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ORIGINAL ARTICLE

Designing the fusion protein of rotavirus VP8 and hepatitis A virus VP1 and evaluating the immunological response in BALB/c mice

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ABSTRACT

Background and Objectives: Rotavirus and Hepatitis A virus are responsible for causing gastroenteritis and jaundice. The current vaccination approaches have proven insufficient, especially in low-income countries. In this study, we presented a novel dual-vaccine candidate that combines the rotavirus VP8 protein and the hepatitis A virus VP1.

Materials and Methods: The VP8*-rotavirus+AAY+HAV-VP1 fusion protein was produced using an *Escherichia coli* expression system. The recombinant protein had a molecular weight of approximately 45.5 kDa and was purified through affinity chromatography. BALB/c mice were injected subcutaneously with the recombinant protein, VP1, VP8 and vaccines for rotavirus and hepatitis A virus, both with and without ALUM and M720 adjuvants. ELISA assays were used to measure total IgG, IgG1, IgG2, and short-term and long-term IL-5 and IFN-γ responses.

Results: The fusion protein, when combined with adjuvants, elicited significantly higher total IgG, IgG1, and IgG2 responses compared to VP1 and VP8 alone, as well as the rotavirus and hepatitis A vaccines. Furthermore, it induced a higher short-term IL-5 and IFN-γ response while demonstrating a higher long-term IL-5 response compared to the rotavirus and hepatitis A vaccines.

Conclusion: This study demonstrates that the VP8*-rotavirus+AAY+HAV-VP1 fusion protein is a promising dual vaccine candidate for immunization against hepatitis A and rotaviruses.

Keywords: Recombinant fusion proteins; Immunoinformatics; Rotavirus; Hepatitis A virus; Bivalent vaccines; BALB/c mouse

INTRODUCTION

Vaccination stands as the foremost and highly effective strategy for controlling a wide range of infectious diseases, playing a crucial role in averting mortality. Conjugate vaccines, within the diverse spectrum of vaccine platforms, have notably reduced infections, particularly among vulnerable populations such as infants and children under two years of age (1, 2). Every year, Rotavirus takes a significant toll on humanity, particularly in low-income countries, by causing high rates of mortality and morbidity in infants through acute gastroenteritis (3). Despite significant efforts, an efficient vaccination approach

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has yet to be developed (4). Currently, there are two FDA-approved attenuated vaccines for rotavirus, both of which have failed to demonstrate sufficient efficacy in low-income countries (5, 6). One issue is that the virulence of the attenuated vaccines could be restored by recombination with other rotaviruses (7, 8). Numerous efforts have been made to devise an improved vaccine for rotavirus. One effective approach involves harnessing the potential of recombinant vaccines. Understanding the structure of the viral genome is crucial in establishing such a vaccine. The genome of rotavirus consists of 11 major segments encoding 6 structural and 6 non-structural proteins (9). The structural proteins, designated as VP1 through VP8, form the capsid of rotavirus, while the nonstructural proteins (NSPs) play essential roles in other tasks, such as replication (9). The VP4 protein undergoes cleavage, producing VP5 and VP8 proteins in the presence of trypsin. VP5 is responsible for facilitating cellular penetration, and VP8 helps the virus mediate the attachment of rotavirus to its target host cells (10, 11). VP8 has shown better solubility and yield in pigs but a poor response in mice (12). Efforts have been made to combine the VP8 of rotavirus and the P2 of CD4+ T cells to produce better immunogenicity in adult seroconversion (13).

Rotavirus vaccines have demonstrated an outstanding safety record and efficacy in preventing fatalities caused by rotavirus gastroenteritis infections (14, 15). A notable divergence is observed among the antigenic epitopes located within the VP8* region of circulating rotavirus A strains across different regions worldwide, highlighting the genetic heterogeneity inherent in this virus (16). The majority of genetic variation is concentrated in the VP8* cleavage fragment of VP4, underscoring its significance in shaping the virus's antigenic makeup (17). Furthermore, structural analysis reveals that certain amino acid differences are present in exposed regions of the antigenic epitopes within the VP8* region, potentially affecting antigenicity (17). As a result, current efforts in the development of rotavirus vaccines have been directed at targeting specific antigenic segments. These targeted segments are expected to induce the production of neutralizing antibodies, addressing challenges posed by the virus's dynamic nature and evolving genetic landscape.

