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Novel approach to porcine epidemic diarrhea vaccine development by reverse vaccinology

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ABSTRACT

Porcine epidemic diarrhea (PED) affects most swine, causing severe diarrhea and rapid death. The mortality rate of piglets reaches up to 80%-100%, which affects the pig industry. This study aimed to develop a PED vaccine using reverse vaccinology applying bioinformatic tools and evaluate epitope-based vaccines in an animal model. The genome of PED virus (PEDV)-CBR1 was retrieved and used, following the prediction of open reading frames (ORFs) by ORF finder. Consequently, protein localization was predicted by the iLoc-Virus program. Target proteins were subjected to adhesin-like prediction followed by B-cell epitope prediction. Finally, three epitope-based vaccines were synthesized and injected in an animal model, and the mucosal antibody response was measured. The candidate vaccine can stimulate mucosal antibodies in different mucosal organs. In conclusion, reverse vaccinology can be applied in PED cases by using different bioinformatic tools and proofing in the animal model.

Keywords: reverse vaccinology; porcine epidemic diarrhea virus; epitope vaccine; mucosal antibody

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1. INTRODUCTION

A novel reverse vaccine design has been discussed, reviewed, and demonstrated to be applicable to viruses, in particular the Herpesviridae family (Bruno et al., 2015; Rappuoli et al., 2016). Porcine epidemic diarrhea virus (PEDV) is a virus in the Coronaviridae family. It causes acute diarrhea, vomiting, dehydration, and high mortality rates in swine (Lee, 2015; Song et al., 2015). The incubation period of PEDV is approximately 2 days, ranging from 1 day to 8 days, and the morbidity approaches 100% in piglets (Lee, 2015). Commercially available vaccines may reduce the prevalence of the disease; however, they fail to induce complete protection (Song et al., 2007). The recurrent emergence of PEDV is due to viral strains and vaccine types. For example, a live attenuated porcine epidemic diarrhea (PED) vaccine was developed from a Korean PEDV isolate in 1998. PEDV has continued to be an unexpected and a major pandemic to the Korean pork industry since March 2014 (Yang et al., 2018). A novel vaccine design, such as the inactivated QIAP1401-p70 Korea PEDV strain, is still required to control pandemic diseases (Lee et al., 2018).

The genetic types of PEDV strains were studied and reviewed. They have been classified following phylogenetic "S gene" studies. PEDV can be genetically separated into two groups: genogroups 1 (G1; classical) and 2 (G2; field epidemic or pandemic) (Jung et al., 2020; Lee, 2015). In Thailand, several outbreaks of severe PEDV infection had S genetic signatures typical of field epidemic

G2 strains and were placed in the cluster adjoining the South Korean and Chinese strains in the G2a or G2b subgroup (Temeeyasen et al., 2014).

In terms of vaccine development against PEDV, antibodies are focused on stimulation because they adopt a first line defensive mechanism (Gerdts and Zakhartchouk, 2017). Newborn piglets are protected by transferring maternal sow milk that was immunized with PEDV, which led to reduced mortality in these animals (Shibata et al., 2001). This finding was later confirmed by the immunoprophylactic effect of colostrum by mucosal antibodies from the maternal sow via colostrum and milk (Lee, 2015). This study aimed to develop a novel epitope vaccine against PEDV by using the reverse vaccinology method. Different bioinformatic tools were studied and investigated for screening of the possible target vaccine. Finally, an animal model was used to evaluate the induction of mucosal antibodies.

2. MATERIALS AND METHODS

2.1 Genetic retrieval of the Thai PEDV genome

Complete PEDV genome sequences were retrieved from the National Center for Biotechnology Information (NCBI) (GenBank) database and published articles. The keywords for the search in GenBank were "porcine epidemic diarrhea virus Thailand complete "genome", "PEDV", "complete PEDV", and "PEDV Thailand". Filtering criteria were focused on epidemics in Thailand and complete genome sequences.

2.2 Prediction expression and localization of proteins

From the selected Thai PEDV sequence, open reading frames (ORF) were predicted by NCBI's ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder). Parameters were assigned as follows: a minimal ORF length of 75 nt, standard genetic code, and ORF start codon "ATG" only.

