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EXPERIMENTAL WORKS

In silico Prediction and in vitro Verification of a Novel Multi-Epitope Antigen for HBV Detection[1]

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Abstract—After years of ongoing endeavors for HBV infection prognosis, diagnosis and treatment, it still remains a major health problem worldwide. About 400 million chronic carriers and an annual death rate as high as one million reflects the seriousness of the problem. Developing novel and more effective diagnostic strategies, using in silico approaches and their subsequent empirical verification, will be helpful in providing for blood supply safety, therapeutics efficacy and disease activity assessment. Exploiting various in silico tools a novel multiepitope detection construct was designed which was consisted of eight linked linear immunodominant HBV epitopes. The designed antigen was expressed in *Escherichia coli* as the host. The detection capability of the designed antigen was tested using Chemiluminescent immunoassay method. Chemiluminescent immunoassay on the expressed antigen revealed that the product may be a credible candidate for simultaneous detection of three main HBV antibodies. All three test samples in two concentrations indicated lower RLU/s in comparison to the positive control which was the direct consequence of HBV antibody detection by the designed antigen. In the present study, employing bioinformatics tools paved the way for rational design of multiepitope antigen in a more cost effective, intelligent and knowledge-based method. The obtained results could be construed as a primary proof of concept that the in silico predictions could be used as primary steps of the biological studies and their subsequent empirical conduction.

Keywords: HBV, protein structure, diagnosis, Chemiluminescent immunoassay

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1. INTRODUCTION

Hepatitis B is one of the most severe and common viral infectious diseases of the liver worldwide and is a particularly serious health threat in Asia and the third-world countries. Approximately two billion people—one third of the world's population—have been exposed to the Hepatitis B virus (HBV) at some time in their lives and an estimated 400 million of them become chronically infected with the virus. Each year over one million people die from HBV-related liver diseases, mainly from cirrhosis and hepatocellular carcinoma (HCC) [1, 2]. HBV is a member of the family of the hepadnaviridae, hepatotropic DNA viruses which are infectious for humans and a few animal species (duck, squirrel and woodchuck) [3]. HBV genome is a 3.2 kb partially double stranded DNA, replicates after an intermediate reverse transcription step, and produces various structural and non-structural peptides, including hepatitis B surface antigen (HBsAg), hepatitis X protein (HBxAg), hepatitis B core antigen (HBcAg), hepatitis B e antigen (HBeAg) and DNA polymerase [4]. HBV causes acute and chronic necroinflammatory disease, and its carriers face an increased risk of developing cirrhosis and hepatocellular carcinoma.

Transfusions of blood and blood products into patients with serious medical conditions or who are deficient in one or more blood constituents, carry risks of immunologic, physiologic and infectious complications in the recipients such as HBV. In such circumstances getting information on either the presence or absence of HBV in the serum of an infected person is vitally important. In this regard, HBV-specific assays are routinely done for several proposes including: (1) the assessment of disease activity in persistent infections, (2) monitoring of therapeutic regimes with antiviral agents and (3) evaluating the infection in a donor's blood to prevent recipients' contamination [5–8]. Five serological HBV markers are used in clinical practices, including HBs antigen (HBsAg), anti-HBs antibodies, HBe antigen (HBeAg), anti-HBe

[1] The article is published in the original.

antibodies, and anti-HBc antibodies (including total anti-HBc antibodies and anti-HBcIgM). In many cases detection (and eventually the quantification) of viral antigens and of specific antibodies in body fluids is based on the use of sandwich enzyme immunoassays (EIAs) [9].

The present commercially available immunoassays detect serological factors individually. So, they may fail because of the time in which an assay is used in the serological course of infection, kinds of mutation occurred in HBV or specific pattern of infection which may not include some of the markers. However, by designing a diagnostic multiepitope antigen which is capable of simultaneous detection of antibodies for all three main antigens of HBV these problems could be effectively overcome. In addition to high epitope density, careful choice of epitopes and use of *E. coli* system for expression provides the potential for the development of an inexpensive diagnostic antigen with high degree of detection accuracy.

The approach of multiepitope antigen design has been used in some recent studies for diagnostic purposes which is of great importance in enhancing the sensitivity and specificity of the detection and showed the potential to solve previous diagnostic problems. Using this approach it would be possible to introduce an effective antibody detection candidate [10–13]. A multiepitope antigen for antibody detection minimizes the amount of repeated testing required for detection and would be able to detect the infection in various patterns and stages of infection and possible mutations.

The present study aims to design and examine a credible multiepitope antigen for simultaneous detection of three main HBV antibodies exploiting in silico design and in vitro verification approach. Therefore, a multi-epitope peptide antigen was designed and its three dimensional (3D) structure was analyzed by molecular modeling and molecular dynamics methods in a way to be certain that it would be an ideal candidate as first step of blood screening among blood donors and blood transfusion centers. In order to design such an antigen different linear immunodominant epitopes of each antigen was taken and combined to form the final multi-epitope antigen. Then, accordingly the designed gene was synthesized and subcloned into the respective expression vector. Ultimately to evaluate accuracy of performed bioinformatic predictions concerning capability of designed antigen to HBV antibody detection, chemiluminescent immunoassay was employed.