Hepatitis A virus (HAV) is another important pathogen that causes hepatitis. This virus is responsible for a wide spectrum of symptoms, including fever, malaise, loss of appetite, diarrhea, nausea, abdominal discomfort, dark-colored urine, and jaundice (18). Similar to rotavirus, HAV is transmitted through the oral-fecal route (19, 20). HAV primarily affects children, but its severity increases with age (21).

The HAV genome is a single-stranded, positive-sense RNA molecule and encodes 4 major structural proteins, VP1 through VP4, and six major non-structural proteins (2B, 2C, 3A, 3B, $3C^{pro}$, and $3D^{pol}$) (22). VP1, responsible for capsid formation, is a structural protein with high immune induction capacity and contains a major immunodominant epitope of HAV, making it a valuable target for recombinant vaccines (23).

Throughout history, efforts to develop HAV vaccines have primarily focused on using inactivated or live attenuated techniques, which have shown considerable success in eliciting protective immune responses. Vaccination with attenuated or inactivated HAV vaccines has been known to stimulate the production of antibodies, particularly in response to structural proteins. However, limited or negligible antibody responses have been observed concerning non-structural proteins (24). The capsid protein VP1, containing crucial HAV epitopes recognized by the immune system, emerges as a vital element for investigation in recombinant subunit protein vaccine candidates. Previous research has successfully produced recombinant forms of VP1 with immunogenic activity in various systems, including *E. coli* and plant systems (14, 24-28).

The conceptualization and development of a dual vaccination strategy offer a strategic response to the common transmission route, providing a comprehensive resolution for mitigating gastroenteritis and hepatitis. Therefore, in the present study, we aimed to express and evaluate the immunogenicity of the HAV-VP1 and the VP8-ROTA constructs as a recombinant dual vaccine candidate against hepatitis A virus and rotavirus.

MATERIALS AND METHODS

Ethics statement. The study was approved by the Ethics Committee of Pasteur Institute of Iran (IR.PII.REC.1398.053).

Plasmids and bacterial strain. pET-24a containing the designed highly conserved truncated sequences of rotavirus strain VP8 * G1P (8) and VP1-HAV promoter (reference sequence NP_740552.1), linked

to a GGGGS linker and a 6xHis tag sequence, by bioinformatic method using amino acid sequence retrieval from GenBank of National Center for Biotechnology Information (NCBI) and using MEGA6.0 ClustalW and Multiple Sequence Alignment (MSA) program to determine the degree of conservation. After alignment using the Bio editing tool, the MSA was visualized and then synthesized by ShineGene Molecular Biotech, Inc (Fig. 1). The *E. coli* DH5α strain was used for cloning and *E. coli* BL21-DE3 was also used for protein expression. The pET24a expression vector containing the desired genes was used to transform the *E. coli* BL21-DE3 strain by the calcium chloride method. These transformed bacterial cells were then cultured in a medium, supplemented with Kanamycin, which served as a selective agent to maintain plasmid stability. Subsequently, the plasmids were extracted from the *E. coli* culture using a plasmid extraction kit (Qiagen, USA) Sanger sequencing was used to ensure the fidelity and accuracy of the cloning procedure (GeneFanavaran, Iran).

Protein expression and purification. Once the recombinant plasmids were validated, they were re-introduced into *E. coli* BL21(DE3) using the calcium chloride method, and these bacterial cells were cultured in a specialized MRS medium containing kanamycin at 37ºC. The addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) induced protein expression. The secreted protein of interest was isolated using a nickel chromatography column (Thermo Scientific™ /USA), ensuring high purity and specificity. The purified protein underwent SDS-PAGE protein electrophoresis on a 12% acrylamide gel. Next, the gel was transferred onto 0.2 µm nitrogen cellulose membrane using the Turbo Transblot Transfer Kit and TurboBlot Blotting System (Bio-Rad, USA). Tris-buffered saline (TBS) containing 5% nonfat milk (w/v) was used to block the membrane. Then, the membrane was incubated at room temperature for 1 h, followed by washing three times with TBS with 0.1% Tween 20 (TBS-T). After that, the membrane was incubated at 4°C overnight with anti-rotavirus and anti-HAV polyclonal antibodies (Merck, Germany). After washing with PBST buffer solution, the membrane was incubated with mouse anti-goat IgG conjugate (Southern Biotech, USA). Immunoreactive proteins were detected using an enhanced chemiluminescence (ECL) assay (Thermo Scientific, USA).