The localization of all possible protein predictions from ORF Finder were predicted using the iLoc-Virus program (http://www.jci-bioinfo.cn/iLoc-Virus) (Xiao et al., 2011). This web-based software can predict six subcellular locations of viral proteins, including secreted protein, cell membrane, cytoplasm, endoplasmic reticulum, capsid, and nucleus. Secreted protein and cell membrane protein localizations were focused on during the search for potential candidate vaccines.

2.3 Prediction of adhesin-like proteins and B-cell epitope

From previous prediction results, the selected cell membrane proteins and secreted proteins were further predicted for the potential adhesin-like property by SPAAN (Sachdeva et al., 2005). The program was run on a Linux OS installed on a Dell computer, with core i5 CPU and 8G RAM. The prediction results were shown as the probability of a protein being an adhesion-like protein (P_{ad}). Under neural network architecture with a multilayer feed-forward topology, most of adhesin proteins had P_{ad} values equal or greater than 0.51.

Following this process, the predicted proteins with P_{ad} values of 0.51 or higher were selected and processed to predict B-cell epitopes using seven bioinformatic tools: ABCpred (Saha and Raghava, 2006), BCPred (Chen et al., 2007), FBCPred (El-Manzalawy et al., 2008), SVMtrip (Yao et al., 2012), COBEPro (Sweredoski and Baldi, 2009), LBtope (Singh et al., 2013), and EPMLR (Lian et al., 2014). The prediction parameters in each tool were determined based on the values proposed by the developer.

2.4 Peptide synthesis and immunization

The peptide sequences were selected based on previous B-cell epitope prediction results. In brief, the total sequencing peptides were scored and sorted for B-cell epitopes. The selected candidate peptides were synthesized by conjugation, in line with a pan HLA DR-binding epitope (PADRE) (Alexander et al., 2000). All peptides were synthesized from Genscript (Piscataway, NJ, USA). The 99% purity and standard analyses were performed following the manufacturers' instructions. The peptides were kept at 4°C until dissolved and used.

Four groups of five 2-month-old female BALB/cMlac mice were obtained from the National Laboratory Animal Center (Mahidol University, Thailand). They were inoculated into the thigh of mice with 0.2 mL (about 1 µg) synthesized peptide dissolved in sterile phosphate buffered saline (PBS) with pH 7.4 (final concentration 5 µg/mL). Four groups were designed as control (group 1, 5% BSA dissolved in PBS) and as peptide groups (groups 2-4, with different amino acid sequences). The injections were carried out for four times at 2-week interval. These procedures were reviewed, and the animal experimental design (approval No. 001/2558) was approved by the Animal Ethic Committee of Faculty of Pharmacy, Silpakorn University.

2.5 Harvest of specimens by perfusion-extraction (PERFEXT) method

The mice were sacrificed 2 weeks after the last injection. Mucosal extracts were obtained in accordance with the PERFEXT method (Decroix et al., 2003) with slight modifications. In brief, the mice were bled under anesthesia with 100 mL 30% chloral hydrate in PBS containing 1% heparin by intraperitoneal injection. The blood was kept, and 50 mL PBS containing 1% heparin was injected into the right-lower area of the heart. The selected organs were collected from exsanguinated mice. The peritoneum and fat were discarded. The organs were extensively washed in PBS to remove the extracellular fluid. All organs were homogenized at 4°C, and the pellets were kept frozen (-20°C) in a PBS solution containing protease inhibitors and 2% saponin. Intracellular molecules were extracted by thawing at 4°C and separated from insoluble components by centrifugation (10,000 xg) for 15 min at 4°C.

2.6 Detection of antibody levels by enzyme-linked immunosorbent assay (ELISA) method

A 96-well microtiter plate (Nunc, Roskilde, Denmark) was coated with 50 µL per well of OVA-linked peptides (10 mg/mL) overnight at 4°C followed by washing with 0.1% v/v Tween 20 in PBS (PBS-Tween) and saturation of free reactive groups with 5% w/v BSA in PBS. The wells were washed with PBS-Tween and incubated with 50 µL diluted samples for 1 h at 37°C. Following additional washes and incubation with 50 µL horseradish peroxidase-labeled goat anti-mouse IgG (Abcam, United Kingdom) or horseradish peroxidase-labeled goat anti-mouse IgA (Abcam, United Kingdom), the bound enzyme was revealed with O-

phenylenediamine. The detection signals were measured under a microplate reader (TECAN, Infini F50, TECAN, Switzerland) at OD 492 nm.