2. METHODS

2.1. Sequence Similarity Search

A BLAST search against non-redundant protein database at http://blast.ncbi.nlm.nih.gov/Blast.cgi has been performed. The only reference sequence available for HBV accession number: NC_003977 at http://www.ncbi.nlm.nih.gov/ served as query to obtain antigen sequences belonging to 4 main serotypes (adr, adw, ayr, ayw) of HBV.

2.2. Multiple Sequence Alignment (MSA)

MSAs were executed to generate consensus sequences for each antigen which are representative of all main HBV serotypes. All MSAs were performed by Megalign tool (Lasergene software). Sequences obtained by blast search were used as query for MSAs. The same was confirmed online using the server PRALINE at http://www.ibi.vu.nl/programs/pralinewww/ and ClustalW program at http://www.ebi.ac.uk/Tools/clustalw/.

2.3. Epitope Selection

2.3.1. Literature review. Several articles were reviewed to find experimentally confirmed immunodominant linear epitopes of HBsAg, HBcAg and HBeAg.

2.3.2. Immunoinformatic analysis. Epitope prediction analysis for each of the 3 HBV antigens was separately performed and novel linear B-cell epitopes were derived from their amino acid sequences. Using the IEDB online server at http://www.iedb.org/ beta-turns, antigenicity, flexibility, surface accessibility and hydrophilicity of the antigens were evaluated under Chou and Fasman, Kolaskar and Tongaonkar, Karplus and Schulz, Emini, and Parker methods respectively. B-cell epitope prediction was done by Bepipred software at the same server. Further B-cell epitope predictions were performed by BcePred software at http://www.imtech.res.in/raghava/bcepred/ and ABCpred software at http://www.imtech.res.in/raghava/abcpred/.

2.4. Synthetic Construct Design

In order to find the best multiepitope antigen exposing all required epitopes, the epitopes were fused together using GSGSG linkers. Various physical and chemical parameters for antigen primary sequence were computed by ProtParam tool at http://expasy.org/tools/protparam.html and SAPS tool at http://www.ebi.ac.uk/Tools/seqstats/saps/.

2.5. Antigen 2nd Structure Prediction

Protein secondary structure predictions were performed using the online servers including Phyre at http://www.sbg.bio.ic.ac.uk/phyre, SOPMA software at http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html and APSSP2 at http://www.imtech.res.in/raghava/apssp2/. Alpha, beta and gamma turns predictions were performed using ALPHAPRED, BetaTpred2 (23) and GAMMAPred software at http://www.imtech.res.in.

2.6. Antigen 3D Structure Prediction and Quality Verification

3D structures of the construct were generated using homology, threading and ab initio modeling methods. Several online servers including: Swiss-model at http://swissmodel.expasy.org/, LOOPP at http://clsb.ices.utexas.edu/web/loopp_server.html, I-TASSER at http://zhanglab.ccmb.med.umich.edu/I-TASSER, Phyre at http://www.sbg.bio.ic.ac.uk/~phyre/index.cgi, SCRATCH online ab initio software at http://www.igb.uci.edu/ and LOMETS meta-server at http://zhanglab.ccmb.med.umich.edu/LOMETS/ were used. They were all employed to model the antigen with different algorithms. Quality of obtained 3D models was verified by QMEAN and Prosa servers at http://swissmodel.expasy.org/qmean/cgi/index.cgi and https://prosa.services.came.sbg.ac.at/prosa.php respectively. Selected (best model) PDB file submitted as input to the Rammpage server at http://mordred.bioc.cam.ac.uk/~rapper/rampage.php for Ramachandran plot mapping.

2.7. Molecular Dynamics and Energy Minimization of Selected Model

Both energy minimization (EM) and molecular dynamics simulation were executed to refine the selected model. MD was performed on the selected model in a vacuum by 50 ns at 310 k to obtain more stable structure and solve some energy barrier problems in the model using GROMAX software. EM profile of SPDB was calculated on the modified model to achieve more reliable results.

2.8. Properties Associated with the Final Protein

Antigen solvent accessibility evaluation of each residue was performed by NetSurf at http://www.cbs.dtu.dk/services/NetSurfP/, VADAR at http://redpoll.pharmacy.ualberta.ca/vadar/ and Discovery Studio Visualizer 2.5.5.

2.9. Construction of Expression Vector

The designed gene was synthesized and delivered to us in a cloning plasmid (between Nde1 and BamH1 restriction sites) by ShineGene Company (China). The plasmid was transformed in to *E. coli BL21* (DE3) to be amplified and extracted using a Plasmid Mini Extraction Kit from BIONEER Company (Korea). The cloning plasmid was digested with *Nco*I and *Eco*RI restriction enzymes (to cleave the gene out of the plasmid), while the pET32a (+) plasmid was digested with the same enzymes. Following the digestions the digested gene was ligated in to the digested pET32a (+) plasmid using T4 DNA ligase to get a recombinant vector. To confirm the sub-cloning process, multiple colonies were prepared, then colony PCR amplifications were performed using universal T7 promoter (forward: 5'-TAATACGACTCACTATAGGG-3') and T7 terminator (reverse: 5'-GCTAGTTATTGCTCAGCGG-3') primers. One of the confirmed colonies was sequenced to confirm the sub-cloned sequence using the same primers.

2.10. Expression and Chemi-Luminescent Assay of the Designed Antigen in E. coli

2.10.1. Protein expression. The confirmed colony was plated on the Luria-Bertani (LB) agar plate supplemented with 100 μg/mL of ampicillin. The plates were incubated at 37°C until the colonies became visible. The transformants were then grown in LB medium at 37°C while shaking, and 1 mM of IPTG was added when the culture reached the OD600 value of 0.4–0.5.

