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Expression of a Novel Fusion Recombinant Protein VP8-VP1 in *Escherichia coli* as a Rota-HAV Vaccine Candidate

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ABSTRACT

Introduction: Rotaviruses (RV) and hepatitis A virus (HAV) are pathogens responsible for more than 2 million hospitalizations, especially in developing countries, due to transmission through the fecal-oral route. Currently, there are several FDA-approved RV and HAV vaccines available which are based on killed or attenuated viruses. However, these vaccines often have side effects and low efficacy in eliciting specific immunity. Therefore, the design of a vaccine based on a recombinant protein, composed of RV and HAV antigens seems essential. **Methods:** We used bioinformatics tools to design and analyze the properties, predict the structure and evaluate the function, immunogenicity, antigenicity, and truncated sequences of HAV VP1 and RV VP8 as a dual vaccine platform. The predicted epitopes were expressed as a recombinant protein in *Escherichia coli* BL21 where half of the VP1 protein was fused with the Rota protein VP8 using pET24a expression vector,. **Results:** The expressed protein was confirmed by SDS-PAGE and Western blotting. Subsequently, high-scale expression, purification, refolding and determination of the protein concentration (~2.4 µg/µl) were obtained. **Conclusion:** Upon completion of the future immunogenicity evaluation through injection into mice, the present fusion protein can potentially serve as a candidate for a recombinant vaccine against both RV and HAV infections.

INTRODUCTION

Both Rotavirus (RV) and hepatitis A (HAV) are transmitted via the fecal-oral route and lead to clinically important diseases in developing countries. The development of recombinant fusion vaccines to induce effective protective immunity against two or more similar diseases is considered a prudent public health strategy [1, 2]. RV is one of the leading causes of severe pediatric diarrhea worldwide and is associated with 125,000 – 200,000 diarrheal deaths each year in under 5-year-old children [3, 4]. The risk of intussusception is high with RV vaccines[5]. Additionally, the attenuated vaccine can reinstall the viral infection and transmit it to unvaccinated persons [6, 7]. Therefore, the development of a more efficient vaccine is needed [8].

The outer capsid protein VP4 is considered as an important target for the development of a recombinant rotavirus vaccine [9] and it is responsible for attachment and penetration of rotavirus, after being cleaved into two fragments, namely VP8* and VP5* by trypsin [10, 11]. The VP8* protein can bind to cell receptors and mediate the attachment of RV. VP8* is the main determinant of rotavirus P serotypes and antibodies against VP8 can block the first step during RV infection. HAV has only one serotype while vaccination against HAV in high-risk communities has globally reduced the rate of the infection [13]. The multiplication of HAV in culture is time-consuming and has poor yields; hence, the production of inactivated vaccines is difficult and expensive [14, 15]. Therefore, the use of recombinant proteins may represent an alternative source of antigens for diagnosis and prevention purposes. HAV capsid protein VP1 is a structural protein with a number of immunodominant epitopes [16, 15]. Several studies have indicated the reaction of HAV with monoclonal antibodies and the use of isolated structural proteins or synthetic peptides for the induction of neutralizing antibodies [17, 15, 18]. Here, two recombinant fusion proteins consisted of VP1 of HAV and VP8 of RV were produced by an

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E. coli expression system for developing subunit vaccine materials. Bioinformatics tools are used to analyze multiple domains, which are essential in generating fusion proteins and identifying immunogenic and conserved residues of interest. These tools have led to the development of new fields in vaccine development, such as *in-silico* epitope prediction to analyze efficacy, safety, toxicity effects, and drug interactions in vaccine designs [19, 20].

The aim of this study was to design for the first time a novel recombinant VP1-AAY-VP8 fusion protein that combines VP4 (VP8*) and the immunodominant regions of VP1 using a flexible linker containing a proteasome-cleavable site. Moreover, this approach helped to develop a bivalent vaccine that can simultaneously confer immunity against both HAV and RV [21]. Pre-procedural study design involved several key steps: 1- Construction of the dataset, alignment of sequences, genome transcoding, and analysis fusion. 2- Prediction of multiple epitopes and codon optimization. 3- Analysis of properties. 4- Prediction of the structure and evaluation of its performance. 5- Immunological analysis for multi-epitope vaccine and disulfide engineering. 6- Molecular binding and molecular dynamics simulation, *in-silico* simulation. 7- Immunity simulation analysis.

