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Engineering and Development of VP4-VP7 Rotavirus Fusion Protein in *Pichia pastoris*

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ABSTRACT

Introduction: Vaccination is the most effective measure to prevent Rotavirus infection in children under five years of age. The most important targets of neutralizing and protective antibodies against this virus are VP4 and VP7 proteins of Rotavirus. Today, the recombinant protein produced in yeast plays an important role in producing highly effective and cost-effective vaccines. **Methods:** The effect of different linkers, including flexible and rigid, were evaluated on the stability and immunogenicity of the protein via *in silico* assays. A suitable linker was selected and expressed in *Pichia pastoris* yeast. Prediction and validation were carried out using bioinformatics tools, including Expasy's ProtPara, Phyre2 online server, I-TASSER server, ElliPro. Moreover, an appropriate linker was selected for cloning into pPICZα and expression in *P. pastoris*. **Results:** The results showed that as a flexible linker, $(GGGGS)_3$ was the best structure to provide stability for VP4-VP7 target fusion protein. The produced recombinant protein was stable after the expression. **Conclusion:** These *in silico* results and expression data on *P. pastoris* suggested that VP4–$(GGGGS)_3$-VP7 construct can potentially serve as a potent immunogenic candidate for recombinant Rotavirus vaccines.

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INTRODUCTION

Rotavirus (RV) infection is the major cause of dehydration and acute diarrhea in young children worldwide. According to the World Health Organization (WHO) estimates, approximately 215,000 infants and young children die each year from RV infections that are preventable by vaccines [1]. RVs are particles with multi-layered icosahedral capsid non-enveloped, double-stranded RNA (dsRNA) genomes [2]. Structural proteins including VP2, VP6, VP4, and VP7 create a three-layered capsid that packages eleven segments of the dsRNA genome with two viral enzymes consisting of a viral polymerase and a capping enzyme [3].

Vaccination is the most successful medical intervention ever developed, and it is cost-effective, too. It has had a considerable impact on global health by reducing the mortality and morbidity of infectious disease [2]. Two vaccines against RV are recommended by WHO, namely Rotarix® and RotTeq® [4]. RV vaccines are introduced in national immunization programs of many countries such as Australia, Belgium, Brazil, Mexico, South Africa and the United States of America. [5, 6]. Live attenuated vaccines are used today; however, the practicability and effectiveness of these vaccines have not yet been established in underdeveloped regions where most RV infant deaths occur [7]. Protective epitopes can be used for designing subunit vaccines. In addition, recombinant proteins can be used to produce non-replicating subunit vaccines. The use of subunit vaccine has significant advantages, including safety, low side effects and capability to induce increased protective immune responses [8].

Two proteins, namely VP4 and VP7 of RV are the neutralizing goals in the immune system. Using VP4 and VP7 proteins of RV leads to a broader production of RV-neutralizing antibodies. In addition, the immunogenicity and structures of these proteins make them good candidates for designing subunit vaccines [9, 10]. Linkers play an important role in binding of the domains and the function of fusion proteins. Linkers in fusion proteins are categorized into three types: flexible, rigid, and cleavable [11]. Flexible linkers are exerted to attach domains that require inter-domain interplays [12, 13]. Today, the recombinant proteins produced in yeasts play an important role in producing highly effective and inexpensive vaccines. Yeasts are important hosts for the production of recombinant proteins. The advantages of these single-celled microorganisms are their rapid growth, ease of genetic manipulation,

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post-translational processes, economic benefits, and microbial safety [14-16].

The aim of this study was to evaluate the effects of various linkers on the stability and immunogenicity of an RV vaccine candidate. In this study, eight different VP4-VP7 fusion protein structures were designed by bioinformatics methods; each VP4 and VP7 design was evaluated for immunogenicity *in silico* and then was compared with a selected fusion protein. The expression of VP4 and VP7 fusion proteins with the correct structure in *Pichia pastoris* yeast was then analyzed in an attempt toward making a new non-proliferative vaccine candidate against RV.

MATERIALS AND METHODS

Search and Collection of Fusion Protein Sequence

The complete amino acid sequences of Human RV A (G1P1A), VP7 (NCBI accession number: P11853.1), and VP4 (NCBI accession number: AGE98621.1) were retrieved from NCBI in FASTA format. The designed constructions VP7: VP4 were linked by 5 flexible and three rigid linkers as presented in Table 1.