2.10.2. Chemi-luminescent assay preparation and study deign. The induced cells (4 h at 37°C) were disrupted by freeze and thaw cycle and a cell disruption buffer (containing 1 mM PMSF, 0.02% sodium azide, 1 mg/mL lysozyme and 1× PBS). Then cellular debris were removed by centrifugation (13000 g for 20 min at 4°C) while the supernatant was saved for chemi-luminescent assay. Chemiluminescent analyses would assess the detection potential of the expressed protein for each HBV antigen. A competitive assay for each antigen was planned and performed using commercial HBsAb, HbcAb and HbeAb detection kits (Autobio Diagnostics Co. China). The chemi-luminescence-based evaluation method simultaneously assessed protein expression and diagnostic potency of resulted protein.

In the case of our tests, the detection procedure was done according to manufacturer's instructions, applying expressed and control cell supernatants instead of serum samples. The HBcAb and HBeAb detection kits contain 96 well HBcAg and HBeAg coated plates, positive control solutions containing respective antigens, negative controls containing no antigens and respective reference antibodies which were HRP conjugated HBV antigen specific antibodies. The tests for the HBcAb and HBeAb were design as follow: the HBcAg and HBeAg are used to coat each well of the plates and the anti-HBc and the anti-HBe antibodies are also provided within the kit. If the reference antibodies are added and there are no interfering molecules (adding negative control solutions), these antigens and antibodies interact with each other and produce the highest Relative Light Units per second (RLU/s). However, if the samples containing our designed antigen are added along with the reference antibodies, the designed protein could bind the reference antibodies and inhibit their interactions with the coated antigens. Therefore, a decrease should be observed in the RLU/s in comparison to negative controls, because a portion of reference antibody is in interaction with the designed antigen and is removed during the washing steps. This decrease indicates that

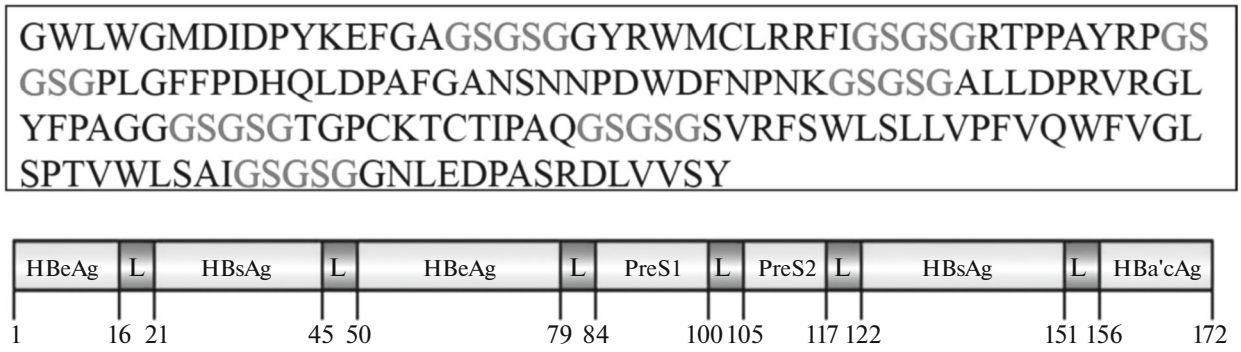

Fig. 1. The construct. The order of the epitopes in the antigen is depicted.

the antigen is expressed and more importantly is capable of detecting anti HBc and HBe antibodies. On the other hands, positive controls contain HBcAg and HBeAg which interact with the respective antibodies and produce the lowest RLU/s due to inhibition of the interaction with the coated antigens. The only difference in HBsAb kit was that the positive control solution contained HRP conjugated HBsAg instead of antibody. Therefore, in the case of HBsAb we designed another competitive assay. If the HRP conjugated HBsAg is added along with the reference antibody a sandwich between the coated HBsAg, reference antibody and the HRP conjugated HBsAg would be formed which results in the highest RLU/s. However, if these three molecules are accompanied with the designed antigen, the designed antigen would compete with the HRP conjugated HBsAg to interact with the coated HBsAg and reference antibody complex. Thus, if the designed antigen is expressed and more importantly interacts with the reference antibody, there would be a decrease in the RLU/s due to removal of unattached HRP conjugated HBsAg during the washing steps. Each sample of test and control contained equal to 0.5 and 5 mg of total protein and were applied in duplicates into the 96 well plates. Then 25 μL of chemi-luminescence substrate A and B were added to each well and incubated for 10 min at room temperature. Finally the RLU/s was measured for each well using Autobio LUMO device (Autobiolabtec instruments Co., Ltd).

3. RESULTS

3.1. Sequence Analysis

Related sequences for 4 serotypes of HBsAg, HBcAg and HbeAg were obtained by BLAST search against non-redundant protein database. This search would help to find all of the reported HBV sequences and therefore to include the most frequent sequences of all antigens. Consensus sequences obtained from each serotype MSAs were used to achieve the final consensus which is the representative of all serotypes. A consensus sequence is the rational way to have the widest coverage of the HBV sequences. MSA analysis of each antigen revealed that all three antigens were relatively conserved.