***In vivo* immunogenicity assessment.** To assess the immunogenicity of the produced protein, a controlled *in vivo* study was conducted using female BALB/c mice aged between 6 to 8 weeks. Subcutaneous administration was employed as the delivery method. The mice were divided into 12 distinct experimental groups (6 mice for each group) based on the antigens administered, the day of inoculation, the dose, and the number of mice in each group. The antigens were administered in three doses with a three-week interval between each dose. The injections were performed subcutaneously and contained an overall quantity of 100 µL. The groups either contained 20 µg of antigen + 100µL PBS, 20 µg of antigen + 70% Montanide ISA 720 (M720), or 20 µg of antigen + 50% Alum. VP8* -rotavirus + AAY + HAV-VP1, hepatitis A vaccine

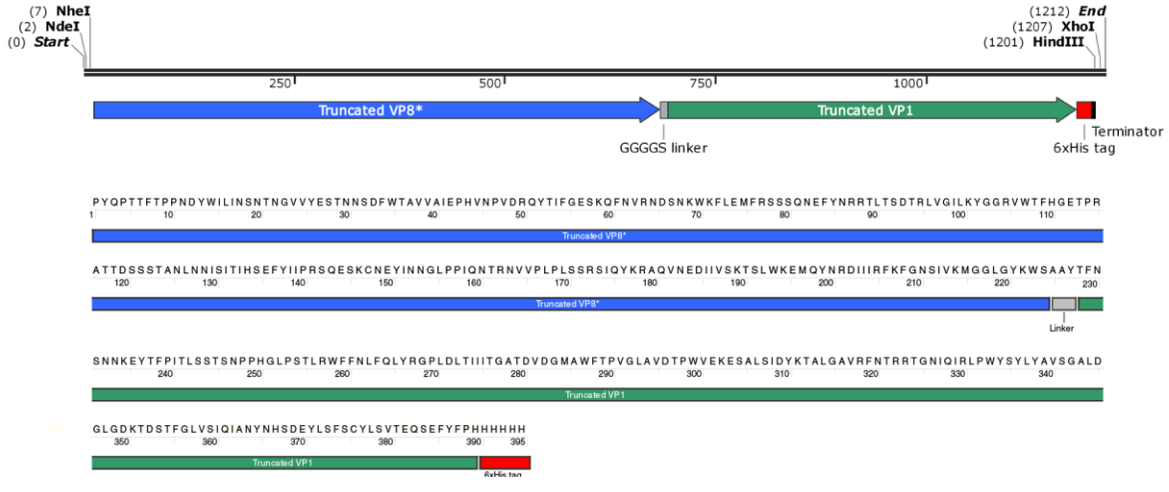

Fig. 1. Schematics of the fusion protein construct.

(VAQTA, Merck, USA), and rotavirus vaccine (ROTARIX, GlaxoSmithKline Biologicals, UK) served as antigens. Consequently, the study included twelve groups structured as follows:

Group 1: 100µL PBS; Group 2: 20 µg of VP8* -rotavirus antigen + 100µL PBS; Group 3: 20 µg of VP8* -rotavirus antigen + 70% M720; Group 4: 20 µg of VP8* -rotavirus antigen + 50% Alum; Group 5: 20 µg of VP1 antigen + 100µL PBS; Group 6: 20 µg of VP1 antigen + 70% M720; Group 7: 20 µg of VP1 antigen + 50% Alum; Group 8: 20 µg of VP8* -rotavirus + AAY + HAV-VP1 antigen + 100µL PBS; Group 9: 20 µg of VP8* -rotavirus + AAY + HAV-VP1 antigen + 70% M720; Group 10: VP8* -rotavirus + AAY + HAV-VP1 antigen + 50% Alum; Group 11: 100µL HAV vaccine; Group 12: 100µL Rotavirus vaccine.

Ultimately, 21 days after the final injection, blood samples were collected from the eye, and the spleen was excised to obtain serum for antibody quantification. Additionally, spleen dendritic cells were isolated and evaluated for stimulation.

Serum total and specific IgG assay. A microtiter plate with multiple wells was prepared for the assay. The wells of the plate were coated with the recombinant protein, and in the subsequent steps, ELISA tests (U-CyTech B.V, The Netherlands) were performed using mouse sera and HRP (Horseradish Peroxidase)-conjugated Goat anti-mouse total IgG, IgG1, and IgG2 antibodies, according to the manufacturer's instructions.

Examination of cytokines. Two to three weeks following the third injection, the spleens of the mice were extracted, and their cells were cultured. The representative cytokines for Type 1 T helper (Th1) and Type 2 T helper (Th2) pathways (i.e., gamma interferon (IFN-γ) and Interleukin-5 (IL-5), respectively were assayed using ELISA (U-CyTech B.V, The Netherlands). To assay long-term immunization, IFN-γ and IL-5 were measured 6 months after the first injection.

