



## Short Communication

# Bacterial Expression and Purification of Antigenic Recombinant Protein Encoded by Hepatitis Delta Virus Antigen of Pakistani Isolate

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## ABSTRACT

HBV is a global burden and co-infection of HBV/HDV causes additional risks and mortality rate. Currently, there is no vaccine available for HDV, therefore, treatment of patients with dual infection is indeed a challenge for clinicians. A timely and accurate diagnosis is critical for treatment in this regard. Expression and purification of HDAg, which is the only protein of HDV of the local isolate is the primary objective of the study. This antigenic recombinant HDAg protein can be useful for both vaccine development and as a diagnostic marker of HDV. After determination of HDAg antigenic region of HDV and its amplification, the fragment was cloned in bacterial expression vector. The expression of recombinant protein was checked by SDS-PAGE and after the purification of protein by using Ni-affinity column. antigenic protein was confirmed with Western Blot analysis. The study demonstrates the expression and purification of HDAg recombinant protein of HDV local isolate in the bacterial expression system. The purified protein has potential to be useful as an antigen in Diagnostic ELISA and as a vaccine candidate.

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## Authors' Contribution

SR and MI planned the proposed study. IA, NA, MAK and TRS contributed to the lab work. IA, MS and SA wrote the article. All authors have read and approved the final manuscript.

## Key words

Hepatitis B virus, Hepatitis delta virus, HDAg recombinant protein, Bacterial expression

Currently, about 3.5% to 6.5% people are infected with Chronic hepatitis B virus (HBV) worldwide (Schmit *et al.*, 2021). Out of these HBV positive patients, about 16.4% are further co-infected with hepatitis D virus (HDV) (Stockdale *et al.*, 2020). HDV is a satellite virus of HBV sharing the same envelope proteins as HBV (Tahaei *et al.*, 2014). Chronic HBV infection has been considered a major cause of liver cirrhosis and HBV/HDV super-infection confers an additional risk which ultimately progress to associated complications like hepatocellular carcinoma (Zhao *et al.*, 2017). Although the epidemiological data reveals that there is a decline in HBV and HDV prevalence particularly in developed countries, but, in developing countries, the situation is worse which accounts for a major contribution on the overall Hepatitis burden in the world. Likewise, according to national survey of Pakistan Health and Research Council, an overall prevalence of HBV is

2.5% in general population (Shafique *et al.*, 2020), whereas the co-infection of HBV/HDV was 14.66% in HBV positive patients with the most prominent HDV genotype 1 (Aftab *et al.*, 2018).

Fast and accurate diagnosis is critical to check the prevalence of disease and for early treatment of the patient. For diagnosis of HDV, mainly two serological markers are used, which include the HDV antigen (HDAG) and anti-HDV antibody. The availability of antigenically active HDV antigen (HDAG), which is the one and only protein of HDV, is crucial for early diagnostic methods like ELISA. For improved quality and better quantity of HDAG, different heterologous systems of protein expression have been used (Rizzetto, 2015). HDAG has two isoforms, the large form L-HDAG (214 amino acids) and the small form S-HDAG (195 amino acids). Previously, both small and large forms of HDAG have been expressed in recombinant baculovirus (Pascarella and Negro, 2011). Likewise, the production of polyclonal anti-HDV antibodies in rabbits has been achieved by purifying small form of HDAG (S-HDAG) from *E. coli* and this has been used in different HDV-specific immune assays (Tunitskaya *et al.*, 2016). Presently, no studies from Pakistan are available for cloning and expression of antigenic recombinant protein (HDAG) of HDV genotype 1 of local isolate as this is an initial step towards vaccine development and for early

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HDV detection. The major objective of this study was to express and purify of diverse antigenic regions located in recombinant HDAg of the local predominant HDV genotype in prokaryotic expression system which will be useful to raise antibodies to block these antigenic spots and for designing effective and low-cost diagnostic in-house assay, in future.

