PREDICTION AND IMMUNOGENICITY TEST OF B-CELL EPITOPES OF PORCINE EPIDEMIC DIARRHOEA VIRUS THAI ISOLATES

By
MR. Woarawut ONIAM

A Thesis Submitted in Partial Fulfillment of the Requirements
for Doctor of Philosophy PHARMACEUTICAL SCIENCES
Graduate School, Silpakorn University
Academic Year 2021

การทำนายและการทดสอบความสามารถในการกระตุ้นภูมิคุ้มกันของบีเซลล์เอพิโทป ของเชื้อไวรัสพีอีดีที่แยกได้จากประเทศไทย

โดย
นายวรวุฒิ อ่อนเอี่ยม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปรัชญาดุษฎีบัณฑิต
สาขาวิชาวิทยาการทางเภสัชศาสตร์ แบบ 1.1 ปรัชญาดุษฎีบัณฑิต
บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร
ปีการศึกษา 2564

PREDICTION AND IMMUNOGENICITY TEST OF B-CELL EPITOPES OF PORCINE EPIDEMIC DIARRHOEA VIRUS THAI ISOLATES

By

MR. Woarawut ONIAM

A Thesis Submitted in Partial Fulfillment of the Requirements
for Doctor of Philosophy PHARMACEUTICAL SCIENCES
Graduate School, Silpakorn University
Academic Year 2021

Title	Prediction and Immunogenicity test of B-cell Epitopes of Porcine Epidemic Diarrhoea Virus Thai Isolates
By	MR. Woarawut ONIAM
Field of Study	PHARMACEUTICAL SCIENCES
Advisor	Assistant Professor Perayot Pamonsinlapatham, Ph.D.
Co advisor	Assistant Professor Suang Rungpragayphan, Ph.D.

Graduate School Silpakorn University in Partial Fulfillment of the Requirements for the Doctor of Philosophy

.....Dean of graduate school
(Associate Professor Jurairat Nunthanid, Ph.D.)

Approved by

.....Chair person
(Assistant Professor Nopphadol Chalortham, Ph.D.)

.....Advisor
(Assistant Professor Perayot Pamonsinlapatham, Ph.D.)

.....Co advisor
(Assistant Professor Suang Rungpragayphan, Ph.D.)

.....Committee
(Assistant Professor Siripan Limsirichaikul, Ph.D.)

.....External Examiner
(Associate Professor Meena Sarikaputi, Ph.D.)

57356801 : Major PHARMACEUTICAL SCIENCES
Keyword : Reverse-vaccinology, PEDV, Mucosal antibody, B cell epitope prediction

MR. WOARAWUT ONIAM : PREDICTION AND IMMUNOGENICITY TEST OF B-CELL EPITOPES OF PORCINE EPIDEMIC DIARRHOEA VIRUS THAI ISOLATES THESIS ADVISOR : ASSISTANT PROFESSOR PERAYOT PAMONSINLAPATHAM, Ph.D.

Porcine epidemic diarrhea (PED) is a virulence disease that effects most swine, causing severe diarrhea and rapid death. The mortality rate of piglets reaches up to 80%–100%, which affects the pig industry. The illegal use of imported PED vaccine caused failure in the control of PED in Thailand. Currently, a new effective vaccine based on the Thai PEDV strain is needed. The objective of this study is aimed to predict B cell linear epitope from the genome sequence of Thailand's PEDV by using reverse vaccinology. The predicted b cell linear epitope will be further evaluated for the potential for IgA and IgG stimulation in mice. In this study, Complete genome of the PEDV-CBR1 were retrieved and used, following the prediction of open reading frames (ORFs) by ORF Finder. Consequently, protein localization was predicted by iLoc-Virus program. Target proteins were subjected to adhesin-like prediction followed by B-cell epitope prediction. Three novel predicted epitopes, BES1(DNKTLGPTANNDVTT), BES2 (LITGTPKPPLEGV) and BES6 (NSSDPHL) were found. They were synthesized, injected in mice and measured mucosal antibodies response.

By using ELISA technique, BES1 and BES2 could stimulate the highest IgA and IgG secreting level in almost all organs respectively. The combination of these two candidated epitopes should be further tested for immune stimulation in pigs. In conclusion, the reverse vaccinology can apply in the B cell epitope prediction of PEDV Thai isolates.