Table 1. Types of flexible and rigid linkers used in this study.

Linker		Ref.
Type	**Sequence**	
Flexible	$(Gly)_{15}$	(Bai and Shen, 2006)
	$(GGGGS)_n$ (n=0,1, 2 and 3)	(Huston et al., 1988) (Bergeron et al., 2009)
Rigid	$(EAAAK)_3$	(Takamatsu et al., 1990)
	$A(EAAAK)_4ALEA(EAAAK)_4A$	(Bai and Shen, 2006)
	AEAAAKEAAAKA	(Zhao et al., 2008)

Prediction of Various Physicochemical Properties

The physicochemical parameters, molecular weight, formula, total number of positively charged residues (Arg-Lys), total number of negatively charged residues (Asp-Glu), theoretical isoelectric point (pI), grand average hydropathicity (GRAVY), aliphatic, and instability index were calculated using Expasy's ProtParam prediction server (http://us.expasy.org/tools/protparam.html).

Structure Analysis

The secondary structure of fusion proteins was predicted using the Phyre2 online server. This tool uses advanced methods to build 3D models, and it specifies the percentage of coils, stands, and helix [17]. I-TASSER server was used for producing high-quality tertiary structure models. It gives an indication of the predicted model's consistency. The modeling validation assays were performed to specify the stability of the model (e.g., RMSD, TM-score, and C score) [18].

Linear and Conformational B-cell Epitope Prediction

The conformational epitopes and B-cell discontinuous epitopes prediction were chosen based on the high-score results of online servers ElliPro [19] and DiscoTope v2.0 [20]. ElliPro provides the score for the average value of each output epitope defined as PI (Protrusion Index).

Cell Strains, Plasmids, and Culture Medium

Escherichia coli Top10 was used as the host for DNA manipulations, and *P. pastoris* strain GS115 was used for protein expression studies. E. *coli* Top10 was cultured on Luria-Bertani (LB) agar medium. Low-salt LB medium was used for zeocin selection of transformants at 37 ˚C. Antibiotics (final concentrations 100 µg. mL^{-1} Tetracycline and 25 µg. mL^{-1} zeocin) were added to LB medium for plasmid selection. *P. pastoris* was cultured on yeast extract peptone dextrose (YPD) agar plates and incubated at 30 ˚C. YPD agar plates were used to grow GS115 cells, and YPD plates containing zeocin were used for selecting positive *P. pastoris* transformants.

Construction of the Expression Vector

The $(GGGGS)_3$ as a potentially suitable candidate linker was chosen based on the results of previous sections and was added to the construction of VP4 – $(GGGGS)_3$ - VP7. The whole fusion protein gene was codon-optimized according to the Pichia codon usage and synthesized by Generay™ Biotechnology (Shanghai, China). The positive recombinant bacteria carrying the construct (named pPICZαA-FuPro) were chosen on low-salt LB plates with 25 µg.mL^{-1} zeocin. Two primer pairs, FuPro-F/FuPro-R and AOX1-F/AOX1-R, were used for amplification of the presence of the fusion protein gene and its completed construction, respectively. Characteristics of primer pairs are shown in Table 2. The PCR conditions were 30 cycles of 95 °C for 1 min, 58 °C for 30 s, and 72 °C for 1 min.

Table 2. Sequences of the primers used in this study.

Primer's name	Sequence
FuPro-F	ATGGCTTCCTTGATTTAC
FuPro-R	GCACGCTTGTATTGAAT
AOX1-F	GACTGGTTCCAATTGACAAGC
AOX1-R	GCAAATGGCATTCTGACATCC

Transformation of *P. pastoris* and Selection of Recombinant Clones

For constructing a competent *P. pastoris* GS115 strain, pPICZαA-FuPro was linearized by *Sac*I, and then competent yeast cells were prepared and transformed with Biorad Gene Pulser according to the manufacturer's protocol [21]. Yeast-positive transformants were analyzed for the presence of pPICZαA-FuPro construct using PCR.