#### Materials and methods

Patient's serum samples positive for HBV were collected from Genome Centre of Molecular Diagnostic Laboratory, Lahore, and further analyzed for HDV by nested PCR by using antisense primer for cDNA synthesis and forward and reverse primers in PCR as described by Butt *et al.* (2014). Genotyping of positive HDV samples was checked by sequencing method by using two sets of primers as described by Aftab *et al.* (2018). The sequencing reaction was run on a 3100 Automated Genetic Analyzer (Applied Biosystems Inc.). The samples having genotype 1 of HDV were further used for the study.

HDAg gene of HDV genotype 1 samples was amplified by using inner sense and antisense as described above. The most conserved region of HDAg gene was considered and the antigenic sites were determined by using software Foldindex (biportal.weizmann.ac.il/fldbin/findex). The software highlighted the folded and unfolded region of the protein sequence. The unfolded region represents the hydrophobic region of the protein and is considered as the proposed antigenic sites of the protein. The larger antigenic part of HDAg gene was amplified with forward primer as 5'-GCCATATGGCCGCCATGCCGGCGACCCTGCTG3' having restriction site of NdeI enzyme on 5' site and reverse primer 5'-GCGGATCCAAAGCGCGGCGGCAG3' having restriction site of BamHI on 3' site.

The amplified region of HDAg-An gene was cloned in pET-28a expression vector. The amplified product of gene was treated with NdeI and BamHI restriction enzymes. The bacterial expression vector pET-28a was also treated with the same restriction enzymes and the digested HDAg-An product was ligated to freshly digested pET-28a vector. The constructed plasmid was cloned in freshly prepared Top10 bacterial competent cells and confirmed by gene specific PCR, restriction digestion analysis, and the right orientation of inserted gene by DNA sequencing.

Transformation of constructed plasmid was carried out using competent Rosetta2 DE3 cells and 1 µl of isolated plasmid of pET-28a harbouring antigenic HDAg gene (HDAg-An), following heat shock for 90 seconds at 42°C. 900 µl of LB medium was added and incubated at 37°C shaker for 1h. Subsequently, about 100 µl of transformant of truncated HDAg-An was spread on LB-agar plates containing kanamycin and chloramphenicol (50mg/ml) each and incubated overnight. Single colonies

were selected for expression studies.

To check the expression of recombinant HDAg-An, Rosetta2 DE3 transformed single colonies were picked and inoculated in 5ml LB media containing antibiotics (kanamycin and chloramphenicol) and then incubated at 37°C in a shaking incubator for overnight. Next day, 1ml of this culture was pipetted out and diluted in the ratio of 1 to 50 in freshly autoclaved 50ml LB medium with selective antibiotics. The culture was allowed to grow for 3-4 h at 37°C shaking incubator to achieve the optical density at approximately 0.6 and for maximum production of truncated HDAg-An gene, the culture was induced with 1mM IPTG for overnight. Next day, the cells were harvested by centrifugation at 5000rpm for 10min at 4°C. After washing twice with 1X PBS, the pellet was resuspended in 100 µl 1X PBS and 100 µl of 2X loading dye with 2-mercaptoethanol was added in it. Then the protein sample was heat shocked at 100°C for 10 min and immediately placed on ice. To check the expression of recombinant HDAg-An protein, the prepared sample was centrifuged at 14,000rpm for 2min at 4°C and 10 µl of supernatant was pipetted out and loaded to run on 12% SDS-PAGE gel for about 45 min. The gel was stained with Coomassie blue R250 dye and then de-stained with de-stain solution containing methanol and acetic acid. SDS-PAGE gel was then visualized for the required protein band by comparing it with unstained protein ladder.

After checking the expression, the truncated recombinant protein was purified with Ni-Sepharose column on AKTA Explorer. Protein supernatant was poured on to the column and allowed to run. After the sample was completely poured, the column was washed with washing buffer (1X PBS) following 0.01M Imidazole to eliminate the unbound protein and then with 0.5M imidazole to eliminate loosely bound proteins. Finally, 2M imidazole was used to elute the required, conjugated His-HDAg-An truncated protein at pH 7.4.