ACKNOWLEDGEMENTS

I would like to gratefully thank my thesis advisor, Assistant Professor Dr. Perayot Pamonsinlapatham and Assistant Professor Suang Rungpragayphan whose encouragement, guidance, patient and support from the initial to the final level enabled me to develop an understanding of the dissertation.

I would be thankful for Assiatant Professor Dr. Busaba Powthongchin for help in the discussion.

MR. Woarawut ONIAM

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I
INTRODUCTION

Thailand is one of the world's most important pig production sources after China, the United States and the European Union. The overall production of Thailand is mainly to meet domestic demand. However, due to the rapid economic expansion of the ASEAN countries, the rising population, the more convenient transportation and the increased product exchange have resulted in Thailand having better pig production and export potential than other countries in the region have a great opportunity to expand export markets in the AEC. Also, Thailand has opportunities to invest in pig farms and pork processing plants in new ASEAN countries, especially Laos and Cambodia. However, there are many damaging diseases for pig farmers. But The disease that affects the pig production industry is quite severe, is a disease that causes severe diarrhea that kill many piglets. Which is most often caused by *E. coli*, *Clostridial enteritis*, *Rotavirus gastroenteritis*, Transmissible Gastroenteritis (TGE) disease, Coccidiosis and Porcine epidemic diarrhea (PED).

Porcine epidemic diarrhea (PED) is a common type of viral enteritis in pigs that is caused by PED virus (PEDV). Consistent with the name of the disease, diarrhea is the major symptom of PED. Additionally, PED presents with various other clinical signs, including vomiting, anorexia, dehydration, and weight loss. PEDV can infect pigs of any age, from neonates to sows or boars; however, the severity of PED in pigs differs according to age. Importantly, PEDV infection in neonatal pigs commonly induces death from watery diarrhea and dehydration. Indeed, in a previous study, researchers stated that over 1,000,000 piglets have died from PEDV infection, with a death rate of 80%-100% (1). Such high death rates are associated with huge economic losses. The spread of PEDV within the farm is caused by ingestion of various secretions, particularly the feces of animals with the disease. There is no specific treatment other than symptomatic treatment of diarrhea and control of secondary infections. PED was first observed in Europe in 1971. During the 1970s and 1980s, the virus spread throughout Europe. After that, it has become an endemic disease in Asian pig farming countries, such as Korea, China, Vietnam, Japan, the Philippines, Taiwan, and Thailand. An outbreak of PEDV infection occurred in the United States in Iowa in

April 2013, and within 1 year, PEDV had spread to Canada and Mexico (2).

As mentioned above, for the severity of the disease and there is no specific cure, vaccine prevention is a highly studied alternative. There are currently a number of vaccines, such as live attenuated vaccines and inactivated vaccines made in China, Japan and South Korea. But these vaccines are not always able to induce immunity (3), especially in Thailand, as infections have still been found in vaccinated piglets. This may be because the strains of the virus found in Thailand are genetically different from those found in other Asian countries (4). Although there have been many studies in the development of subunit vaccine, no vaccine has been reported to provide comprehensive protective effects. The fact that current vaccines are unable to immunize comprehensively may be because the qualifying protein-based antigens to be developed into the PED vaccine have not yet been discovered through traditional studies. The focus of this research is to develop a vaccine against PED using a reverse vaccinology technique, which uses bioinformatics tools to analyze the viral genome to select an immune-inducing epitope. The selected epitope was then subjected to immuno-stimulation test in mice for further development of the vaccine. This reverse vaccinology technique will enable the study of the total protein of the target strains through computer simulation and prediction, which will significantly save time and costs in vaccine development compared to the traditional vaccine development process, which had to be initiated from live microorganism and can studied only in a small fraction of the total protein present. If a vaccine is developed and produced for domestic use, in addition to reducing the loss of disease, it can also help reduce the import of vaccines from abroad. The developed vaccine can be sold to neighboring countries, which will have a profound impact on pig farming development and promote the export of both pigs and vaccines.

Objective of research

1. To predict B cell linear epitope from the genome sequence of Thailand’s PED virus.

2. To measure IgA and IgG response in mice that were stimulated by predicted epitopes.

Scope of the study

Predict B cell linear epitope from the genome of the PED virus isolated in Thailand using bioinformatics tool and test the ability of predicted epitope to stimulate immune response in mice.