Evaluation of the diagnostic value of the recombinant protein. To evaluate the binding ability of the fusion antigen to specific antibodies, we performed an ELISA assay (U-CyTech B.V, The Netherlands) using proven positive sera samples of Rotavirus and HAV. These samples were obtained from the sera archives of the Hepatitis and AIDS Department of Pasteur Institute of Iran. A total of 100 µl (5-20 µg/ml concentrations) of the fusion protein was coated onto ELISA plates (Flat bottom 96-well polyvinyl chloride plate-Nunc, Denmark) and incubated at 4°C overnight. Next, 100 µl of each serum sample was added to the associated well. Following a one-hour incubation at 37°C, washing and blocking steps were performed. Then, 100 µl of 1:10000 dilution of Goat anti-human total IgG-HRP abs (Sigma, USA) was added, and the plates were incubated for one hour. At the end, 100 µl of Tetramethylbenzidine (TMB) (Sigma, Aldrich) was added. Finally, the optical absorbance was measured at 450 nm.

Statistical analysis. Statistical analysis was conducted using GraphPad Prism 9 to examine group differences, as determined by the computed P-values. The graphs were created using R v 4.3.1. The differences between results were statistically analyzed by one-way ANOVA. Tukey's post hoc test was performed to adjust the p-values. The significance threshold of $P < 0.05$ was utilized to determine the statistical significance of the findings.

RESULTS

The Iranian population's highly preserved VP1-HAV and VP8* rotavirus areas were chosen. The variable sections were removed to produce the most appropriate shortened version of HAV, and as a result, amino acids 99 to 259 in the VP1 alignment comprised the most conserved amino acids throughout the clades as mentioned elsewhere (25). Additionally, the viral VP8* amino acids 68 to 277 were used (29). This study led to the selection of VP8* -rotavirus + AAY + HAV-VP1 for further evaluation.

Protein expression and purification. Full-length fusion sequences of VP8 (P4 and P8 genotypes) and VP1 (HAV), connected by a GGGGS linker, were successfully cloned into an *E. coli* expression vector. The expressed His-tagged recombinant protein was identified as a single band in the total protein extract by SDS-PAGE and was confirmed via Western blot analysis using an anti-His tag antibody (Fig. 2A and B). The molecular mass of the recombinant protein was consistent with the calculated values for the fusion protein, validating the successful expression and fusion of the VP8 and VP1 sequences in *E. coli.*

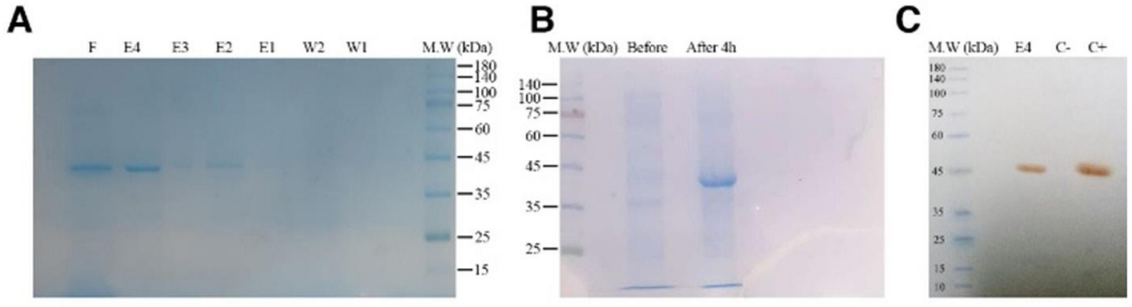

Fig. 2. Purification and expression of VP1-AAY-VP8 fusion protein from *E. coli* BL21. Total protein extracts were collected from *E. coli* BL21 (DE3) cells harboring expression vector pET24a-VP8-VP1. (A) His-tagged recombinant VP1-AAY-VP8 was purified from soluble protein extracts by a Ni-NTA affinity chromatography. The purities and identities of the purified recombinant VP1-AAY-VP8 was verified with SDS–PAGE followed by Coomassie brilliant blue. W indicates wash, E indicates elution and F indicates follow through. (B) Expression of recombinant VP1-AAY-VP8 fusion protein before and 4 h after the induction with IPTG verified with SDS–PAGE. (C) The expression of the recombinant VP1-AAY-VP8 fusion protein (45.5 kDa) was verified by Western blotting. E indicates elution and C indicates control.

