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Improving Access to Anti-HDV Testing: Development and Validation of an Affordable In-House ELISA Assay

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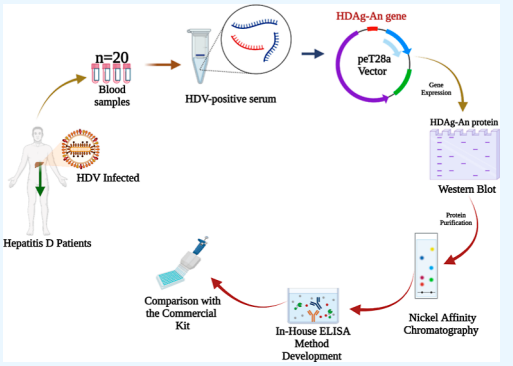
ABSTRACT: In certain low-income nations, the hepatitis Delta virus and hepatitis B virus (HBV) pose a serious medical burden, where the prevalence of hepatitis B surface antigen (HBsAg) is greater than 8%. Especially in rural places, irregular diagnostic exams are the main restriction and reason for underestimation. Utilizing serum samples from a Pakistani isolate, an internal ELISA for the quick identification of anti-HDV was created, and the effectiveness of the test was compared to a commercial diagnostic kit. HDV-positive serum samples were collected, and a highly antigenic domain of HDAg antigen was derived from them. This antigenic HDAg was expressed in a bacterial expression system, purified by Ni-chromatography, and confirmed by SDS-PAGE and Western blot analysis. The purified antigen was utilized to develop an in-house ELISA assay for anti-HDV antibody detection of the patient's serum samples at very low cost. Purified antigens and positive and negative controls can detect anti-HDV (antibodies) in ELISA plates. The in-house developed kit's efficiency was compared with that of a commercial kit (Witech Inc., USA) by the mean optical density values of both kits. No significant difference was observed (a *P* value of 0.576) by applying statistical analysis. The newly developed in-house ELISA is equally efficient compared to commercial kits, and these may be useful in regular diagnostic laboratories, especially for analyzing local isolates.



1. INTRODUCTION

Hepatitis delta infection (hepatitis Delta virus (HDV)) disease may be a noteworthy therapeutic burden, particularly in less affluent nations of the world, but epidemiological information is, to a great extent, deficient and arranged. Since its discovery in 1977 in Italy, HDV has been documented as an infectious agent worldwide.[1] HDV is a satellite virus that can infect and multiply only in the presence of Hepatitis B virus (HBV) surface antigens. HDV is the least important human virus, although coinfection (when HBV and HDV are present at the same time) and superinfection (when both viruses are present) may cause serious health problems. HDV infection in chronic HBV patients[2] is a severe form of hepatitis that often ends in liver cancer, cirrhosis, and death.[3] Estimates place HDV as the cause of 20% of HBV-related hepatocellular carcinoma (HCC) and 18% of liver cirrhosis.[4] It is estimated that 300 million people have chronic HBV infection globally,[4] and the number of annual deaths caused by viral hepatitis is 1.34 million, of which 66% are credited to HBV infection alone.[5] According to the WHO 2021 report, almost 5% of HBV-positive patients are further coinfected with HDV, which leads to disease severity. A global survey reported that 48 to 60 million individuals are infected with HDV, but low-income countries, particularly in Asia and Africa, are badly affected, as a study conducted in 2017 reported the presence of 7 million HDV infections in the sub-Saharan Africa region alone.[6]

HDV infection is declining due to successful HBV vaccination programs, particularly in the urban populations of high-socioeconomic nations, because the most effective way to prevent HDV infections is through HBV vaccination, which works by reducing the number of HBsAg carriers. So far, where the incidence of HBsAg is decreasing, the prevalence of HDV is also decreasing, and thus the protection of younger generations is increasing.[7] Only 0.02% of the 52,00 HBsAg carriers in the United States are also anti-HDV positive;[8] nevertheless, HDV prevalence rates as high as 30% and as low as 43% have been observed in Tunisia and Upper Egypt, respectively.[9] In low-income countries like Pakistan, where