3. RESULTS

3.1 Complete Thai PEDV genome sequences

The Thai PEDV genome sequences were retrieved from GenBank and research articles. In GenBank, four complete Thai PEDV genome sequences include those with GenBank accession numbers KR610991.1, KR610992.1, KR610993.1, and KR610994.1 of which two PEDV genomes, namely, KR610991.1 (EAS1) and KR610993.1 (CBR1), were detected in the eastern region of Thailand (Cheun-Arom et al., 2015). All genome sequences were selected and compared for multiple-alignment genome sequences using the EMBL web service (Lassmann and Sonnhammer, 2006). CBR1, CRB2, EAS1, and EAS2 strains showed more than 96% nucleotide sequence similarities. Moreover, the CBR1 strain had a nucleotide sequence of a spike gene similar to that of the frequently found Chinese and Thai PEDV, with similarities between 94.2% and 98.5%. For this reason, in this study, the PEDV clone CBR1 (GenBank: KR610993.1) complete genome was selected as a candidate for Thai PEDV epitope for reverse vaccine design.

3.2 Fifty-four proteins selected for further comprehensive analysis

By using NCBI's ORF Finder, 190 ORFs were predicted under the selection criteria with nucleotide lengths of more than 75. Six ORFs already known from the complete CBR1 strain and within 3 ORFs were identified by protein location as surface-exposed proteins (spike, envelope, and membrane proteins) (Cheun-Arom et al., 2015). The localization of proteins in 184 ORFs was unknown.

Surface-exposed and secreted proteins are recommended for the development of vaccines by the reverse vaccinology method (Rappuoli et al., 2016). Thus, 184 ORFs from the previous step were predicted for their cellular locations. A total of 128 ORFs were excluded, and the remaining 56 ORFs were predicted by the iLoc-Virus program. From the prediction results obtained from the program (Table 1), 9 ORFs might be membrane proteins, and 1 ORF might be a secreted protein.

Table 1. Ten surface-exposed proteins predicted using the iLoc-Virus (PSSM: position-specific scoring matrix)

Position of protein	Genomic position	Protein length (Amino acid residues)	Prediction method
Cell membrane	35 to 304 Frame +2	89	PSSM
	10948 to 11250 Frame -2	100	PSSM
	15298 to 15555 Frame +1	85	PSSM
	17716 to 17895 Frame +1	59	PSSM
	18232 to 18453 Frame +1	73	PSSM
	20850 to 21221 Frame +3	123	PSSM
	26505 to 26735 Frame -3	76	Gene Ontology
	26544 to 26732 Frame +3	62	Gene Ontology
	26644 to 26916 Frame -2	90	PSSM
Secreted protein	12998 to 13204 Frame -1	68	PSSM

3.3 Total 13 epitope targets from adhesin-like proteins and B-cell epitopes

From the previous step, 10 ORFs were predicted by iLoc-Virus program, and 3 ORFs already known as spike, envelope, and membrane proteins were predicted for their adhesin-like properties. SPAAN is a Linux OS-based software that can identify adhesin-like proteins. Adhesins are good vaccine targets because they mediate adherence to host cell receptors for successful colonization. The prediction results were shown as P_{ad} values. Two ORFs with genomic positions between 20342-24499 (spike protein) and 26505-26735 (hypothetical protein) had P_{ad} values of more than 0.51 (0.60 and 0.74, respectively).

For B-cell epitope prediction, we selected bioinformatic tools that can be accessed via the internet and are free to use. From the previous step, two selected ORFs, namely, a spike protein (SK1) and a hypothetical protein (S7), were selected for further B-cell epitope prediction. Seven B-cell epitope prediction tools that were accessible via the internet were selected: ABCPred, BCPred, FBCPred, COBEpro, SVMtrip, LBtope, and EPMLR. They showed prediction results as scored points. Peptide chains with a high prediction score had a high probability to be an epitope. Table 2 shows the two sequences with the highest prediction score from each tool.