Expression of the Fusion Protein

Single yeast colonies of zeocin-resistant transformants were grown overnight in 25 mL of BMGY medium, shaking at 30 °C at 250 rpm to reach an OD_{600} of 2-6. The cells were then centrifuged for 5 min at 3000 ×g. The pellets were re-suspended in 50 mL BMMY medium to an OD_{600} of 1 and grown at 30 °C with shaking at 250 rpm for inducing protein expression. After adding 63 µL fresh %100 methanol every 24 h (to compensate for evaporating and losing metabolites), the culture supernatants of the transformants were examined by SDS-PAGE and Western blotting to detect the recombinant protein.

SDS-PAGE and Western Blot Analyses

The supernatant culture was analyzed with electrophoresis on a 12% polyacrylamide gels. The gels were stained with blue R-250 Coomassie. Western blot assay was performed with a hyperimmune rabbit antiserum (1:50) raised against Wa (P[8]) strain according to standard protocols [22].

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RESULTS

Collection of Protein Sequences and PDB Structures and Prediction of Various Physicochemical Properties

The fusion protein sequence was retrieved as FASTA format from the NCBI database and was linked together by the above-mentioned flexible and rigid linkers. Results of the designed constructs with flexible and rigid linkers are showed in Table 3. The isoelectric point (pI) was computed as 5.46-48 for all the constructs. The instability index was used for estimating the stability of the proteins *in vitro*. The instability index of all the constructs was more than 40, and the constructs were found to be stable with their high aliphatic index. The GRAVY values of all the constructs had the best hydrophilicity pattern for better interaction with water (Table 3).

Table 3. Properties of the designed constructs with linkers determined by Expasy's Prot Param.

Protein	VP4 – $(G)_{15}$ - VP7	VP4 - VP7	VP4 - GGGGS - VP7	VP4 – $(GGGGS)_2$ - VP7	VP4 – $(GGGGS)_3$ - VP7	VP4 - AEAAAK EAAAKA- VP7	VP4 - $A(EAAAK)_4ALE A(EAAAK)_4A$- VP7	VP4 - $(EAAAK)_3$ - VP7
Molecular weight	125705.21	124849.43	125164.72	125480.01	125795.29	125932.64	129140.23	126261.01
Theoretical pI	5.46	5.46	5.46	5.46	5.46	5.47	5.45	5.48
Total no. of negatively charged residues (AspþGlu)	112	112	112	112	112	114	121	105
Total no. of positively charged residues (ArgþLys)	99	99	99	99	99	101	107	102
Formula	$C_{5596}H_{8725}N_{1477}O_{1729}S_{42}$	$C_{5566}H_{8680}N_{1462}O_{1714}S_{42}$	$C_{5577}H_{8697}N_{1467}O_{1720}S_{42}$	$C_{5588}H_{8714}N_{1472}O_{1726}S_{42}$	$C_{5599}H_{8731}N_{1477}O_{1732}S_{42}$	$C_{5612}H_{8758}N_{1476}O_{1730}S_{42}$	$C_{5749}H_{8990}N_{1516}O_{1778}S_{42}$	$C_{5626}H_{8782}N_{1480}O_{1735}S_{42}$
Instability index	32.77	31.51	31.75	31.98	30.65	31.28	32.22	31.51
Aliphatic index	85.99	87.17	86.77	86.38	86.45	86.95	85.99	86.80
GRAVY	-0.248	-0.246	-0.247	-0.249	-0.244	-0.244	-0.250	-0.248