3.2. Epitope Selection

The most important features of B-cell epitope sequences were assessed to find the best epitopes of the antigens. These analyses would find the high scored epitope regions which are not included in previous studies. Although HbeAg and HbcAg have a close sequence similarity, epitopes on HbcAg are primarily conformational while HbeAg have three main epitopes two of which are linear (HbeAg-β and HbeAg-1) and HbsAg like HbcAg mostly contain conformational epitopes. Among all these reported epitopes the linear ones with no post-translational modifications were chosen for simple and more cost effective expression. Finally six reported immunodominant linear epitopes (41–45) and 2 predicted epitopes of 3 HBV antigens were selected (Fig. 1). The two predicted epitopes had the highest scores and are not overlapped with the reported ones; the results of which are summarized in Table 1.

3.3. Synthetic Gene

Checking several possible arrangement of the epitopes would provide an opportunity to find out the best order of the epitopes for detection purposes. A synthetic gene encoding multiepitope diagnostic antigen was designed based on *E. coli* codon bias. Primary sequence analysis of the synthetic construct suggests a theoretical pI of 7.91 and a molecular weight of approximately 17.9 kD.

3.4. Protein Structure Predictions

The structural predictions of the designed protein could reveal the detailed intricacies of the novel protein. This would help to analyze if the designed protein is of proper structure. Secondary structure prediction for the multiepitope antigen revealed that it mainly

Table 1. Selected epitopes. All reported and predicted epitopes with their relative position and sequence are shown

	Sequence	Position	Antigen	Refernces
1	GYRWMCLRRFI	245–255	HBsAg	[41]
2	TGPCKTCTIPAQ	292–303	HBsAg	[42]
3	PLGFFPDHQLDPAFGANSNNPDWDFNPNK	21–49	PreS1	[43]
4	ALLDPRVRGLYFPAGG	130–145	PreS2	[43]
5	RTPPAYRP	127–134	HBeAg	[44]
6	GNLEDPASRDLVVSYV	74–90	HBcAg/HBeAg	[45]
7	SVRFSWLSLLVPFVQWFVGLSPTVWLSAI	341–369	HBsAg	Bepipred, BcePred, ABCpred
8	GWLWGMDIDPYKEFGA	6–22	HBeAg	Bepipred, BcePred, ABCpred

contains alpha helix, extended strand and random coil. Alpha and gamma turns occurred within the coiled stretches of sequence while beta turns did not occur in the structure. The overall statistics of antigen showed that over 50 percent of secondary structure is random coil while extended strand by 30% place as second and third place is for Alpha helix and Beta turn with 8% (Fig. 2).

Homology modeling method failed to build proper models, but several models were built by other two methods. A model predicted by I-TASSER was selected due to its highest score of accuracy determined by QMEAN and Prosa software. Ramachandran plot confirms the quality of the model, as the majority of the residues were within the favorable regions (Fig. 3).

3.5. Molecular Dynamics Refinement

Molecular dynamics run provides a platform in which the protein could have its natural behavior within a period of time, while and energy minimization helps the structure to solve the energy barrier and clashes which are existing in the 3D structure. Ultimately these calculations resulted in more stable state with less residual errors and the final free energy for selected structure was calculated to be-4544 kj/mol (Fig. 4).

3.6. Final Protein Properties

To have a better concept about the properties of the designed protein, the structure should be further inspected. Data from NetSurf and VADAR online servers showed that less than 50% of residues are exposed (the threshold was set to be 25% of exposure). Moreover, the analysis with Discovery Studio Visualizer 2.5.5 revealed that about 20% of residues were hydrophilic and 30% were hydrophobic (Fig. 5).

3.7. Construction of Expression Vector

The finally achieved gene should be inserted in to an expression vector, to be overexpressed in a host cell. The designed gene was successfully synthesized and sub-cloned in to pET32a vector which expresses a thioredoxin tag in fusion to inserted gene. Sequencing results confirmed the accuracy of the cloning and synthesis process.

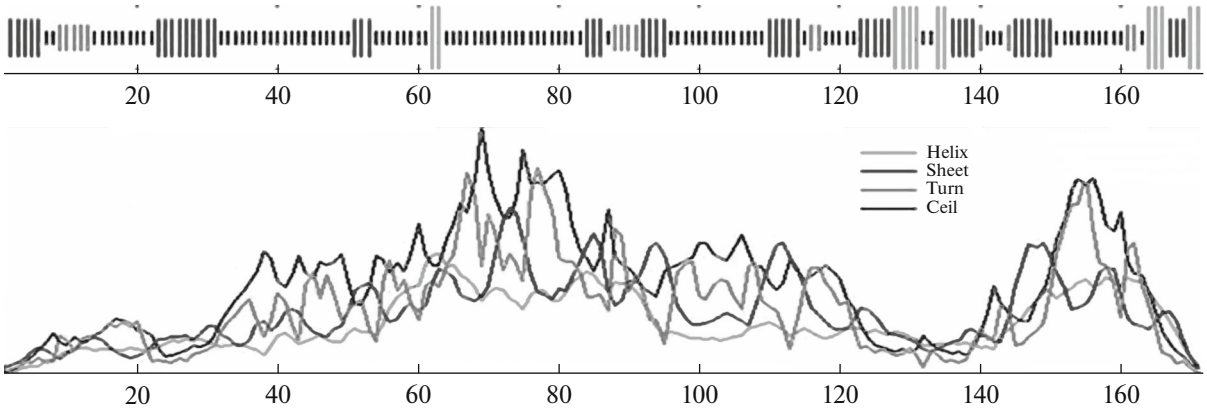

Fig. 2. Graphical illustration of antigen secondary structure.