The top ten prediction results of each B-cell epitope prediction tool were compared, and the regions of peptide chains predicted as epitopes by most tools were selected as candidate epitopes. The summary results showed six possible epitopes from genomic positions between 20342 and 24499 (spike protein) and between 26505 and 26735 (hypothetical protein) (Table3). BEH7 and BEH8 were nucleocapsid proteins that were unsuitable for vaccine development. BES1-BES6 were selected and compared with four types of commercial vaccines (CV777, 83P-5, DR13, and SM98) from China, Japan, and Korea. Three novel candidate epitopes vaccines (BES1, BES2, and BES6), being different from commercial vaccines, were used to evaluate the animal model.

Table 2. Two highest scores from B-cell epitope prediction tools of S7 and SK1

Prediction tool	Hypothetical protein (S7)		Spike protein (SK1)	
	Sequence	Score	Sequence	Score
ABCPred	TGYETCYCYYYCLLCS	0.89	GELITGTPKPLEGVTD	0.96
	SEKLHDPGYFHDSVNY	0.87	PEVIPDYIDVNKTLDE	0.96
BCPred	SVNYRGTGCCLCYYSGIWTC	0.90	ECVKSQSQRYGFCGGDGEHI	1.00
	HLTGFCSWTGYETCYCYYYC	0.86	PDNKTLGPTANNDVTTGRNC	1.00
FBCPred	DPGYFHDSVNYRGT	0.95	HSNDGSNCTEPVLV	1.00
	YSGIWTCCCHCHDS	0.85	GFKGEGIITLTNSS	1.00
COBEpro	CHDSCY	0.79	GKDGISY	0.84
	LHDPGY	0.72	PTVGDF	0.84
SVMtriP	FHHGHLTGFCSWTGYETCYC	1.00	FSGCCRGPRLQPYEAFEKVH	1.00
	VNYRGTGCCLCYYSGIWTCC	0.80	LPGVVDAEKLHMYSASLIGG	0.76
LBtope	CHDSCYVNLHVK	1.07	NTLVDLEWFNRVETY	1.36
	FCSWTGYETCYCYYY	0.76	NSSDPHLTTFAIPLG	1.06
EPMLR	FHDSVNYRGTGCCLC	1.00	VGITWDNDRVTVFSD	1.00
	TGYETCYCYYYCLLC	1.00	GCCGACFSGCCRGPR	1.00

Table 3. Peptide sequences having potential B-cell epitope from ORFs 20342-24499 (SK1) and 26505-26735 (S7)

Position of protein	Name	Sequence	ORFs position	Epitope position	Note
Spike protein	BES1	DNKTLGPTANNDVTT	20342 to 24499	130-144	New
	BES2	LITGTPKPPLEGV	20342 to 24499	627-638	New
	BES3	SNDGSNCT	20342 to 24499	738-745	In market
	BES4	VKSQSQRYGFCGGDG	20342 to 24499	1123-1137	In market
	BES5	FSGCCRGPRLQPYE	20342 to 24499	1364-1377	In market
	BES6	NSSDPHL	20342 to 24499	351-357	New
Hypothetical protein	BEH7	TGYETCYCYYYC	26505 to 26735	15-26	Nucleocapsid protein
	BEH8	SVNYRGT	26505 to 26735	42-48	

3.4 Mucosal antibody responses from different candidate peptides

The mucosal antibodies were extracted by using the PERFEXT method, and the IgA and IgG levels that were secreted in the jejunum, ileum, cecum, colon, spleen, and serum were measured by ELISA. The results showed that peptide BES1 (PADRE-DNKTLGPTANNDVTT) had a high IgA-antibody response in various mucosal tissues, such as the jejunum, ileum, cecum, and colon, in contrast to a moderate IgG-antibody response in these mucosal tissues. The BES2 (PADRE-LITGTPKPLEGV) peptide had the highest IgG-antibody response in the jejunum, ileum, cecum, and colon but exhibited a slight IgA-antibody response in these mucosal tissues. The BES6 (PADRE-NSSDPHL) peptide showed a high IgA-antibody response in the cecum and colon but a slightly IgG response in other mucosal tissues (Figure 1). Between-group comparison using the Wilcoxon signed-rank test revealed that the IgG- and IgA-antibody responses of mucosal tissues were significantly higher than those of the negative control ($p<0.05$).