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14.6% of HBsAg-positive people are also infected with HDV, HBV remains a serious health concern.[10]

The key factor leading to underestimating the HDV prevalence rate is the lack of rapid and extensive anti-HDV testing in HBsAg-positive individuals.[11] Currently, there is a wide variation in the sensitivity of various HDV RNA assays, and there is no global uniform detection standard for similar assays between laboratories. Furthermore, lack of access to healthcare in low- and middle-income countries often results in underdiagnosis of the disease, preventing early detection and effective treatment.[12] HDV significantly enhances the risk of HCC and is the key determinant in the rapid progression of cirrhosis in HBsAg-positive people compared to HBV alone.[13] Promoting data collection and identifying HDV's exact epidemiology and clinical implications requires raising public knowledge of anti-HDV screening, especially in HBsAg-positive patients. In routine diagnostics, two serological strategies have been utilized to detect HDV in serum, including anti-HDV antibodies and HDAg antigen in ELISA. Among other types of hepatitis, HDV diagnostics are not widely available, and it is considered a neglected human pathogen, although HDV infection is much more prevalent in women, particularly in pregnant women in Pakistan.[14] Pakistan is an underdeveloped country with very limited health facilities and faces a huge burden of infectious diseases, including viral hepatitis. These people, instead of keeping a check on their health conditions, try to struggle for their family's survival. These realities motivate us to develop an in-house diagnostic tool to detect HDV infection in a local population at a low cost.

To establish a quick and cost-effective in-house assay for detecting HDV local isolates, a recombinant HDAg antigen has been cloned and expressed in a prokaryotic system. This purified antigen was utilized in a particular and sensitive ELISA method to diagnose anti-HDV antibodies. This newly developed in-house ELISA was compared to a commercially available kit for detecting anti-HDV antibodies.

2. MATERIALS AND METHODS

2.1. Patients and Samples. At first, five HDV-positive serum samples with high viral titers confirmed by polymerase chain reaction (PCR) were obtained to derive HDAg antigenic gene fragments using an online tool to clone and express them in a bacterial expression system and purified. Furthermore, 20 anti-HDV positive serum samples were collected to detect anti-HDV on an in-house ELISA microtiter plate. Two negative controls of healthy individuals confirmed negative for HBV and HDV infections and one HDV-positive control were taken on an ELISA plate along with the patient's sera. All of the sera were collected from Genome Centre for Molecular Diagnostics and Research, Lahore.

2.2. Production of Recombinant HDAg Gene. HDV-positive samples were utilized to produce antigenic recombinant HDAg (named HDAg-An) by using antigenic-specific primer pair 5′GCCATATGGCCGCCATGCCGGC-GACCCTGCTG3′and 5′GCGGATCCAAAGCGCGGCGG-CAG3′ by PCR using specific conditions. This HDAg-An gene fragment of 402bp was excised and eluted from agarose gel using a GeneJET Gel Extraction Kit (Catalog number: K0691) and cloned in the bacterial expression peT28a vector. The clone was confirmed by restriction digestion analysis using the restriction enzymes NdEI and *Bam*HI and by Sanger sequencing.

2.3. HDAg-An Protein Expression. The constructed clone harboring HDAg-An in the peT28a vector was transformed in the bacterial expression system of Rosetta2 DE3 cells and then induced with isopropyl β- d-1-thiogalactopyranoside (IPTG), and SDS-PAGE checked the expression. HDAg-An protein was then purified using Ni-affinity chromatography and eluted in 2 M imidazole at pH 7.4. This purified HDAg-An protein was confirmed by Western blot analysis by running on 12% SDS-PAGE and by treating the nitrocellulose membrane with mouse anti-His6 monoclonal antibodies and IgG secondary antibodies conjugated with alkaline phosphatase. The membrane was at that point treated with the BCIP/NBT (Sigma Fast) substrate and, after that, visualized.

