express the proteins effectively. Continued research and innovation in this area hold promise for enhancing our ability to combat HBV effectively (Wu et al., 2018).

Tables and figures:

Original HBsAg sequence	Altered HBsAg sequence
MENITSGFLGPLLVLQAGFFLLTRILTIPQSL DSWWTSLNFLGGTTVCLGQNSQSPTSNHSP TSCPPTCPGYRWMCLRRFIIFLFILLLCLIFLL VLLDYQGMLPVCPLIPGSSTTSTGPCRTCMT TAQGTSMYPSCCCTKPSDGNCTCIPIPSSWA FGKFLWEWASARFSWLSLLVPFVQWFVGL SPTVWLSVIWMMWYWGPSLYSILSPFLPLL PIFFCLWVYI *Highlighted in Yellow= Transmembrane domains sequences.	MENILTIPQSLDSWWTSLNFLGGTTVCLGQ NSQSPTSNHSPTSCPPTCPGYRWMCLRRDY QGMLPVCPLIPGSSTTSTGPCRTCMTTAQG TSMYPSCCCTKPSDGNCTCIPIPSSWAFGSV IWMMWYLWVYI

Table 1. *Left- Original Amino sequence of HBsAg (226 AA). Right- HBsAg sequence after removal of transmembrane parts (132 amino acids).*

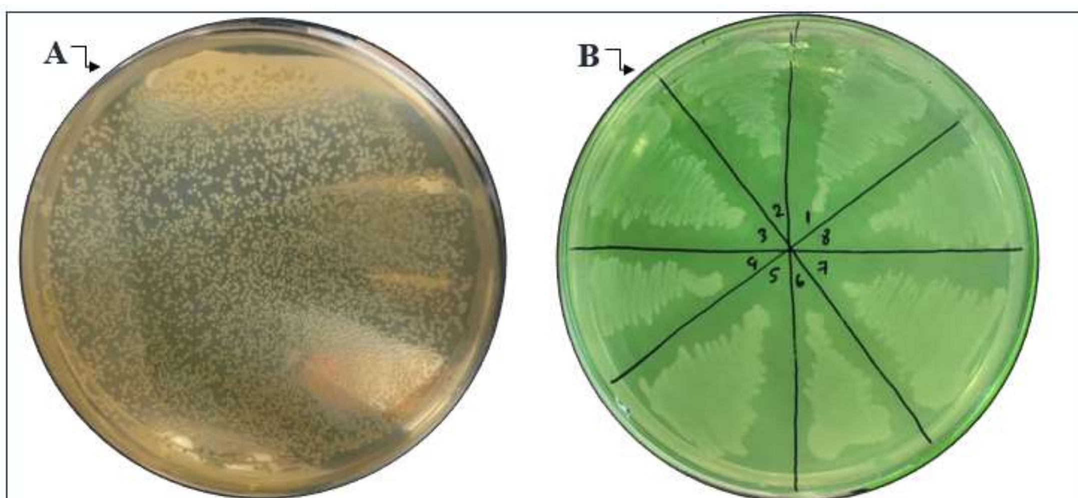

Figure. 1. *Transformation and selection of clones.* ***(A)*** *Plate showing transformed colonies on LB Agar + ampicillin plate.* ***(B)*** *Image showing single colonies restreaked from Plate A.*