Total serum and specific IgG assay. As indicated in Fig. 2, the immunogenic potential of the fusion protein, comprised of VP8 and VP1 proteins from rotavirus and hepatitis A virus, respectively, was examined using BALB/c mice, subcutaneously immunized with the fusion protein, VP8, VP1, alone and combined with vaccine adjuvants ALUM and M720, as well as rotavirus and hepatitis A virus, and PBS, as negative control. The serological response, assessed by ELISA indicated the total IgG optical density (OD) at two antigen concentrations (1:400 and 1:100). The results indicated a significant increase in absorbance over the PBS control at both concentrations ($P<0.0001$) in all groups. In addition, the fusion protein alone, and in conjunction with the adjuvants, demonstrated a significantly more pronounced increase in OD than VP1 alone at both concentrations. Although the fusion protein alone did not show a difference compared to VP1 combined with the adjuvants, the fusion protein with adjuvants yielded a significantly higher OD than VP1+ALUM and VP1+M720 at the 1:400 concentration. At the 1:100 concentration, the fusion protein combined with either adjuvant showed significantly greater OD compared to VP1+ALUM. Furthermore, the fusion protein alone or combined with ALUM and M720 elicited significantly higher OD than VP8 at the 1:400 concentration. In addition, when compared to VP8 with adjuvants, the fusion protein with either adjuvant was significantly more effective; however, the fusion protein alone did not significantly differ in OD from VP8 combined with ALUM or M720. The fusion protein, both alone and with adjuvants, demonstrated a significantly higher OD than both vaccines at the 1:400 concentration. The results of ANOVA and Tukey's *post-hoc* test are summarized in Supplementary S1.To analyze the IgG subtype response, we detected serum IgG1 and IgG2 levels using ELISA (Fig. 3). All groups showed a significantly higher response, compared to the control samples for both antibodies ($P<0.0001$). The fusion protein alone did not show a significantly higher IgG1 or IgG2 response compared to the vaccines, but when combined with either adjuvant, it showed significantly higher IgG1 and IgG2 responses. VP1, VP8, and FP containing adjuvants showed a higher IgG1 and IgG2 response compared to their adjutant-free antigens. VP8+M720 showed a significantly higher IgG1 response, compared to the VP1 group. When combined with either adjutant, both VP1 and VP8 did not show a significantly different response in IgG1 and IgG2, compared to the vaccines.

Cytokine examination. To examine the Th1 and Th2 responses, we assayed IFN-γ and IL-5 secretions by ELISA, respectively (Fig. 4). All groups showed significantly higher levels of IL-5 and IFN-γ compared to the PBS control ($P<0.0001$). Although no significant difference was found in IL-5 levels between VP1 and VP1+ALUM, VP1+M720 showed a significantly higher IL-5 level compared to VP1 and VP1+ALUM. No significant difference was observed between VP8 alone and VP8 combined with either of the adjuvants. Moreover, the fusion protein did not show significantly higher IL-5 levels compared to VP1, VP8, and the vaccines; however, its combination with either of the adjuvants resulted in a significantly higher response compared to all the antigens,

except VP1+M720. For IFN-γ, in the VP1 group, VP1+ALUM showed a significantly higher level, compared to VP1 alone and the rotavirus vaccine, but not compared to VP1+M720 and the Hepatitis A vaccine. VP8+M720 triggered a higher IFN-γ response compared to VP8 alone and all the VP1 combinations; however, there was no significant difference in IFN-γ between VP8 combined with either of the adjuvants. When combined with either adjuvant, the fusion protein did not induce significantly higher levels of IFN-γ compared to the fusion protein alone, VP8+M720, and VP8+ALUM but it induced a significantly higher IFN-γ response compared to both vaccines, VP1, VP1+Alum and VP1+M720.

Furthermore, to assess long-term response, we evaluated IL-5 and IFN-γ levels 6 months after the initial immunization in BALB/c mice (Fig. 5). All groups exhibited a significantly higher response than the PBS control. The fusion protein, when combined with either adjuvant demonstrated a significantly higher IL-5 response compared to the fusion protein alone. However, the response was not significantly higher compared to VP1, VP8, or the vaccines. In terms of the IFN-γ response, all groups showed a significantly higher response compared to the control. Both VP1 combined with adjuvants and VP8 combined with adjuvants showed a significantly higher response compared to VP1, VP8, and fusion protein