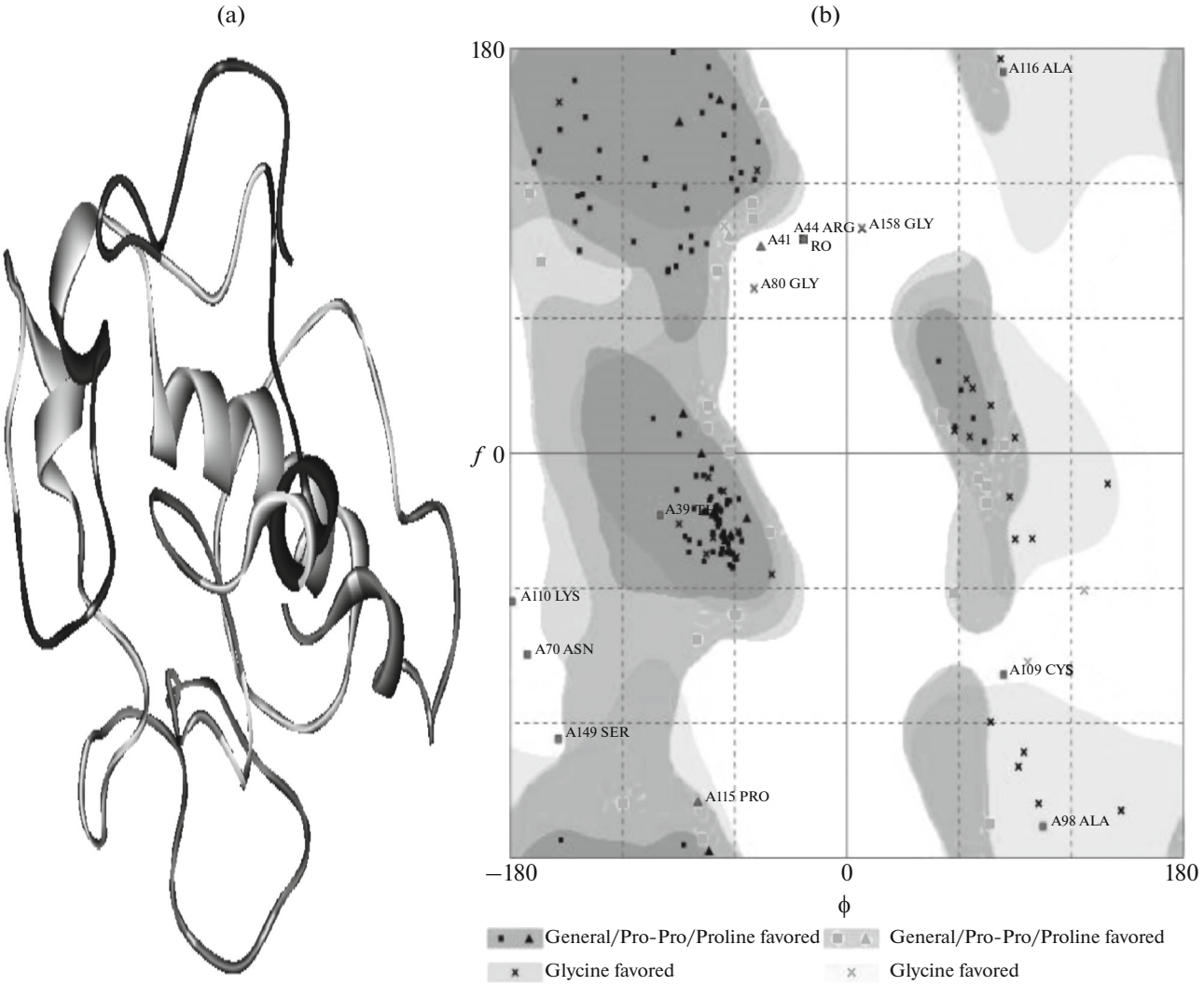

Fig. 3. (a) Graphical view of selected model predicted by I-TASSER software. Each color is representative of a single epitope (white colors are for linker sequences). (b) Ramachandran Plot for selected model which the Number of residues in favored region 131 (77.1%).