MATERIALS AND METHODS

Designing the *vp8-vp1* Construct and Immunoinformatics Methods

The full-length gene encoding the VP8 protein of LLR strain of RV and VP1 of HAV was obtained by immunoinformatic methods. Antigenic proteins were studied and T cell epitopes were selected. Antigenicity, sensitization and toxicity of selected epitopes were evaluated using VaxiJen 2.0, AllerTOP and ToxinPred servers, respectively. The affinity of the proposed vaccine to MHC I and II molecules was measured in a molecular binding study. The resulting vaccine candidate had antigen, high stability and half-life compatible with use in vaccination programs, which was evaluated after sequencing and expression. To design the recombinant immunogen, the optimized nucleotide sequence with the selected *vp8-vp1* construct was selected [26] and appropriate restriction enzyme sites were added to facilitate the cloning process. The final DNA fragment was commercially synthesized by Shine Gene Molecular Biotech, Inc. (China), presented in a pUC57 vector.

Subcloning the *vp8-vp1* Gene into pET-24a (+) Expression Vector

The *vp8-vp1*gene fragment (without stop codon) was excised from pUC57- vp8-vp1 using *Nde*I/*Xho*I (Thermo Fisher Scientific, USA) restriction enzymes and subcloned into the same enzymatic sites of a pET24a (+) prokaryotic expression vector using T4 DNA ligase (Thermo Fisher Scientific, USA). The ligation product was transformed into competent *E. coli* DH5α strain. The recombinant pET24a(+)-*vp8-vp1* vector was extracted from kanamycin-resistant *E. coli* colonies. The concentration and purity of the DNA construct were determined by a Nano Drop spectrophotometer (Thermo Fisher Scientific, USA). The correct cloning procedure was validated by colony-touch PCR using the following thermocycling program: initial denaturation at 94 °C 5 min, denaturation at 94 °C 20 sec, annealing at 56°C and 20 sec, extension at 72°C and 45 sec, and final extension at 72°C and 5 min.

Expression of the Recombinant *vp8-vp1* Construct

A kanamycin-resistant clone of *E. coli* BL21 (DE3) as well as a Rosetta clone harboring pET24a(+)-*vp8-vp1* construct were each cultured in 5 mL LB medium containing 50 µg/ml kanamycin at 37°C overnight in a shaker incubator (150 rpm). After incubating 100 µl of each overnight culture in 50 ml fresh TY2x medium (Peptone 1.6%, Yeast 1%, NaCl 0.5%) in separate tubes, the protein expression was determined under different conditions, including optical density (OD_{600}), isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration, temperature and

incubation time after IPTG induction. The cultures were incubated at 37°C for 3 - 4 h with constant shaking until the OD_{600} reached 0.4, 0.6, and 0.8. The protein expression was induced by adding IPTG at final concentrations of 0.25, 0.5, and 1 mM, followed by incubation for 2, 3, 4, and 16 h at 30 or 37°C. Afterward, the bacterial pellet was harvested by centrifugation at 1000 g, and the protein expression pattern was analyzed.

Purification of the Recombinant Protein

Based on Qiaexpressionist (Qiagen, Germany) protocol, the disruption of the bacterial biomass by sonication in lysis buffer (Tris/NaCl, pH8) was performed. The resulting pellet and supernatant were separated by centrifugation. According to the manufacturer's instructions for purification under denaturing conditions (i.e. 8M urea buffer and pH 4.5), VP8-VP1 recombinant protein was purified by affinity chromatography using HisPur Ni-NTA resin (Thermo Fisher Scientific, Germany).

Expression Analysis of the Recombinant VP8-VP1 Recombinant Protein

SDS-PAGE and Western blotting analyses were performed to detect His-tagged VP8-VP1 Recombinant Protein. For Western blotting, cell pellets from each sample was mixed with sample loading buffer (0.5 M Tris-HCl (pH 6.8), 5% SDS, 10% glycerol, 0.25% bromophenol blue, 5% mβ-mercaptoethanol), and incubated for 5 min at 100°C for cell disruption. The samples were loaded onto 12.5% SDS-PAGE gel and then were electrotransferred to a nitrocellulose membrane (Whatman, UK). TBS buffer (Tris-buffered saline including 3% bovine serum albumin) was used for blocking the membrane. The membrane was immersed in 1:10000 dilutions of peroxidase-conjugated anti-His-tag monoclonal antibody (Abcam, UK) for 2 h at room temperature. Subsequently, the protein band was visualized using 3, 3' Diamino benzidine (DAB)/H_2O_2 substrate solution (Roche, Germany). The BL21 (DE3) and Rosetta bacterial strains carrying the empty pET24a (+) vector were used as the negative controls.

RESULTS

Confirmation of the Expression and Purification of VP8-VP1 Recombinant Fusion Protein

Fig. 1 shows the confirmation of colonies harboring vp8-vp1 construct by colony-touch PCR.