Purified HDAg-An protein was run on 12% SDS-PAGE for confirmation of required protein band and compared it with unstained protein marker. The confirmed protein sample was further used for western blot analysis. The protocol followed for western blotting was same as in SDS-PAGE, but the pre-stained protein marker was used to compare the results. Protein was run on the gel and then transferred to a nitrocellulose membrane by sandwiching the membrane between blotting papers. Semi-dry blotting apparatus (Biorad, USA) was used to transfer the protein at 12V for 45min. After that the membrane was blocked with 5% BSA (bovine serum albumin) at 4°C overnight. Next day, the nitrocellulose membrane was washed twice with PBST for 5 min and then incubated with mouse anti-His6 monoclonal antibodies at 4°C for 2h. The membrane was washed again with PBST and secondary antibody

IgG was used in (1:10000) dilution for 1 h at room temperature. Secondary antibody was conjugated with alkaline phosphatase (AP). The membrane was then treated with BCIP/NBT (Sigma Fast) substrate for 15 min at 37°C, dried and then photographed.

### Results

*HDag* gene fragment of size 642bp was confirmed by sequencing analysis and the sequences were submitted in GenBank at NCBI with accession no. MW176094-MW176096A. Antigenic gene fragment of HDag-An of size 402bp was amplified by PCR by using antigenic specific primers (Fig. 1) and this PCR product have restriction sites on both 5' and 3' directions and a 6xHis-tag (Hexa Histidine tag) residue on C-terminal.

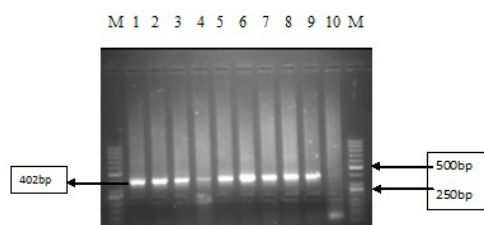


Fig. 1. PCR amplification of *HDV* gene fragment, Lane 1-9, HDV DNA positive of 405bp and Lane 10 showing negative control, Lane 11, DNA marker of 50bp.

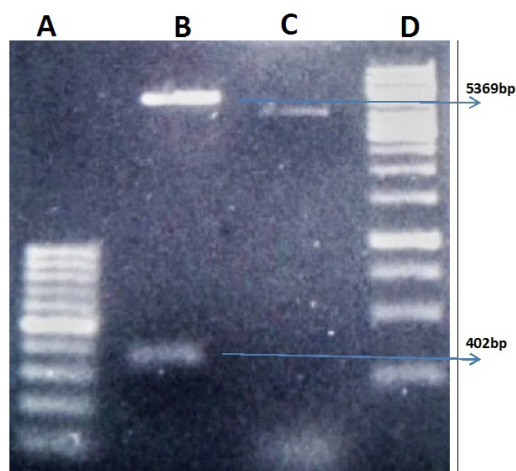


Fig. 2. Restriction digestion analysis of clone in expression vector: Lane A, DNA marker of size 100bp; Lane B, first band digested of peT28a vector of size 5369bp and second band of *HDag-An* gene fragment of size 402bp. Lane C, undigested clone; Lane D, ladder 1Kb.

Figure 2 shows the two bands of 402bp and 5369 bp in 1% agarose gel after digestion of recombinant peT28a-HDAg-An clone was double digested with *NdeI* and *BamHI*

restriction enzymes. To confirm the right orientation of *HDag-An* gene fragment in the vector, sequencing was performed by using T7 universal primers and a clone construct of size 5726bp was oriented having fragment of inserted gene (Supplementary Fig. 1). The linearized recombinant plasmid peT28a-HDAg-An was then transferred into bacterial expression host Rosetta 2 DE3 cells and the expression was confirmed by SDS-PAGE.