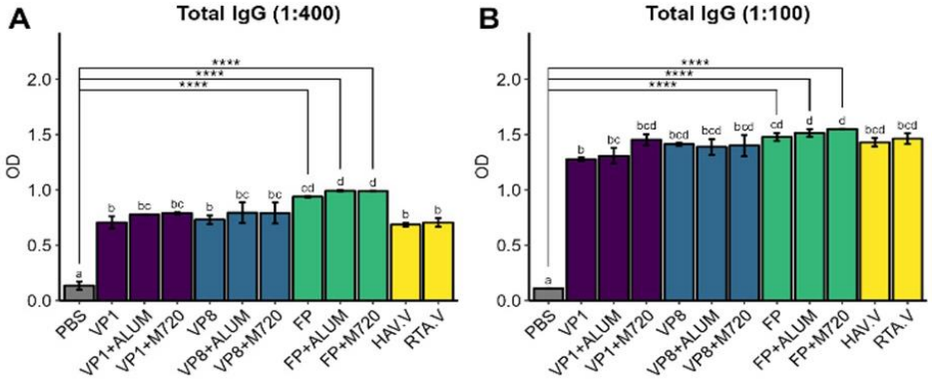

Fig. 3. Total serum IgG analysis against recombinant VP1-AAY-VP8 fusion protein. The sera were collected after 3 months from BALB/c mice, immunized subcutaneously. The results are shown at two concentrations: (A) 1:400, and (B) 1:100. Different letters represent statistical differences according to Tukey's test, with a confidence level of 95%. Values represent means ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. FP: fusion protein; PBS: phosphate buffer saline; HAV.V: Human hepatitis A virus vaccine; RTA.V: Rotavirus vaccine.

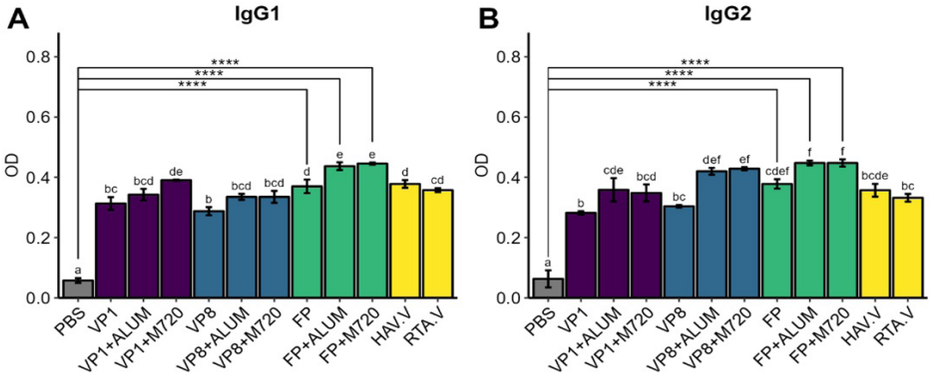

Fig. 4. Evaluation of antibody subtype responses. (A) IgG1, and (B) IgG2) against recombinant VP1-AAY-VP8 fusion protein. Sera were collected after 3 months in BALB/c mice post subcutaneous immunization. Different letters represent statistical differences according to Tukey's test, with a confidence level of 95%. Values represent means ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. FP: fusion protein; PBS: phosphate buffer saline; HAV.V: Human hepatitis A virus vaccine; RTA.V: Rotavirus vaccine.

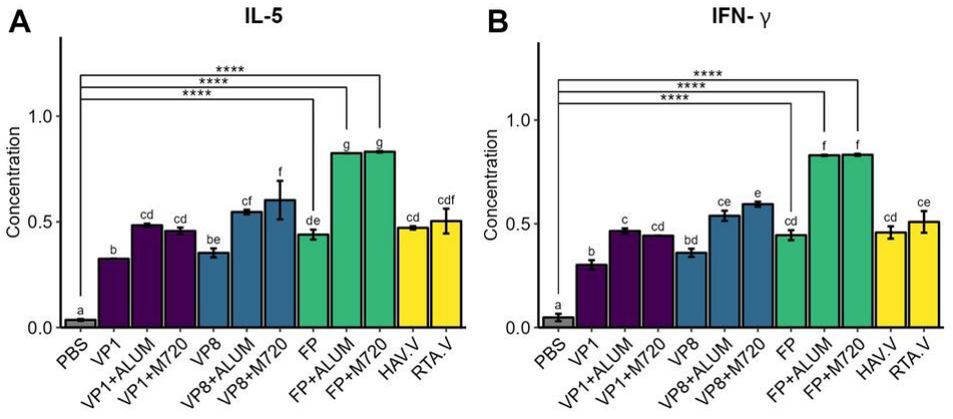

Fig. 5. Evaluation of long-term cytokine responses. ((A) IL-5, and (B) IFN-γ) against recombinant VP1-AAY-VP8 fusion protein after 6 months from BALB/c mice immunized subcutaneously. Different letters represent statistical differences according to Tukey's test, with a confidence level of 95%. Values represent means ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. FP: fusion protein; PBS: phosphate buffer saline; HAV.V: Human hepatitis A virus vaccine; RTA.V: Rotavirus vaccine.