CHAPTER II
LITERATURE REVIEWS

2.1 General characteristics of PEDV

PEDV, the etiological agent of PED, is a positive -sense large-enveloped single- stranded RNA virus, which is a member of the family *Coronaviridae*. Swine coronaviruses can be divided into respiratory (PRCoV) and enteropathogenic coronaviruses such as transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV) and porcine deltacoronavirus (PDCoV). The family *Coronaviridae* is currently divided into four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus*. TGEV and PEDV belong to the *Alphacoronavirus* genus, whereas PDCoV belongs to the genus of *Deltacoronaviruses*. PEDV was placed in the *Arteriviridae* family in the order *Nidovirales* based on similarities in genome organization and replication strategy.

2.2 Genetic Structure and Characteristics of PEDV

PEDV is an enveloped, single-stranded RNA virus. It has an approximately 28 kb long genome and consists of seven open reading frames (ORF) encoding three nonstructural proteins (replicase 1a,1b and ORF3) and four structural proteins (spike (S), envelope (E), membrane (M) and nucleoprotein(N)). The genome arrangement is in this order; 50 untranslated region - replicase (1a/1b)–S-ORF3–E–M–N–30 untranslated region. The polymerase gene consists of 2 large ORFs, 1a and 1b, that cover the 50 two-third of the genome and encode the non-structural replicase polyproteins (replicases 1a and 1b). Genes for the major structural proteins S (150–220 kDa), E (7 kDa), M (20–30 kDa), and N (58 kDa) are located downstream of the polymerase gene. The ORF3 gene, which is an accessory gene, is located between the structural genes. It encodes an accessory protein, the number and sequence of which varies among different coronaviruses (1, 2, 5-7).

The PEDV S protein is a type I glycoprotein composed of 1,383 amino acids (aa). It contains a signal peptide (1–18 aa), neutralizing epitopes (499–638, 748–755, 764–771, and 1,368–1,374 aa), a transmembrane domain (1,334–1,356 aa), and a short cytoplasmic domain. The S protein can also be divided into S1 (1–789 aa) and S2 (790–1,383 aa) domains based on its homology with S proteins of other coronaviruses. Like other coronavirus S proteins, the PEDV S protein is a

glycoprotein peplomer (surface antigen) on the viral surface, where it plays a pivotal role in regulating interactions with specific host cell receptor glycoproteins to mediate viral entry, and stimulating induction of neutralizing antibodies in the natural host. Moreover, it is associated with growth adaptation in vitro, and attenuation of virulence in vivo. Thus, the S glycoprotein would be a primary target for the development of effective vaccines against PEDV(1, 2, 6, 7).

The PEDV M protein, the most abundant envelope component, is a triple-spanning structural membrane glycoprotein with a short amino-terminal domain on the outside of the virus and a long carboxy-terminal domain on the inside. The M protein not only plays an important role in the viral assembly process but also induces antibodies that neutralize the virus in the presence of its complement. The M protein may play a role in a-interferon (a-IFN) induction (8). Coexpression of M and E proteins allowed the formation of pseudo particles, which exhibited interferogenic activity similar to that of complete virions. Additional work on the M glycoprotein should increase our understanding of the genetic relationships between, and the diversity of PEDV isolates and the epidemic situation of PEDV in the field (1, 2, 5-7).

The N protein, which binds to virion RNA and provides a structural basis for the helical nucleocapsid, is a basic phosphoprotein associated with the genome. As such, it can be used as the target for the accurate and early diagnosis of PEDV infection. It has been suggested that N protein epitopes may be important for induction of cell-mediated immunity (CMI) (1, 2, 5-7).

Whereas the genes encoding the structural proteins have been thoroughly investigated for most coronaviruses, little is known about the functions of the accessory proteins, which are not generally required for virus replication in cultured cells. On the contrary, their expression might lead to decreases of viral fitness in vitro, and mutants with inactivated accessory genes are easily selected during serial passage through cell cultures. In general, accessory genes are maintained in field strains, and their loss mainly results in attenuation in the natural host. In the case of PEDV, the only accessory gene is ORF3, which is thought to influence virulence; cell culture adaptation has been used to alter the ORF3 gene in order to reduce virulence, as has been done for TGEV (9). Differentiation of ORF3 genes between the highly cell-adapted viruses and field viruses could be a marker of adaptation to cell culture and attenuation of the virus.