2.4. HDAg-An Protein Purification. *2.4.1. Biomass Production and Lysis.* The contents of two more LB flask media (inoculated with confirmed clones and activated with IPTG) were mixed in two 2 L flasks, and the resulting biomass was incubated in an ON culture at 370 °C in a shaker. The cells were extracted from the culture by centrifugation at 3500 rpm for 5 min the next day. After collecting the biomass, we dissolved it in 1× PBS at pH 7.4 and subjected it to 1.5 mPa of pressure from a "Constant Cell Disruption System" to lyse the cells. To separate the supernatant from the inclusion bodies, the lysate was spun at a high speed (12,000 rpm).

2.4.2. Nickel Affinity Chromatography. After the Ni-sepharose column on the AKTA Explorer was equilibrated with 1× PBS at pH 7.4, the supernatant was injected into the column. Second, the column was washed with buffer A, which was 1× PBS at pH 7.4. After UV stabilization, a second wash in 1× PBS at pH 7.4 containing 10 mM imidazole was done, and finally, elution was accomplished in 1× PBS at pH 7.4 containing 500 mM imidazole. The protein content and purity were determined by the Bradford assay, and SDS-PAGE and Western blot analyses were performed using a commercial Ab. HDAg (HDVGP2) antibody (Cat no. ABIN1606178).

2.5. In-House ELISA Method Development. Overnight at room temperature, a 96-well flat-bottom microtiter plate was coated with 100 μL of pure HDAg-An and 100 μL of 1× PBS for use in an in-house ELISA. The next day, after keeping at 37 °C for two h, the wells were blocked with a 5% BSA solution, and the plate was rinsed three times with PBS to get rid of any remaining blocking buffer. After incubating the plates for an hour at 37 °C, the wells were washed three times with washing buffer, and 100 μL of human blood samples (anti-HDV antibodies) were added to each well (both for patients and the negative control). Secondary antibody conjugate, including anti-delta (anti-HDV) antibodies that were horseradish peroxidase (HRP)-conjugated, was added to each well at a concentration of 50 μL. After 1 h in a 37 °C incubator, the plate was read. The plate was washed four times with at least 380−400 μL in each well and then aspirated after soaking in the same washing solution for 40 s. Each well received 100 μL of the active 3,3',5,5'-tetramethylbenzidine (TMB) substrate combination. The plate was then placed in the dark at 18 to 24 °C for 20 min. SpectraMax Plus 384 spectrophotometer readings were taken at 450 nm after 50 μl of a 0.75 M/L sulfuric acid solution was used to stop the process.

2.6. Comparison with the Commercial Kit. Following the manufacturer's instructions, we compared the findings of our in-house ELISA with those obtained using a commercial kit for the detection of antibodies to the HDV in human serum (EIA-ANTI-HDV; Witech Inc., USA, ref# D-152/1.0.).

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Samples were run in triplicate by ELISA on both kits to confirm the reproducibility of the results. The in-house and commercial ELISA kits' mean optical density (OD) values were observed for further comparison.

A valid test requires an OD value for the positive control to be >0 8 and an OD value for the negative control to be less than 0.2. The sample is considered positive if the OD value is higher than or equal to the threshold value.

2.7. Statistical Analysis. The in-house ELISA kit and the commercial kit were compared to one another by using the paired T-test to see whether there were statistically significant differences in the OD values of the patients. All statistical analysis was performed in IMB SPSS version 22.[15]

3. RESULTS

3.1. HDAg-An Antigen. HDAg-an gene fragment of size 402bp (after purification from PCR gel) was cloned in the peT28a expression vector, and the Rosetta2 DE3 cells were utilized as the host system for efficient bacterial expression. The expression was confirmed by analyzing protein samples on 12% SDS-PAGE, and the ∼14 kDa protein bands in all positive clones were observed (Figure 1).