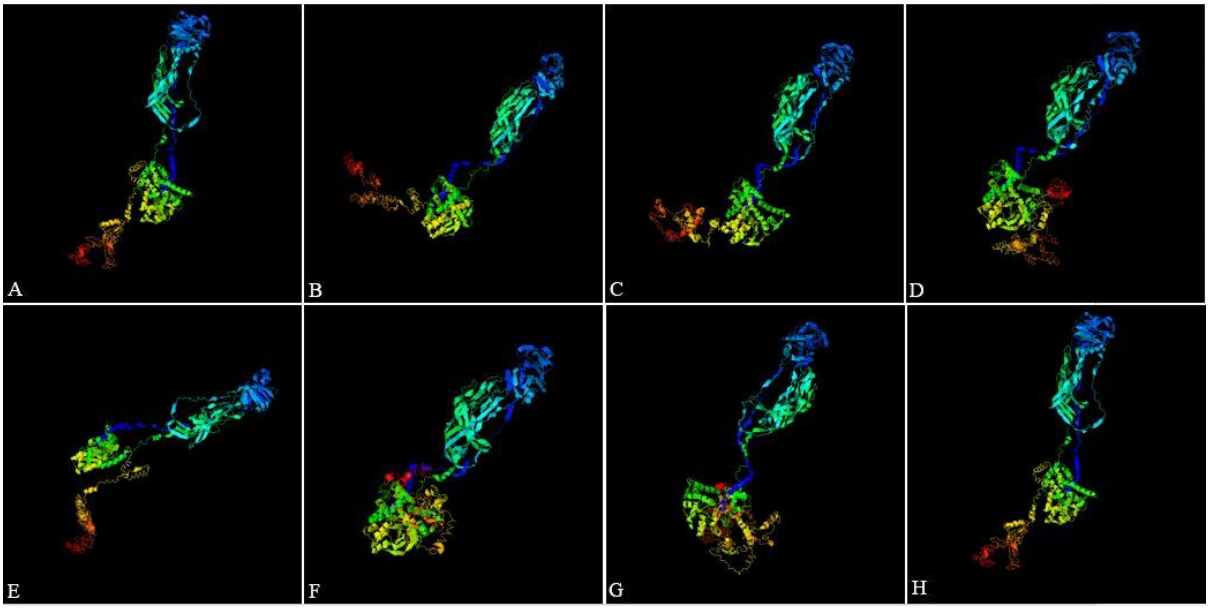


Fig. 1. The 3D structure of the designed constructs with flexible and rigid linker. **(A)** VP4 - VP7**(B)** VP4 – $(G)_{15}$ - VP7 **(C)** VP4 - GGGGS - VP7 **(D)** VP4 – $(GGGGS)_2$ - VP7 **(E)** VP4 – $(GGGGS)_3$ - VP7 **(F)** VP4 -$(EAAAK)_3$ - VP7 **(G)** VP4 - AEAAAKEAAAKA- VP7 **(H)** VP4 - $A(EAAAK)_4ALEA(EAAAK)_4A$ - VP7.

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Structural Analyses

The analysis of secondary fusion protein structure by Phyre2 is shown in Table 4. The appropriate fusion structure consists of the highest α-helixes.

The value of the C-score for predicting the quality of the models is shown in Table 4. C-score is a confidence score for estimating the quality of models predicted by I-TASSER. A higher value of the C-score shows a high confidence model. The fusion protein construct linked by $(GGGGS)_3$, which was accepted as the best 3D structure model, had the highest C-score predicted. Glycine-rich peptides provide flexibility, allowing domains to move independently while maintaining individual 3D shapes (Fig. 1).

Linear and Conformational B-cell Epitope Prediction

Ellipro's results showed high scores for VP4 –$(GGGGS)_3$-VP7 residue with a score of 0.972 (Table 5). The mean entropy values for each predicted epitope provide data in terms of the vaccine coverage. The epitope VP4-$(GGGGS)_3$-VP7 exhibited higher identified residues of the B-Cell epitope and could thus be used in this study (Table 5).

Table 5. Results of predicted linear and discontinuous epitopes and identified B-Cell epitope residues out of total residues, for each fusion protein.

Proteins	Predicted Linear Epitopes		Predicted Discontinuous Epitopes		Identified B-Cell epitope residues
	1 Number of Epitope(s)	Best score	2 Number of Epitope(s)	Best score	
VP4 - VP7	10	0.840	3	0.831	235
VP4 - GGGGS - VP7	11	0.864	11	0.832	271
VP4 – $(GGGGS)_2$ - VP7	18	0.821	10	0.811	228
VP4 – $(GGGGS)_3$ - VP7	16	0.878	3	0.972	277
VP4 – $(G)_{15}$ - VP7	17	0.851	4	0.816	235
VP4 -A$(EAAAK)_4$ALEA$(EAAAK)_4$A-VP7	13	0.853	6	0.846	256
VP4 - AEAAAKEAAAKA- VP7	19	0.838	8	0.929	246
VP4 -$(EAAAK)_3$ - VP7	10	0.840	3	0.831	235

Preparation of Fusion Protein Construct and Transformation of *P. pastoris*

The construct (pPICZαA-FuPro) was linearized by *SacI* restriction enzyme and transformed by electroporation into *P. pastoris* GS115 cells. PCR amplification of genomic DNA using the above-mentioned specific primers confirmed the presence of a 740-bp fragment for the positive transformants. PCR analysis by AOX1 primers amplified a 3895-bp DNA fragment. Also, an amplicon produced by AOX-F/FuPro-R and FuPro-F/AOX-R primers showed that our target gene was properly transformed into the *P. pastoris* genome (Fig. 2).