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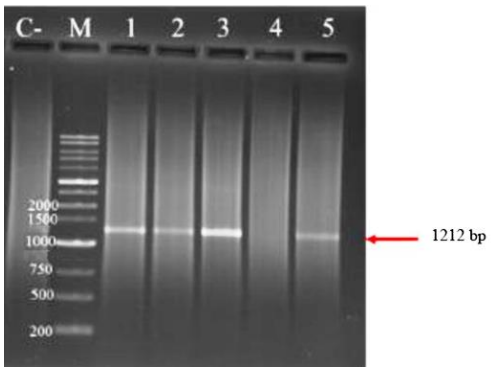

Fig. 1. Electrophoresis of amplicons due to amplification of *vp8-vp1*(1212 bp) fragment. Wells 1 to 5 represent PCR products of the recombinant colonies of *E. coli* Bl21(DE3), harboring pET24a (+)-*vp8-vp1* construct.

Following the expression (induced by 0.5 mM IPTG and grown at 18°C overnight) and purification of the fusion protein, it was analyzed by SDS-PAGE. The result confirmed that the *vp8-vp1* construct was overexpressed in *E. coli* BL21 (DE3) (Fig. 2 A) where a clear band of ~45.5 kDa at overnight induction could be observed, compared to 4 h incubation times. Similar results were obtained with Rosetta cells as protein expression hosts (data not shown). A single similar size band of ~45.5 kDa could be observed after purification of the protein under the denaturing conditions (Fig. 2 B).

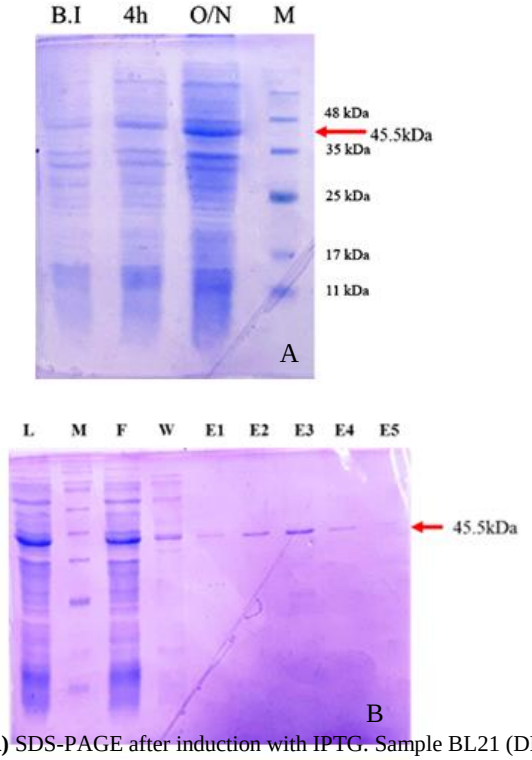

Fig. 2. (A) SDS-PAGE after induction with IPTG. Sample BL21 (DE3) pET24a-VP8-VP1 in the conditions of 37°C IPTG (1mM). B.I: Colony sample number 1 before the Induction. 4h: Four hours growth after induction with IPTG. O/N: Overnight incubation after adding IPTG. M: Protein marker (10-180 kDa; Sinaclone). **(B)** SDS-PAGE result from purified protein (Denaturing phase) on the Ni-NTA column. L: Sample before purification in the insoluble phase. M: Protein weight indicator 10-180 kDa, Sina Clone). F: The sample removed from the column after oading or Flow. W: Washed sample with Wash Buffer Denature pH 5.9. E1 to E5: Samples eluted from the column with Elution Buffer pH 4.5.

Western Blotting and Assessment of Yield of the Recombinant Protein

Western blot analysis using anti-His tag antibody detected the His-tagged purified polypeptides (Fig. 3) of the same expected size (~45.5 kDa), indicating the proper expression of the recombinant protein by the prokaryotic expression system (pET24a). To refold the denatured protein, the purified polypeptides were dialyzed against phosphate buffer saline (PBS) 1X using a dialysis membrane (10 kDa, Thermo Fisher Scientific, Germany), and their concentration and purity were assessed by the Bradford method and NanoDrop spectrophotometer (Thermoscientific, USA) to be 2.4 µg/µl.