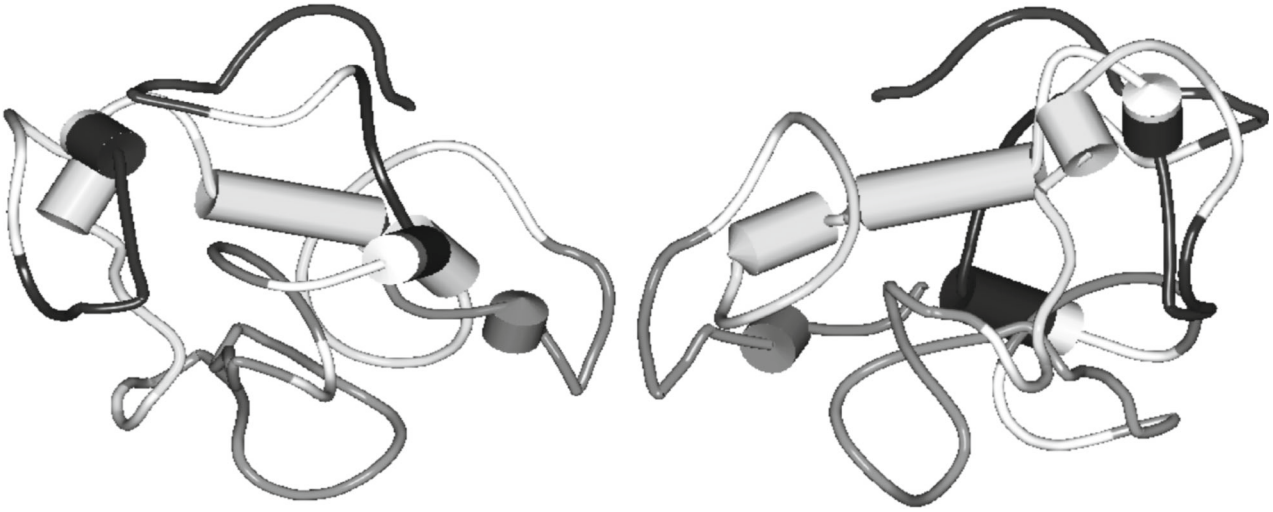

Fig. 4. Illustration of antigen structure after dynamics and energy minimization run. (a) Front view, (b) Rear view and each epitope is in specific color (white colors belong to linker sequences).

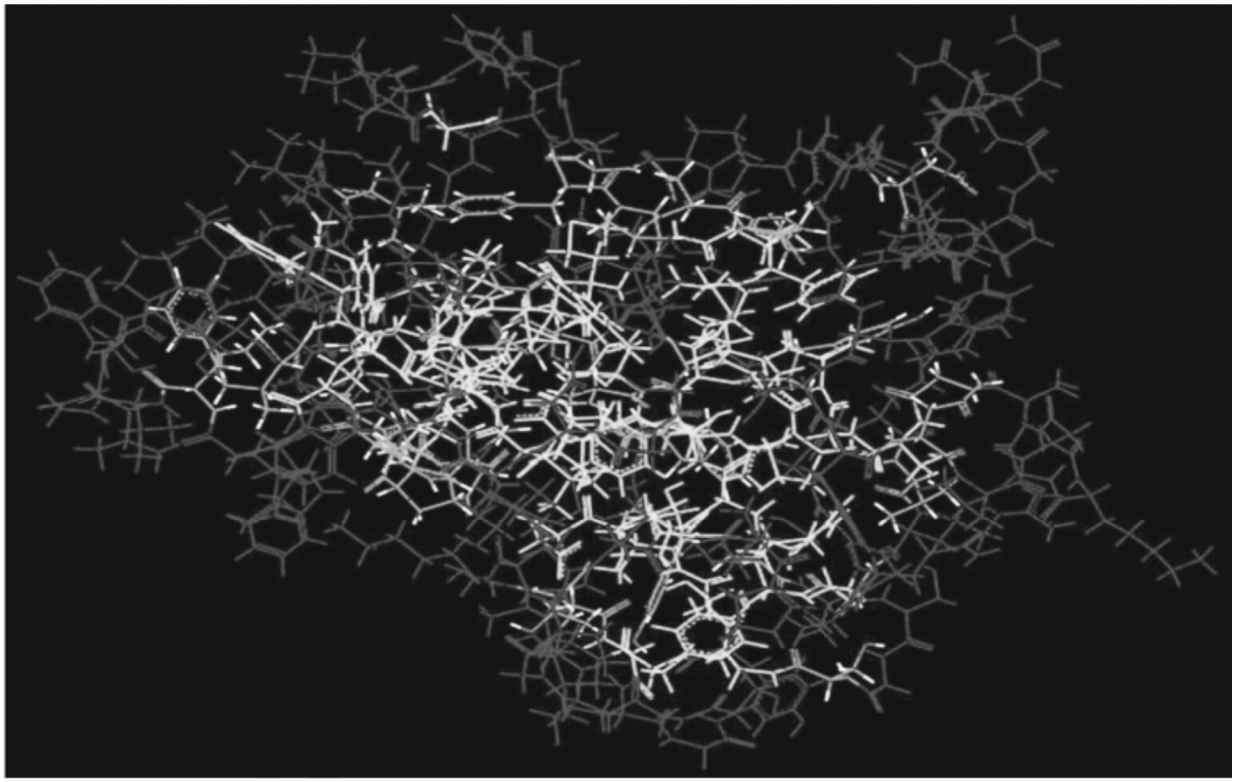

Fig. 5. Solvent accessibility. Solvent accessible residues are in blue and buried ones are in green.

3.8. Protein Expression and Chemi-Luminescence Assay

In order to evaluate the protein expression and its performance in HBV antibody detection employing a highly sensitive method, the chemi-luminescence assay was selected. All three tests indicate that the positive control samples have the lowest RLU/s, the positive control samples have the highest RLU/s, the pET32a without the designed gene control samples have in between RLU/s and the test samples containing the designed antigen have lower RLU/s than the empty pET32a controls. According to the study designed explained in the method section, these results indicated that the expression was successfully done and the resulted diagnostic protein is capable of detecting all three HBV antibodies. Each test was performed in quadruplicate, results were reported as mean ± SD of four tests, error bars indicate SD and the results were statistically significant in comparison to controls with a P value of <0.05. (Fig. 6).