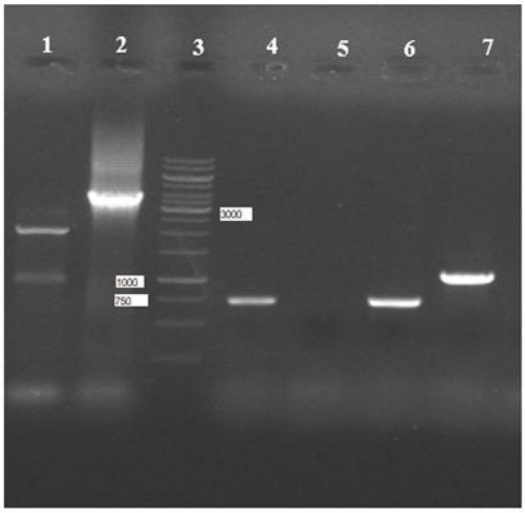


Fig. 2. PCR amplification of transformants genomic DNA by specific primers.
Lane 1: FuPro-F and AOX-R (2350 bp), Lane 2: AOX-F and AOX-R (3895 bp), Lane 3: 1 Kb molecular ladder, Lane 4: FuPro-F and FuPro-R (740 bp), Lane 5: yeast clone transformed with pPICZαA plasmid (negative control), Lane 6: RepetitiousFuPro-F and FuPro-R (740 bp), Lane 7: AOX-F and FuPro-R (1093 bp).

Expression and Detection of the Fusion Protein in *P. pastoris*

A 4-day culture suspension was collected and analyzed with Western blotting and a ~120-kDa protein band was observed (Fig. 3).

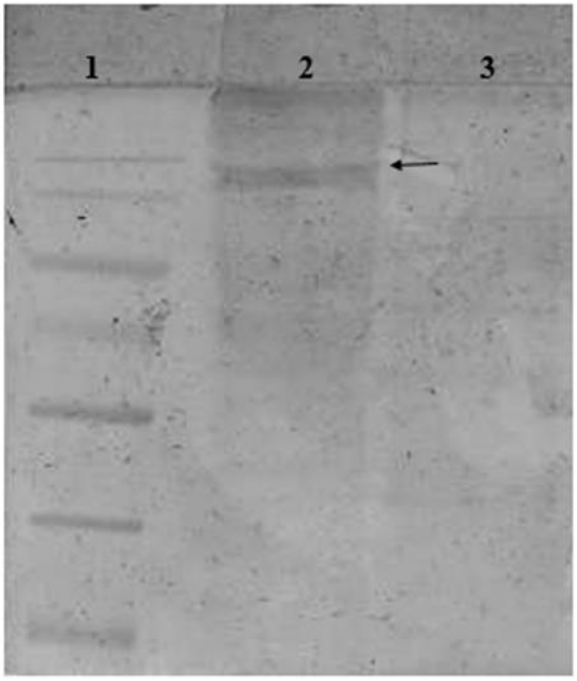


Fig. 3. SDS-PAGE analysis reaction with FuPro.
Lane 1: molecular weight marker (11–180 kDa), lane 2: recombinant GS115/pPICZαA-FuPro and lane 3: negative control transformant with empty plasmid (GS115/pPICZαA).

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The supernatant was analyzed by a Western blot using a hyperimmune rabbit antiserum to confirm the expression of the fusion protein. The findings showed that in the fusion protein cultures, a hyperimmune rabbit antiserum interacted positively with a protein band of the target molecular weight band. Moreover, in the negative control, no related band was found (Fig. 4). These results demonstrated the effective expression of the fusion protein target in the *P. pastoris* system.