4. DISCUSSION

The development of a PED vaccine remains a challenge, especially when outbreaks continually occur in Asia, including China, Japan, Korea, and Thailand. Currently, two types of PED vaccines are available in the market: inactivated and live attenuated vaccines (Song et al., 2007). Inactivated vaccines are more stable, easier to transport, and cheaper than live attenuated vaccines. However, they have been less effective and often require booster shots. Live attenuated vaccines are usually very effective, and a single dose is often sufficient to produce long-lasting immunity. However, they cannot be used in pigs with poor health condition, and the attenuated reversion to the virulent form may possibly occur. In such a case, PED subunit vaccines developed by reverse vaccinology may be a better choice than live attenuated and inactivated vaccines. The reverse vaccinology method can accelerate vaccine development and can possibly be used to identify epitope vaccines (Rappuoli et al., 2016).

In this study, the genome of PEDV was retrieved and analyzed by using bioinformatic tools in three major steps. First, ORF Finder was used to predict the possible proteins that can be replicated from the viral genome. The structure of a virus is important for the re-design of vaccines because it is the first contact between the immune host cell and viral antigen. When developing a vaccine using the reverse vaccinology method, only surface-exposed and secreted proteins have a high potential to become an effective antigen. Various tools, such as iLoc-Virus and Virus-mPloc, can be used to predict the locations of virally produced proteins. The iLoc-Virus program was selected in this study because of its higher accuracy (78.2%) than Virus-mPloc (60.3%). However, as a limitation of this software, peptide chains of less than 60 amino acids may be unpredictable (Xiao et al., 2011). Thus, the identification of the adhesin-like property with a high sensitivity of 89% by

SPANN software was used, and only ORFs that had a P_{ad} value more than 0.51 were selected (Sachdeva et al., 2005). Finally, B-cell epitope prediction tools help in identifying possible peptides that can be a good candidate vaccine (Rappuoli et al., 2016). Each tool has a different prediction accuracy value. ABCPred and LBtope had prediction accuracies 66.41% and 86%, respectively. Meanwhile BCPred, COBEpro, EPMLR, and SVMtrip had area under the receiver operating characteristic curve values of 0.758, 0.829, 0.728, and 0.702, respectively. In addition, these tools are used to develop new vaccines for other diseases, such as COVID-19, nocardiosis disease, and onchocerciasis (Hwang et al., 2021). However, these B-cell epitope prediction tools were developed by using different mathematical principles and databases. Thus, for the best prediction result, all the above tools were used, and the possible epitopes were selected from repeated sequence discovery in different tools. B-cells produce neutralization antibodies IgG and IgA and play an important role to neutralize pathogenic molecules at the first line of contact from viral diseases.

Normally, peptide antigens produce a relatively weak immune response and thus require the use of immunostimulants (adjuvants) for optimal efficacy. The PADRE-linked peptide can promote the stimulation of the mucosal immune response as a carrier with a theoretical B-cell binding peptide (Alexander et al., 2000). The mucosal antibody response in mice was determined after four times of injections of three selected peptides (BES1 (PADRE-DNKTLGPTANNDVTT), BES2 (PADRE-LITGTPKPLEGV), and BES6 (PADRE-NSSDPHL)) at 2-week intervals. The IgA and IgG levels stimulated by the three selected peptides were evaluated. BES 1 could stimulate the highest IgA secretion level in almost all organs except the colon. However, when the stimulation level of IgG was examined, BES2 caused the stimulation of the highest level of IgG secretion in 4 out of 6 organs (jejunum, ileum, cecum, and colon). Therefore, in the development of the PED vaccine, BES1 and BES2, which could stimulate high levels of IgA and IgG in mice, should be used for further testing of immune stimulation in pigs. To ensure the plausibility of this developed vaccine in PEDV prevention, scholars can challenge piglets that have become immune to PEDV with wild-type or virulence PEDV and observe the clinical signs of diarrhea and mortality. The antibody response against PEDV will be studied during the trial period. To date, the proposed molecular mechanisms imply that nearby antigen-presenting cells are induced by parenteral injection and circulated into the lymphoid system before a homing mechanism to produce mucosal antibodies at the site of injection (Bouvet et al., 2002; Li et al., 2020).

The beneficial use of an epitope vaccine is the safety from viral pathogens. The vaccine dose for injection can also be calculated and is easy to prepare. In large-scale pig industries, the cost will decrease due to simplified synthesis. In the next step, the candidate vaccine should be tested for PED protection in swine.