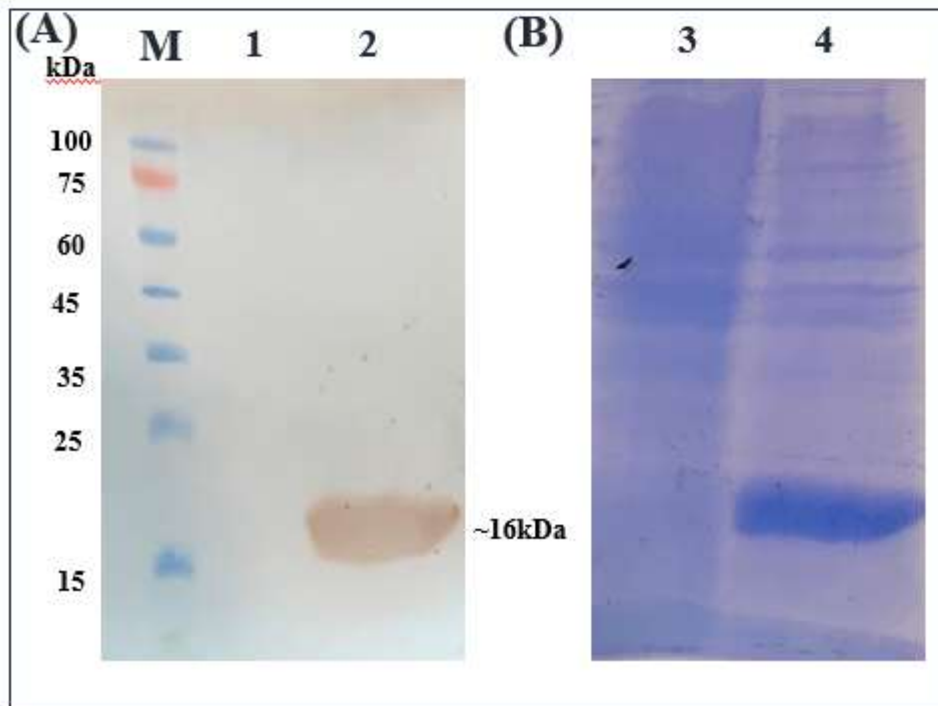

Figure. 2. ***SDS-PAGE profiles of expressed HBsAg in E. coli BL21 (DE3).*** *The plasmid containing bacterial culture was induced with IPTG and without IPTG. (A) Lane 1 and lane 2 shows uninduced and induced sample detected with Anti-his tag antibody, respectively, and lane 3 and lane 4 shows uninduced and induced samples after Coomassie blue staining.*

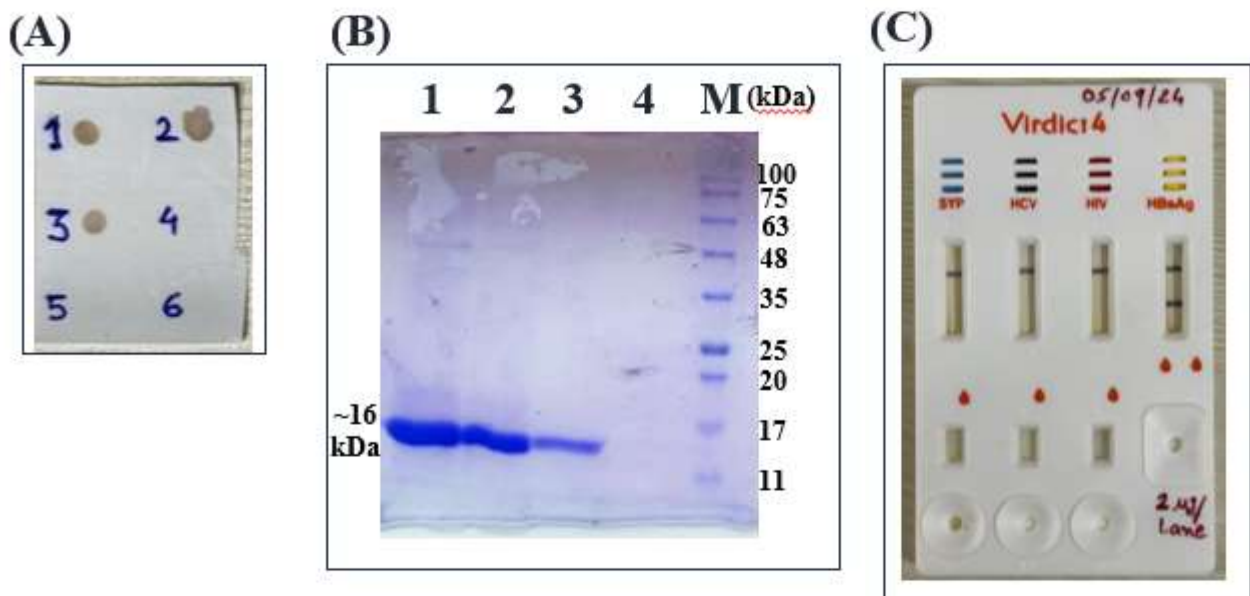

Figure 3. (A) ***Western blotting analysis of purified HBsAg*** *(No. 1-6: 2µL sample loaded from each Eluted fraction of HBsAg from Ni-NTA column). Sample was loaded on NCM and treated with anti-his secondary antibodies before developing with diaminobenzidine (DAB) substrate in Western blotting.* **(B)** ***SDS-PAGE results showing expression levels of soluble HBsAg****. Various concentration of purified antibody loaded onto 12% polyacrylamide gel and visualised after Coomassie blue staining. Lane 1= 5µg Sample, Lane 2= 2 µg sample, lane 3= 1 µg sample, Lane 4= No sample and M= Molecular Marker.* **(C)** ***Protein reactivity check.*** *2 µg sample was loaded onto each sample loading place on Virdict 4 and developed according to manufacturer's instruction. Sample was found to be reactive against HBsAg and no other cross reaction was observed.*

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