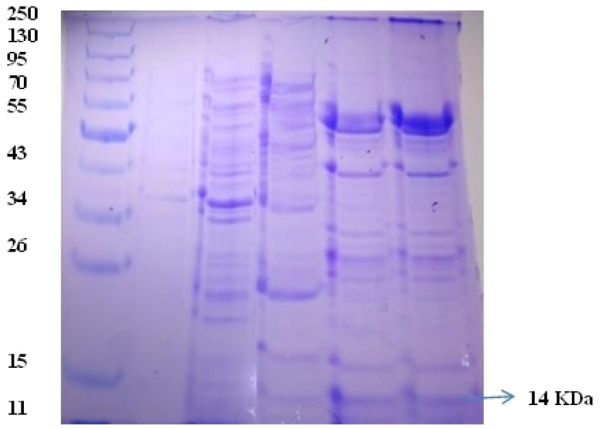

Figure 1. Lane 1 is a prestained protein ladder (Thermo Scientific 26,635), Lane 2 is a negative control, and Lanes 3, 4, and 6 show the expression of a protein with a molecular weight of 14 kDa (as measured by SDS-PAGE).

3.2. Purification of HDAg-An Antigen. The HDAg-An protein was isolated and purified via immobilized metal affinity chromatography and Ni-affinity chromatography. Total HDAg-An recombinant purified protein was 98 mg/L of bacterial culture with a purity of 95%, and the overall yield was 33%. A summary of the chromatography results is provided in Table 1.

The purified protein was run on 12% SDS-PAGE and shifted to a nitrocellulose membrane for Western blot analysis. After the nitrocellulose membrane was treated with HDV-specific primary and IgG secondary antibodies and then with the substrate, a clear band of ∼14 kDa molecular weight was observed. The purified HDAg-An protein was utilized for the development of the in-house ELISA assay.

3.3. In-House ELISA for Anti-HDV and Its Comparison. To check the antigenic response in human serum against anti-HDV by ELISA, positive and negative serum samples were used in a microtiter plate, which was already coated with the purified antigenic protein HDAg-an. The same procedure was followed for the commercial ELISA kit. The antibodies were detected in both an in-house ELISA assay plate and a commercial ELISA kit plate. ELISA was run in triplicate, and the mean OD values of both patients and controls were obtained, as shown in Table 2. A graphical line representation of the OD values of samples P1−P20 of both the in-house kit and the commercial kit can be observed in Figure 2.

Table 1. Purification Summary of HDAg-An Protein via Ni-Affinity Chromatography

step	total protein[a] (mg)	HDAg-An protein[b] (mg)	purity[c] (%)	step yield[d] (%)
biomass	435 (+3.2)	333 (+2.1)	40 (+2.7)	92 (+4.0)
lysate	413 (+3.7)	301 (+2.9)	51 (+2.7)	96 (+2.6)
IMAC purification	375 (+2.4)	98 (+2.3)	95 (+3.2)	33 (+3.3)

[a]Total protein was determined by Bradford method. [b]HDAg-An was calculated by quantitative RP-HPLC. [c]Purity was assessed by SDS-PAGE. [d]Step yield was calculated based on difference in out/input step.

Table 2. Comparison of Data of the In-House ELISA Kit and Witech Inc. USA Kit

samples	in-house ELISA (OD Values)	Witech Inc (OD values)
N1	0.134	0.053
N2	0.144	0.055
PC	2.482	2.721
P1	3.12	2.686
P2	3.091	2.552
P3	2.858	2.389
P4	3.183	2.304
P5	2.913	3.665
P6	3.06	2.621
P7	3.087	3.845
P8	3.066	2.575
P9	3.169	2.58
P10	2.941	2.845
P11	3.432	2.952
P12	2.652	2.765
P13	2.544	2.654
P14	2.771	3.042
P15	2.986	2.983
P16	3.453	3.764
P17	3.231	3.453
P18	2.663	2.843
P19	2.876	2.901
P20	2.921	3.564

3.4. Statistical Analysis. Patients' OD values were compared between the in-house kit and a commercial kit using a paired *t*-test to see whether there was a statistically significant difference. Tables 3 and 4 show results that are quite comparable across the two kits; hence, there was not a significant difference between them (*P*-value = 0.576).