alone. While the fusion protein alone did not elicit a more significant response compared to the vaccines, VP8 and VP1 alone, when combined with either adjutant, it induced a significantly higher IFN-γ response compared to both vaccines and the all-other tested antigens, suggesting a longer-lasting Th2 response.

Detection of anti- VP8* and anti-VP1 antibodies by the recombinant protein. As shown in Fig. 6, the specific binding to the positive Rotavirus or HAV samples was detected through ELISA assay. The antibody response was more intense as the fusion protein increased. The fusion protein did not show any significant difference in the production of anti-hepatitis A or anti-rotavirus antibodies when 5µg and 10µg of the protein were coated. However, when 15µg and 20µg of the protein were used, a stronger response against hepatitis A antibodies was observed compared to rotavirus.

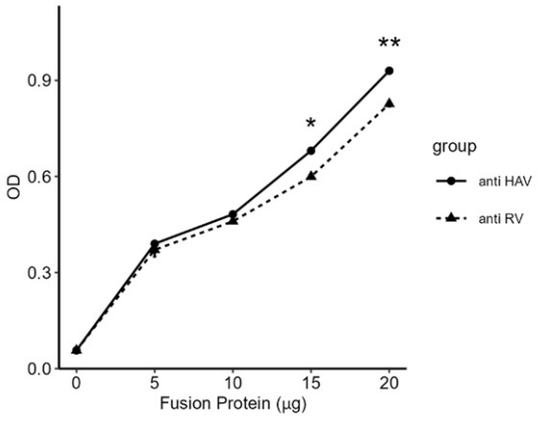

Fig. 6. Human antibody assay against the fusion protein. The test was performed at different protein concentrations (0 µg, 5 µg, 10 µg, 15 µg, and 20 µg). PBS was used for 0 µg concentration. Values represent means ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. FP: fusion protein; PBS: phosphate buffer saline; HAV.V: Human hepatitis A virus vaccine; RTA.V: Rotavirus vaccine.

DISCUSSION

Rotavirus vaccines have demonstrated remarkable safety and efficacy in preventing fatalities caused by rotavirus gastroenteritis infections (14, 15). There is a notable divergence among the antigenic epitopes within the VP8* region of circulating rotavirus A strains across different regions worldwide, highlighting the genetic heterogeneity inherent in this virus (16). The majority of genetic variation is concentrated in the VP8* cleavage fragment of VP4, underscoring its importance in shaping the virus's antigenic makeup (17). Additionally, structural analysis reveals certain amino acid differences in exposed regions of the antigenic epitopes within the VP8* region, potentially affecting antigenicity (17). Consequently, current efforts in the development of rotavirus vaccines have been aimed at targeting specific antigenic segments, with the expectation that these segments will induce the production of neutralizing antibodies, addressing challenges posed by the virus's dynamic nature and evolving genetic landscape.

Previous studies have indicated the presence of conserved epitopes in the VP8* (30). Subsequent studies have examined the immunogenicity of VP8 to determine its appropriateness as a candidate for a subunit vaccination. A modified variant called ΔVP8* plays a vital role in inducing a potent immune response against rotavirus. It generates substantial amounts of both homotypic and heterogeneous rotavirus-neutralizing antibodies (31, 32). Preliminary examination of the immunogenicity of the VP8* peptide confirms its inherent immunogenic properties. A recent study has discovered that VP8* antigens are present in larger protein complexes, suggesting that increasing the number of peptide copies during immunization, as previously suggested, could potentially improve the immune response to the peptide (33). Furthermore, ΔVP8* has a significant ability to boost the immunogenicity of the antigen components when combining two or three proteins. This highlights its potential as a beneficial subunit vaccine (34).