4. DISCUSSION

The lack of effective medicine or treatment that can cure the HBV infection, results in roughly one million deaths annually [14, 15]. Therefore, effective detection and monitoring of HBV is extremely important for treatment purposes and safety of blood transfusions and supplies [16]. However, due to its error prone RNA reverse transcription and different viral infection profiles, HBV detection could be associated with some complications.

In the present study, we employed the in silico strategies as an evolving field to grasp a better understanding of designed protein and decrease the extent of required experimental attempts [17–20]. In this regard, using the consensus sequence, derived from MSA analyses, as representative of all HBV serotypes provides an opportunity to have the widest range of detection targets. Immunoinformatics tools are widely used for epitope prediction and their accuracy is examined in vitro [21]. The confirmation of epitopes, specified in previous experimental studies, indicates the reliability of prediction tools and identified novel high scored epitopes. Final decision for epitope prediction was made by considering several B-cell epitopes properties like antigenicity, flexibility, surface accessibility and hydrophilicity. The approach of homology modelling as a technique capable of predicting most reliable models of high quality over a wide range of sizes, was employed to arrive at the best protein model [22]. However, due to lack of any matching templates the threading and ab-initio methods were employed for protein modeling. Among the predicted models, a model predicted by I-TASSER server was selected as framework for the resumption of analyses. Energy minimization cannot solve the energy barrier problems of the predicted model [23]; thus molecular dynamics (MD) was performed to

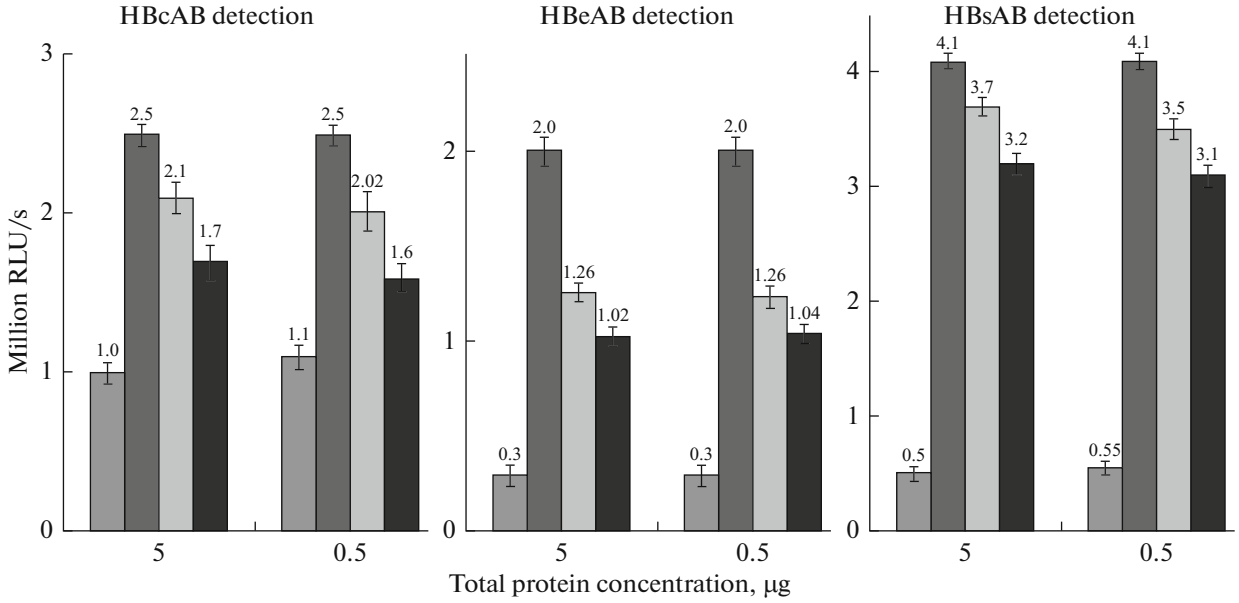

Fig. 6. Chemi-luminescence immunoassay. In the case of HBcAg and HBeAg plots: Cntl1 is the positive control which HBcAg and HBeAg containing solution is added, Cntl2 is the negative control which the solution lacking HBcAg and HBeAg is added, Cntl3 is the pET32a vector expression without the design gene and Tst is the expression of designed gene. In the case of HBsAg plot: Cntl1 is the negative control which the HRP-conjugated HBsAg antigen (without antibody) is added, Cntl2 is the positive control which HRP-conjugated HBsAg antigen (with antibody) is added, Cntl3 is the pET32a vector expression without the design gene and Tst is the expression of designed gene. Each data bar represents the mean of quadruplicate samples; error bars represent standard deviations ($P < 0.05$).

achieve a more stable conformation. Surface accessibility analysis and visualization tools indicated that although there were buried amino acids within the structure of the protein, all epitopes were exposed on the surface and none was totally buried at the core of antigen which was in favor of better detection of antibodies. Final gene was sub-cloned into pET32a vector, designed for cloning and high-level expression of peptide sequences fused with the 109aa Trx · Tag^{TM} thioredoxin protein. Thioredoxin tag is a solution for soluble protein expression, circumventing common *E. coli* expression drawbacks [24].