2.3 Pathology of PED

When pigs infected with PEDV by oral, the virus will grow and replicates in enterocytes covering the villi of the middle and distal part of small intestine and some areas of the large intestine resulting in degeneration of enterocytes leading to shortening of the villi. This causes clinical manifestations of the disease including watery diarrhea. Intestinal mucosa cells are infected in 12-18 hours after ingestion and increase the number to the maximum in the 24-36 hours, which is consistent with clinical symptoms that begin to develop diarrhea within 12 hours after infection and are most common in the last 36 hours. The infection of the cells results in the degeneration of villi cells, their appearance is shortened, resulting in the absorption and digestion of food in the small intestine. The food that remains in the intestine is of a high concentration, thus drawing water from the surrounding cells, causing the symptoms of watery diarrhea. When non-weaned piglets that infect with PEDV, feed on the milk. The indorsed milk causes more water from the surrounding cells to be extracted than other groups of pigs, resulting in more dehydration. Therefore, the mortality rate is higher than in other groups. The autopsy lesions are often found to have a bulging small intestine and fluid inside the intestinal wall. The intestinal wall looks thinner and found milk curd in the stomach (10).

The main symptom of the disease is watery diarrhea, with symptoms starting in the 12-24 hours after infection, may include vomiting and anorexia. Pig morbidity rates on farms with PEDV transmission may be as high as 100 %. The severity of symptoms in pigs of all ages is not equal, weaned pigs may show a slight diarrhea for a short period of 1-2 days. Pigs will return to normal within 2-3 days with or without treatment. This resulted in a low mortality rate of only 1-3%, which was different from the non-weaned piglets that were more likely to show severe illness. The infected non-weaned piglets are more often shown more severe illness with yellow watery diarrhea. The stools have a fishy smell and have undigested milk fragments, weight loss and dehydration. They usually have symptoms for about 7-14 days, with very high mortality rates ranging from 80 to 100%. Piglets under 1 week of age usually die within 2-7 days after symptoms, while surviving piglets older than 3 weeks showed lower growth rates and body weight than normal piglets (11).

2.4 Epidemiology of PED

The spread of this virus within the farm is caused by ingestion of various secretions, particularly the feces of animals with the disease. The natural infection starts after oral uptake of the virus. The fecal-oral route is the major route of virus transmission. The spread of infection between farms is usually caused by the movement of infected animals from one farm to another. Or PEDV may contaminate materials, equipment or workers traveling between farms or pig trucks. After an outbreak has occurred on a breeding farm, the virus may disappear but it may also persist if sufficient litters of pigs are produced and weaned so that the virus is maintained by infection of consecutive litters which have lost their lactogenic immunity at weaning. PEDV may then be a cause of recurrent weaning diarrhea in 5-8-week-old pigs.

There is currently no specific treatment for the disease. Infected pigs will be treated by symptomatic treatment and prevention of second infection. Most adult pigs recover without treatment within 7-10 days unless secondary infections occur. Reinfection can occur in pigs with low immunity (10).

Several measures have been taken to prevent PEDs with varying degrees of success. Strict biosecurity is the most effective measure to prevent infection and spread of the virus, especially, introduction of healthy pigs, controlling the movement of pigs, materials and workers in farm, disinfection of vehicles and equipment and proper disposal of dead pigs. All-in-all-out practice has proven effective in breaking the transmission cycle within the farm. Another measure is the use of the PED vaccine, which is available and used in many countries (12).

2.5 Outbreak of PEDV

PEDV was firstly observed among English feeder in 1971 and was identified as coronavirus-like strain CV777 from pigs with watery diarrhea in Belgium and the United Kingdom in 1978. After that the disease widely spread throughout many swine-raising countries in Western Europe: Hungary (1981); Germany (1981); to Asian countries: Japan (1982); South Korea (2000); Taiwan (2013) and North America (2013). During the 1970s and 1990s, a few severe PED outbreaks have been reported in Europe. Nevertheless, PEDV infection nowadays has become epidemic in Asia pig industries, consist of China, Japan, South Korea, Vietnam, Thailand, the Philippines and Taiwan. In China, a large-scale diarrhea outbreak was reported in the end of 2010 with the confirmation of PEDV in the pig population that over 1,000,000 piglets

died with a mortality rate of 80%-100% and resulted in enormous economic losses. In South Korea, the PEDV was firstly described in 1992 and re-emerged as a severe outbreak during 2013 with considerable variants that were different from previous Korean isolates or vaccine strains. A massive PED outbreak suddenly occurred in the North American pig farm in April 2013 and rapidly spread across the country also to countries sharing the same border: Canada and Mexico; causing high rates of mortality and huge economic losses. (5)