HDag-An protein (confirmed by SD-PAGE analysis by size) was purified by Ni-affinity chromatography and confirmed by running protein sample on 12% SDS-PAGE. The purified HDag-An truncated protein of size 14KDa was visualized (Fig. 3A). Protein was further confirmed by western blot analysis, for identification of the conjugated His-HDAg-An truncated protein and a size of 14KDa was observed (Fig. 3B).

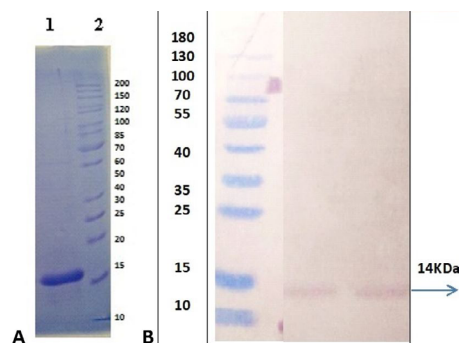


Fig. 3. A, SDS-PAGE of purified protein showing expression of 14KDa protein fragment. B, Western Blot representing 14KDa protein fragment of HDag-An purified protein.

### Discussion

Rapid, accurate, and economical diagnosis always matters for the early treatment of disease. The serological diagnosis of HDV is mainly based on two types of serological markers, i.e., HDV antigen (HDAg) and anti-HDV antibody (anti-HDV) (Shattock and Morgan, 1984). Purification after expressing a recombinant HDag can be a candidate protein in the diagnostic ELISA application for the HDV infections. As HDag comprises of two isoforms, L-HDAg and S-HDAg and both forms of HDag has been expressed previously in recombinant baculoviruses in order to further understand the structure and biological properties of HDag (Pascarella and Negro, 2011). The S-HDAg protein, which is smaller in size than L-HDAg but contains all the HDV antigenic epitopes which proves its ability as diagnostic marker (Ding *et al.*, 2014). The production of polyclonal anti-HDV antibodies in rabbits has also been achieved by purifying small form of HDag (S\_HDAg) from *E. coli* and this has been used in different

HDV-specific immune assays (Tunitskaya *et al.*, 2016). But, on the other hand, the expression and purification of S-HDAg are not very effective (Chiang *et al.*, 2006) because of deficiency of post-translational modification, if expressed in *E. coli* (Calogero *et al.*, 1993; Sheu and Lai, 2000) and early degradation of protein, if expressed in baculovirus or insect cells (Hwang and Lai, 1993). These limitations may lead to the partial characterization and immunological assessments of S-HDAg. For detection of anti-HD in human serum, we have utilized, for the first time, a specific recombinant HDAg having antigenic spots and cloned from local strain of HDV.

In comparison to eukaryotic system, prokaryotic expression systems especially *E. coli*, have more advantages like cost-effectiveness, fast, and a trouble-free production. Previously, *E. coli* system has been used for effective preparation of foreign proteins and for expression studies of S-HDAg protein (Ding *et al.*, 2014), in another study S-HDAg has been expressed and purified in *E. coli* to generate polyclonal anti-HDV rabbit antibodies, the study demonstrate that these antibodies could be used in different types immune assays for HDV detection (Tunitskaya *et al.*, 2016). Considering these studies, we also used prokaryotic system for expression of recombinant antigenic HDAg derived from a local predominant strain of HDV genotype 1 and purified it using Ni-affinity chromatography. This recombinant antigen can be a milestone for further study in development of in-house assay for detection of antibodies as well as a candidate for vaccine development against HDV.

### Conclusion

The purified antigenic recombinant protein of local isolate has a potential to be use a serological marker for in-house assay development against HDV local predominant genotype and it can also be useful to raise antibodies in animal models.

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### Supplementary material

There is supplementary material associated with this article. Access the material online at: <https://dx.doi.org/10.17582/journal.pjz/20210712110746>

### Statement of conflict of interest

The authors have declared no conflict of interest.

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