Recognizing the significance of VP1 and VP8, our analysis specifically targeted the immunodominant regions of VP1-HAV and VP8-ROTA. We obtained total protein extracts from *E. coli* BL21 (DE3) cells containing expression plasmids (pET24a-VP8-VP1). Subsequently, the polyhistidine-tagged recombinant VP8* -rotavirus + AAY + HAV-VP1 was isolated from soluble protein extracts using Ni-NTA affinity chromatography. The purities and identities of the resulting purified recombinant VP1-AAY-VP8 were assessed using SDS–PAGE followed by Coomassie brilliant blue staining. To confirm the expression of the recombinant VP8* -rotavirus + AAY + HAV-VP1 fusion protein, SDS–PAGE was performed both before and 4h after induction with Isopropyl β-D-1-thiogalactopyranoside.

To assess the immunogenicity of the fusion protein, we subcutaneously immunized BALB/c mice with the fusion protein, VP8-ROTA, and VP1-HAV, individually and in combination with adjuvants Alum and M720. We used either M720 or Alum adjuvants to enhance the vaccine's capacity to stimulate the immune system. Our findings showed that the VP1-HAV and VP8-ROTA vaccines, as well as the fusion protein, whether combined with M720 or Alum adjuvants, induced significantly higher responses compared to control samples, in terms of total IgG, IgG1, and IgG2. Notably, the fusion protein induced a higher total IgG response at a 1:400 concentration and demonstrated higher IgG1 and IgG2 responses compared to the vaccines. In a related study, Jang et al. focused on the recombinant subunit HAV protein, generating VP1-His, 3D2-His, and VP1-3N-His recombinant polypeptides using the *E. coli* expression system. Administering these polypeptides to mice prompted the production of specific IgG antibodies against the targeted antigens in both sera and cytokine secretions from spleen cells (13, 17).

In the subsequent stages, at 3 and 6 months following subcutaneous immunization in BALB/c mice, we evaluated Interleukin 5 (IL-5) and Interferon gamma (IFN-γ) to examine the short- and long-term responses of Type 1 T helper (Th1) and Type 2 T helper (Th2) cells. IL-5 and IFN-γ play pivotal roles in modulating immune responses, with IL-5 primarily promoting short-term humoral immunity by activating B-cells and facilitating antibody production, while IFN-γ is associated with boosting short-term cellular immunity, including the activation of cytotoxic T cells and macrophages (35-37). Our findings indicated that the fusion protein with either adjuvant demonstrated significantly higher short-term IL-5 and IFN-γ levels compared to all other groups. In the long-term response, the fusion protein with either adjuvant demonstrated a significantly stronger IFN-γ response. However, concerning the IL-5 response, despite being higher, the response was not significant compared to other groups.

Our findings demonstrated the fusion protein's capability to neutralize human antibodies produced against both viruses, showing its effectiveness in inducing an immune response against both rotavirus and hepatitis A viruses. Interestingly, at higher concentrations (15μg and 20μg), a more robust response was demonstrated, which was significantly stronger towards hepatitis A antibodies compared to rotavirus antibodies. Moreover, Jang et al. concluded in their study that sera from mice immunized with VP1-3N-His, VP1-His, and 3D2-His could effectively inhibit HAV propagation, highlighting recombinant VP1 as a promising candidate for HAV subunit vaccines. In a separate exploration of VP1, bioinformatics analysis unveiled conserved regions, indicating that the most preserved residues are internally situated and interact with other capsid proteins. Arginine emerged as the most prevalent conserved residue, followed by proline (17, 25, 38).

The utilization of live-attenuated rotavirus vaccines has presented challenges stemming from potential recombination events between the vaccine

strain and circulating strains within the population, the logistical burden of maintaining a cold chain, and the risk of intestinal obstruction (39). On the other hand, interest in producing recombinant viral subunit vaccines has increased due to the absence of genetic components of the virus, higher safety, elimination of the need for a cold chain during vaccine transport, and markedly reduced production costs.

One limitation of the study is the fact that the results on mice may not fully reflect human immune responses. Moreover, although we assessed immune responses and antibody production, this study did not address other aspects of efficacy such as durability of protection, potential adverse effects, or effectiveness across various strains of rotavirus and hepatitis A virus. Therefore, there is a need for more comprehensive studies and clinical trials to provide a more comprehensive assessment of the efficacy of the recombinant rotavirus and HAV vaccines.

CONCLUSION

We developed a fusion structure of the rotavirus VP8 protein and the hepatitis A virus VP1 as a dual vaccine candidate. This fusion protein has demonstrated the ability to induce both short-term and long-term immunity against rotavirus and hepatitis A virus, showing great promise as a vaccine candidate. Future research should prioritize the examination of the efficacy, safety, and long-term immune response of this recently developed dual vaccine candidate in clinical settings. This will require conducting comprehensive clinical trials and in-depth mechanistic studies to assess its potential for broader vaccine usage.

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