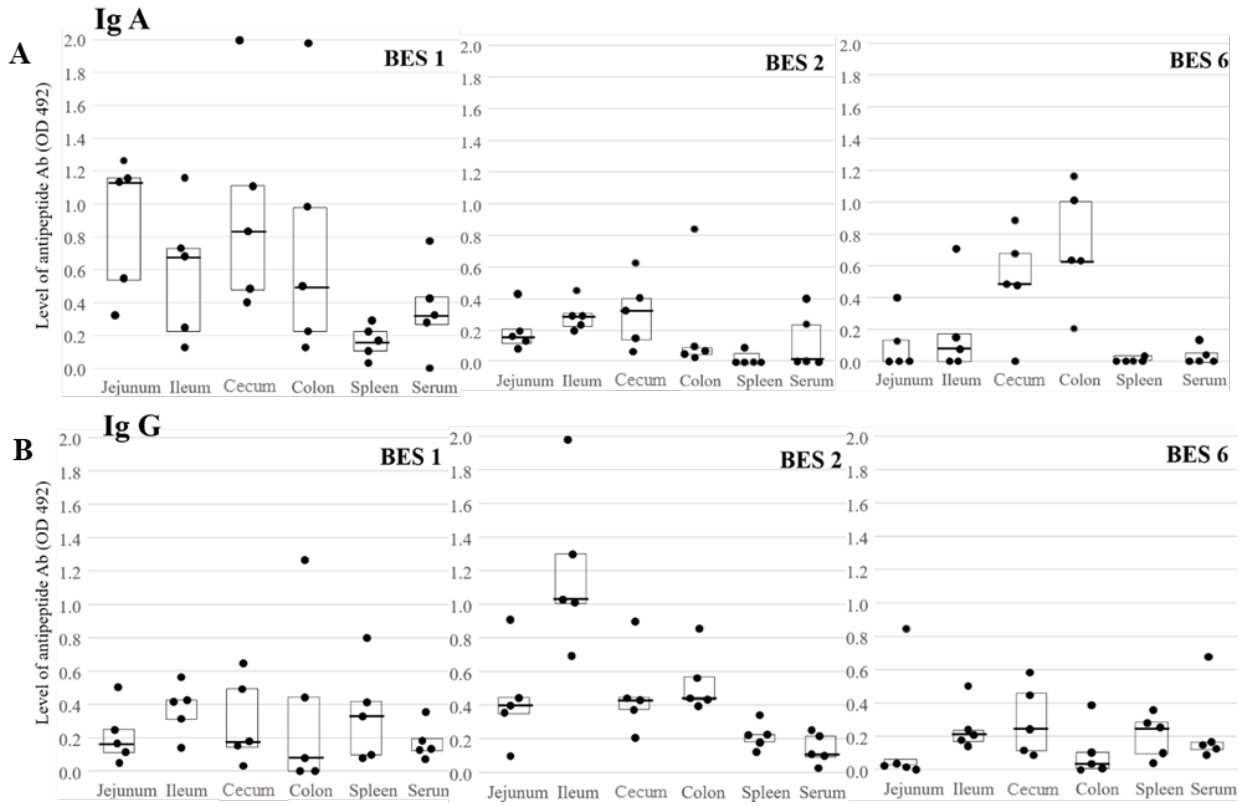

Figure 1. Mucosal antibody response to each epitope (BES1, BES2, and BES6); A) Mucosal IgA response, and B) Mucosal IgG response
Note: For each organ, the median value of five animals is represented by a horizontal line within a box enclosing the interquartile.

5. CONCLUSION

Candidate epitope vaccines from a reverse vaccine design demonstrated the framework of theoretical calculation to search for a small subunit epitope that stimulates mucosal antibodies in the animal model. The combined advanced immunological and information technologies are a powerful tool to accelerate vaccine development. In our study, we developed and tested an epitope-based PED vaccine, whose dose can be controlled and which can be conveniently administered to animals. Using the reverse vaccinology method, only viral genome and bioinformatics tools were used for vaccine development. Following this article, a suitable vaccine based on target epitopes can be predicted for PED. The selected peptide candidates, which were synthesized by conjugation in line with PADRE, stimulated mucosal IgA and IgG in mucosal organs. The candidate epitope-based vaccine may be a possible way to control the widely spread PED through stimulation of the mucosal antibodies, which are the first line of basic immune defense mechanism.

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