The first Thailand PED case was reported in Trang province, in 1995. After that, PED had an epidemic periodically. According to the Department of Livestock development's laboratory reports from 1995 to 2004, 10 outbreaks were found, all from pig farms in the southern and western regions. However, after 2004, there is still a period of outbreaks in which the severity of each outbreak may not be equal. For example, in 2007, the disease re-emerged in Nakhon Pathom that had an impact to local economic and spread throughout the country. This event cause more than 90% of Thai swine farms being infected. Since then, PED has become endemic and causes sporadic outbreaks in which outbreaks of one or two times a year have been reported. (4)

2.6 Genetic heterogeneity of Thailand’s PEDV

In Thailand, Temeeyasen et al (13) studied the differences in PEDV S genes found in Thailand between 2008-2012 and compared them with other Asian and European countries. The complete sequence of all PEDV S genes to be studied was used to create a phylogenetic tree as shown in Figure 1. Letters of different colors mean Country-specific PEDV: Pink refers to China, Blue refers to Thailand, Violet refers to Korea, Green refers to European countries. From the phylogenetic tree, all PEDVs isolated from Thai samples were included in cluster 1 with PEDV isolated from Chinese and Korean samples. When measuring the nucleotide and amino acid sequence identity of PEDV in cluster 1 ranged from 94.4–99.9% and 93.1–99.9%, respectively. And when examining the amino acid sequence of PEDV S genes isolated from Thailand during 2008-2012, it possesses a unique and common genome characteristic defined by the insertion of 4 amino acids (GENQ) between positions 55 and 56, a 1-aa (N) insertion between positions 135 and 136, and the deletion of 2 amino acids between positions 155 and 156. These unique isolates were genetically related to or identical to previously reported Korean KNU-serial isolates and isolates that were responsible for recent severe outbreaks in China. In addition, the Thai PEDV isolates displayed

a high degree of genetic heterogeneity, especially in the neutralizing epitope region. This finding may explain why the current illegal use of the PEDV vaccine in Thailand, which is based on old seed stock including strains from China and KPED9 from Korea, has not resulted in successful PED control. Confirmation of this explanation could lead to the development of a new vaccine that is more suitable for PEDV outbreaks in Thailand. (13)

2.7 Current PED Vaccine

For the prevention of PEDV infection, several Commercial PEDV vaccines, including live attenuated and inactivated vaccines, have been reported in Asian countries. Some of these vaccines have been combined with vaccines for TGEV (a bivalent vaccine) and porcine RV (a trivalent vaccine) and used in China and South Korea. Moreover, an attenuated virus vaccine using cell culture-adapted PEDV has been administered to sows in Japan since 1997. Oral vaccination with a cell-attenuated vaccine has been used in South Korea since 2004 and in the Philippines since 2011. Although these commercial vaccines are considered effective and have been widely used, not all animals develop solid lactogenic immunity. Examples of vaccines for PED are shown in the Table 1. (14)