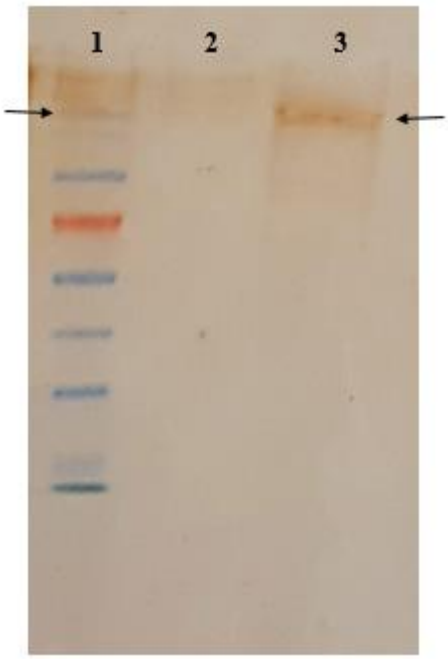


Fig. 4. Western blotting analysis reaction with the fusion protein. Lane 1: Molecular Weight marker (11–180 kDa), lane 2: negative control transformant with empty plasmid (GS115/pPICZαA) and lane 3: recombinant GS115/pPICZαA-FuPro.

DISCUSSION

Vaccination plays a critical role in prevention of infectious diseases. A vaccine should be cost-effective and highly efficacious to protect the population. Although vaccine production technology is advanced, vaccine design is still a broader challenge for vaccine development. In this study, eight different models of a fusion protein composed of VP4 and VP7 proteins of RV were designed by two different groups of hard and flexible linkers. To save time and resources, bioinformatics studies were applied to select potentially the best model for producing the desired fusion protein. Hence, physicochemical properties, including instability index and hydrophilicity of a protein structure, were investigated. The results showed that the fusion protein VP4-VP7 with linker $(GGGGS)_3$ has acceptable stability. The molecule with more hydrophilicity properties has a higher surface area available for contact with the antibody, and the most important criteria for determining epitope behavior is the availability of the region to be bound to the antibody [23]. Moreover, prediction and analysis of the secondary structure of the fusion protein with linkers showed that VP4-VP7 fusion protein with $(GGGGS)_3$ linker has the highest number of α helices compared to other models. Other studies have shown the stability of fusion protein by flexible linkers. These linkers enable specific degrees of movement; thus, flexible linkers with medium size, the same as $(GGGGS)_3$ or $(GGGGS)_4$, are assumed to provide stable structures [24, 25].

An RT vaccine candidate protein must also contain suitable epitopes for B-cell lymphocyte receptors. Based on the available information in the literature [27], the bioinformatics tools used in this study identified antibodies and epitopes which can identify linear and space epitopes. The results showed that the VP4-VP7 fusion protein with $(GGGGS)_3$ linker had more antigenic epitopes than other designed models.

VP4 and VP7 RV proteins are important immunogenic options that have been investigated in other studies for the development of a recombinant vaccine. The VP4 is a key target for developing a recombinant RV vaccine because it interferes with the attachment and penetration of RV [28]. Li and colleagues have demonstrated that the truncated VP4 could increase immunogenicity and immunoprotective RV vaccine candidate and could be considered a viable candidate for further development as a RV vaccine [28]. Studies have shown that the use of VP7 protein stimulates the production of RV-neutralizing antibodies, but the use of this antigen alone is not sufficient to protect against RV [29]. This antigen has been used as a vaccine candidate in various studies. The protein structure of VP7 is glycosylated; therefore, this antigen needs a eukaryotic expression system for complete and correct expression [30, 31]. Eukaryotic cells like Saccharomyces cerevisiae and *P. pastoris* are notable hosts for the expression of recombinant products. The high quality and native folding of expressed proteins in *P. pastoris* have made it the best choice in many studies [32-34].

In this study, a secretory VP4-VP7 fusion protein was achieved by cloning the gene of interest into pPICZαA vector. The expression of the protein fusion in *P. pastoris* host was confirmed by SDS PAGE and Western blotting. *P. pastoris* has been previously used to express RV antigens as vaccine candidates [35, 36]. Other studies have shown that the subunit protein produced by yeast can be used as a suitable vaccine candidate [16, 37]. Further studies are required to assess the immunogenicity of this fusion protein in animal models of RV infection.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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