Although HBV has been detected from various sources including dried blood/plasma spots, hepatocytes, ovarian tissue, cerumen, saliva, parotid tissue, renal tissue, oocytes and embryos, cholangiocarcinoma tissue, etc. (reviewed in [25]), serological methods (using serum or whole blood) of detecting HBV infection are among the most common, rapid and cost effective methods. In this regard, chemiluminescent enzyme immunoassay is a valid serological method to detect HBV markers. Can Liu et al. compared test results from different serological detection methods based on different principles. Their evaluations revealed that, gold immunochromatographic assay (GICA) is only suitable for the preliminary screening of HBsAg positive individuals and enzyme-linked immunosorbent assay (ELISA) can be applied to the qualitative diagnosis of HBsAg, while both chemiluminescent based assays (e.g. chemiluminescent microparticle immunoassay (CMIA) and electrochemiluminescent immunoassay (ECLIA)) are suitable for the quantitative determinations [26]. In a separate study, Khadem-Ansari et al. reported the HBV detection sensitivity and specificity of chemiluminescent enzyme immunoassay (CLEIA/ CLIA) to be 96 and 100% in comparison to PCR method which is assumed as gold standard method with 100% sensitivity and specificity [27]. The HBV detection sensitivity of the CLIA could be enhanced even more by incorporating different modifications [28–30]. CLIA showed advantages over other conventional methods: since the light signal is generated by a chemical reaction "in the dark," thus low or negligible nonspecific signal is produced, it does not need long incubations and there is no need for any reaction termination [31, 32]. Therefore, CLIA chose to be the method of preference for this study. Our results indicated that in the case of HBcAb and HBeAb detection, to bind respective HRP conjugated antibodies, expressed protein successfully competed with the coated HBcAg and HBeAg on the well. Since the designed antigen is capable of reacting with HBcAb and HBeAb, less HRP conjugated antibody bound to coated antigens and consequently RLU/s values decreased. On the other hands, in the case of HBsAb detection, expressed antigen successfully competed with HRP conjugated HBsAg to bind reference HBsAb. Since the expressed antigen is capable of detecting HBsAb, less HRP conjugated HBsAg bound to reference HBsAb and consequently less sandwiches

with coated HBsAg formed and RLU/s values decreased. Apparently designed antigen is capable of binding all three HBV antibodies. This means that B cell epitopes for each tested antibody is exposed on the surface of the finally folded state of the antigen, therefore antibodies were able to bind the antigen through respective epitopes. These data verifies the robustness of harnessed bioinformatics approach, implying the well-designed nature of the antigen.

Carman et al. first described the HBV Immune Escape Mutants (IEM) [33] as an adaptive response of the HBV virus to selective pressure exerted by drugs and immune system. Several years later, Faleye et al. reported the first Nigerian IEM case with mutations in the "a" determinant [34], implicating the presence and circulation of HBV IEMs in different populations. Since the IEMs encompass mutated forms of the antigen, their antibody response could not be detected using conventional tests. Moreover, atypical serological profiles in hepatitis B virus infection is a well-established phenomenon due to factors related to the agent (infection by a new strain or viral serotype—antigenic variant) and/or to the host (immune tolerance, cellular immune response, and immunosuppression) (reviewed in [35]). These atypical serological profiles could lack any of the serological markers, resulting in false negatives. However, since the designed antigen is capable of detecting all three main HBV antibodies, any of the aforementioned complications in the conventional detection kits of the HBV could be circumvented using this antigen. Although using multiepitope antigens too diagnose various disease became a widespread approach [36–39], Queiroz de Souza et al. reported the only recombinant multiepitope antigen to be used for diagnosis of hepatitis B [40]. However, their study was designed only for anti-HBc diagnosis. Therefore, to the best of our knowledge this study is the sole multiepitope approach encompassing three main HBV antigens. The multiepitope approach offers several advantages over conventional methods; it eliminates the need for whole virus antigen (thus contributing to safety due to omission of virus production in tissue culture or suckling mouse brain) which is used in some diagnostic tests, prevents the possible cross-reactivity towards antibodies against other unrelated infectious agents, permits a high epitope density (sensitivity), enables careful choice of unique specific epitopes (specificity), and *E. coli*-based overexpression (cost-effective antigen production). To completely cover all current variants of HBV, the detection antigen should encompass all differentially evolved epitopes, which is impossible to have such an antigen due to size limitations. In this regard, we designed an antigen that is comprised of several immuno-potent epitopes belonging to all three antigens of the HBV. This antigen is not capable of detecting the antibodies belonging to all of the variants of per se HBsAg. However, due to presence of the epitopes from two other HBV antigens it would detect the mutated HBV infection; because the epitopes of the other antigens could compensate for the failure in detection of the antibodies of the mutant HBaAg. This means that, although the antibodies of each individual antigen could escape from the detection but the infection itself would be detected by the other two epitopes if the corresponding antibodies are present in the sample depending on the stage of the disease.

CONCLUSIONS

In conclusion, using a novel in silico approach a multiepitope antigen was designed and successfully assessed for its ability to detect HBV antibodies. This antigen would be potentially capable of solving detection drawbacks associated with conventional detection agents. It would be able to detect HBV infection during the period of infection course in which the antibodies for any possible antigens or mutation forms are not available. To the best of our knowledge it is the first report that in silico features of a hepatitis B diagnostic antigen is designed and predicted in a through information based strategy and then verified in vitro. In addition to introduction of a novel antigen showing promising insight for hepatitis B detection, the correlation between a thoroughly conducted in silico study and the empirical confirmation of predictions were established performing an integrated strategy. The obtained results could be construed as a primary proof of concept that the in silico predictions could be used as primary steps of the biological studies.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

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