4. DISCUSSION

Many infectious diseases, including viral hepatitis, have struck developing countries with inadequate healthcare resources. Infection with the HDV is more prevalent than infection with other types of hepatitis, particularly among pregnant women in countries like Pakistan, Indonesia, and Saudi Arabia.[14,16] Among other countries, Pakistan has been confronting budgetary limitations since the Covid-19 pandemic. In

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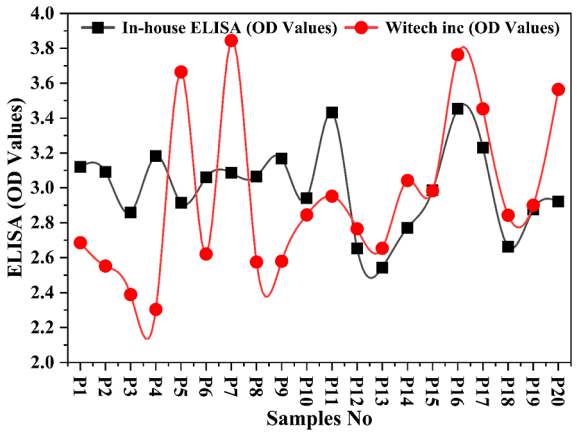

Figure 2. On the graph, the line represents the average OD values of samples P1−P20, measured with the in-house and commercial (Witech Inc. USA) kits.

Table 3. Comparative Analysis of OD Values from Two Kits (Witech Inc. USA and In-House ELISA Kit)

kit name	minimum	maximum	mean	std. deviation	std. error mean
Witech Inc. USA	2.54	3.54	3.0053	0.24948	0.05579
in-house ELISA	2.30	3.84	2.9455	0.46489	0.10395

Table 4. In-House ELISA Kit OD Values Compared to Those from Witech Inc., USA (Paired T-Test)

paired sample	mean difference	degree of freedom	T-test value	std. error mean	*P*-value
Witech Inc. USA vs in-house ELISA	0.05985	19	0.569	0.10522	0.576

particular, lower-middle-class individuals, rather than keeping a check on their well-being conditions, attempt to battle for their family's survival. These considerations encourage us to develop an inexpensive in-house diagnostic technique for detecting HDV infection in the local populace.

An accurate and cost-effective diagnostic assay is imperative to check the prevalence of infection on a large scale for the early treatment of patients. During infection, HDV is known to express only its known protein, HDAg. Obtaining extremely pure and antigenic HDAg recombinant protein may be necessary to develop an ELISA-like diagnostic method for anti-HDV antibodies, particularly if the titer is about 1:1000.[17] There has to be a lot of particular and minimally antigenic viral protein available for the creation of enzyme-linked immunosorbent tests[18] and radioimmuno assays.[19] Previous HDAg production has been difficult for diagnostic purposes because of the possible biohazards involved in using human serum, biopsy samples, or animal HDAg.[20]

The extraction and purification of HDAg from the bacterial expression system ensure the total and harmless production of antigens, which could be utilized for anti-HDV antibody detection.[21] Purified, recombinant HDAg shows a good antigenic response and high affinity toward anti-HDV in HDV-positive serum.[17] The *Escherichia coli* system has been utilized in previous studies to rapidly prepare foreign proteins and in expression studies of the S-HDAg protein.[22] S-HDAg has also been expressed and purified in *E. coli* to generate polyclonal anti-HDV rabbit antibodies, and the results demonstrated that these antibodies could be utilized in different types of immune assays for HDV detection.[23] These findings corroborate our decision to use a rapid, cost-effective, and biohazard-free bacterial expression technique to produce a highly antigenic region of HDAg-an and purify it to provide a sufficient supply of antigen.