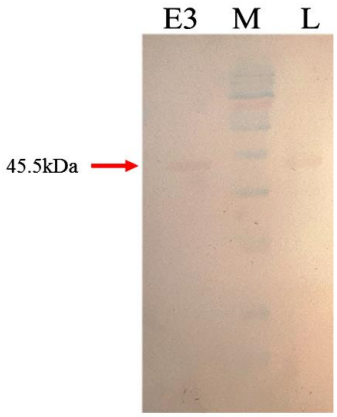

Fig. 3. Analysis and confirmation of purified VP8-VP1 recombinant protein by Western blotting; M) protein molecular weight indicator (10-180 kDa, Sinaclone). L and E3) Examination of the purified and unpurified VP8-VP1 protein with anti-His tag antibody, respectively.

DISCUSSION

Human enteric viruses, such as RV and HAV, are major causes of severe dehydrating gastroenteritis and hepatitis infections [22]. Natural infection by RV reduces the prevalence and severity of the subsequent episodes, suggesting that vaccination could control RV diarrhea. The capsid protein of RV, known for its strong vaccination properties, may theoretically be used as carriers for epitopes from other pathogens [8, 23]. Although the use of recombinant RV vaccines is currently limited [24], , they possess the potential advantage of being cost-effective and producible in large quantities. Recombinant vaccines represent a new generation of highly immunostimulant and specific vaccines against viral HAV and RA, with known advantages over the live attenuated vaccines. In some cases, these vaccines can also be used as carriers of epitopes of other pathogens. The growing knowledge of truncated pathogen genomes and the availability of efficient systems for producing large quantities of recombinant proteins allow the potential use of recombinant proteins as vaccines [25].

The role of capsid proteins in the immune response against the virus in form of new recombinant vaccines against RA and HAV infections have been studied [26]. In this regard, a vaccine, expressed by a pET24a-VP8-VP1 construct from the human hepatitis and RA strain (G1P8A) produced in *E. coli* is considered as the most successful recombinant vaccine candidate. Interestingly, RV VP8 capsid protein is not a glycoprotein and can be well expressed in *E. coli* [27, 28].

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Generally, the yield of recombinant protein vaccine candidates prepared from the *E. coli* expression systems is significantly higher than those prepared from other expression systems, including baculovirus-insect cells and mammalian cells expression systems. Prokaryotic expression systems such as *E. coli* systems costs less due to their fast growth rate and high transformation efficiency [29]. Among the common hosts in such systems is *E. coli* BL21 (DE3) [30, 31]. Also, Rosetta strain is a BL21 derivative which is designed to enhance the expression of eukaryotic proteins containing the rare codons used in *E. coli* [32]. In this study, both E. coli BL21 (DE3) and Rosetta strains were used to evaluate the expression of the recombinant protein and similar results were obtained. The truncated VP1-AAY-VP8 fusion protein is shown to trigger high titers of neutralizing antibodies, representing a promising advancement in the evolution of recombinant RV and HAV vaccines [33]. However, much more work is needed to develop a recombinant human RV and HAV vaccine.

Previously, Li *et al* have shown that the truncated VP4 (aa26-476) could be expressed in soluble form in *E. coli* and could be purified into homogeneous trimers. They have also indicated that the truncated VP4 could induce high titers of neutralizing antibodies when aluminum adjuvant was used which conferred high protective efficacy in reducing the severity of diarrhea and RV shedding in stools, in animal models [8]. Moreover, multiple antigen peptides (MAP) have been revealed to be efficient immunological candidates for the induction of immune responses to a variety of infectious agents. The capsid proteins of HAV (mainly, VP1 and VP3), are the immunodominant targets for a protective antibody response [34, 35].

Chuanfeng Li *et al*. have cloned an optimized *vp1* gene in *E. coli* and have subcloned it into pET32a(+) vector to increase its expression. The recombinant VP1 fusion protein has been purified from the inclusion bodies by Ni^{2+} affinity chromatography using His-Bind Resin. The expression of the codon-optimized *vp1* gene in *E. coli* was remarkably increased when compared to the wild-type *vp1* gene, showing at least a 17-fold increase [36]. Wen *et al.* have developed a novel recombinant subunit parenteral RV vaccine, which may be more effective in low-income countries and is shown to prevent the potential problem of intussusception. They have indicated that the truncated recombinant VP8* (VP8*) protein of human RV strain Wa P, DS-1 P, or 1076 P expressed in *E. coli* was highly soluble and could be generated in high yields [37].

In conclusion, we designed a *vp1-vp8* construct using immunoinformatics software and obtained sufficient expression of the resulting protein in a prokaryotic expression system while achieving an acceptable degree of purity and yield, following purification with Ni-NTA method. Upon completion of the future animal and challenge studies, it is envisaged that this fusion protein can be considered as a vaccine candidate against both RV and HAV infections.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interests.

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