Patients with HDV have been demonstrated to have a unique humoral response to HDAg, and their viremia only persists for a short period of time (a few weeks).[24] So, checking for HDAg in the patient's serum is a viable option. However, studies of nucleotide sequences have shown that HDV's genome has an exceptionally diverse RNA sequence,[25] For this reason, it would be more convenient and accurate if the HDAg was produced from a strain that is often found in the region.[21]

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).[26] In a recent study, prototype serological (IgG) and molecular (RNA) assays were developed for HDV infection and checked for high-throughput testing on Abbott m2000 and ARCHITECT systems, respectively. Although, both prototype assays were sensitive and specific enough for HDV detection, in this case, the peptide length is more crucial for reactivity, as the smaller scanning peptides cannot cover some specific regions.[27]

There are also a few healthcare challenges confronted by low-income countries in the case of viral hepatitis, particularly HDV-like. Molecular detection and genotype analysis are not routinely performed, and in this manner, important data are exceptionally constrained. Furthermore, the testing frequently included possibly one-sided populations, such as medical outpatients or health-care workers.[28]

As the HDV antigen (HDAg) has two isoforms, both of these forms of antigen, small or large, are capable of stimulating antibody production and can be used as a serum biomarker for HDV detection, regardless of coinfection or superinfection. S-HDAg is necessary for HDV RNA replication, but on the other hand, L-HDAg inhibits HDV replication but is crucial for the assembly of virions.[12] In light of these results, we used a local strain of HDV genotype-1, which is the only prevalent genotype in Pakistan,[29] and it was observed that the domestically produced recombinant HDAg-an, which was obtained from the most conserved region of HDV, would be more helpful in creating an in-house ELISA test. The newly designed ELISA test may have more potential and specificity for screening anti-HDV antibodies than the presently available kits.

5. CONCLUSIONS

Developing a cost-effective in-house diagnostic tool to detect HDV infection in underdeveloped countries is essential. An accurate and cost-effective diagnostic assay is necessary to identify the prevalence of infection on a large scale for early patient treatment. Obtaining highly purified and antigenic HDAg recombinant protein is crucial for developing diagnostic methods such as ELISA for anti-HDV antibodies. The extraction and purification of HDAg from a bacterial expression system are rapid, inexpensive, and biohazard-free, making it ideal for the safe and unlimited production of antigens. The study concluded that the recombinant HDAg-An derived from a local strain was more useful for the establishment of an in-house ELISA, and utilizing the same antibodies and other reagents (as used in the commercial kit) in the in-house ELISA assay showed more specificity. This

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newly developed assay has more potential for inexpensive screening as the system we tried to develop is indigenous with a very low cost per sample, i.e., 100 times cheaper than the available commercial kits, and is equal in sensitivity to a commercial kit. This HDV ELISA assay can be commercialized in the future and will offer assistance to reduce the financial burden that the government confronts in importing detection kits for HDV infection, and it can improve the diagnosis of HDV on a large scale, particularly among the poor people in Pakistan.

6. LIMITATIONS OF THE STUDY

Although the newly developed indigenous system is efficient and cost-effective, some limitations can also be faced by using this system. The antigen concentration obtained after purification is not always appreciable and needs to be improved. Especially if a person is in the gray zone, then this assay will not detect HDV infection. In order to overcome this limitation, a dual detection system for the diagnosis of both antigens and antibodies should be developed.

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Author Contributions

IA, SR, AS, and MI planned the proposal of the study. IA, SR, and MS drafted the manuscript. IA, SA, NH, ST, and MW contributed to the lab work, and RM, IA, MS, and AME analyzed the data., AA, AS, MS, and MI critically reviewed the manuscript. All authors have read and approved the final manuscript.

Notes

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