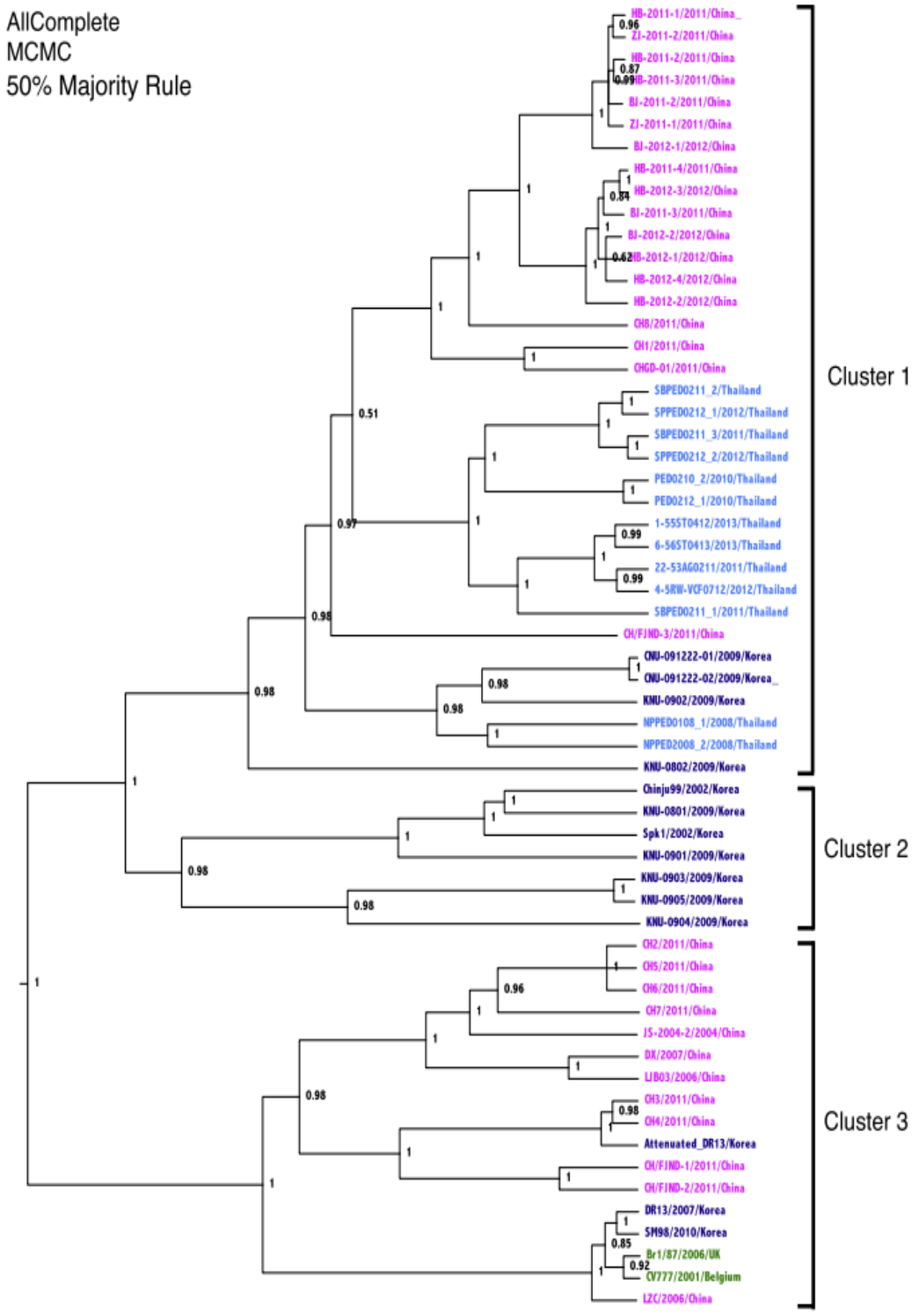


Figure 1 *Phylogenetic tree analysis was created from PEDV's S gene information.(13)*

Table 1 Examples of PED vaccines types, and strain currently available in Asia.(14)

Product Name	Type	Strain/Subtype	Adjuvant	Licensed Countries
SuiShot® PED	Live	Not Available	Not Available	South Korea
PRO-VAC® PED-K	Killed	SM98P	Montanide IMS-1313 NPR	South Korea
Nisseiken PED Live Vaccine	Live	P-5V	None	japan
PED Vaccine (Qilu Animal Health Products Factory)	Killed	Not Available	Not Available	China

2.8 Reverse vaccinology

By the end of the 20th century, great advances in genetics and technology gave scientists the ability to in little time determine the order of nucleotides in the DNA of living organisms and cost less. As a result, during this time there were numerous databases that were storing information about the DNA sequencing of various pathogens. Examples of important database (15), such as:

1. **Genbank** is a database of nucleotides of the United States, sponsored by the National Institute of Health (NIH), which has been operating since 1992. The URL of website is http://www.ncbi.nlm.nih.gov/genbank.
2. **EMBL** is a European database of nucleotide sequences. Created by an international collaboration, GenBank of the United States and DDBJ of Japan. The URL of website is http://www.ebi.ac.uk/embl.
3. **DDBJ** is Japan's database of nucleotide sequences which is supported by the Japanese government. It opened for the first time in 1986. The URL of website is http://www.ddbj.nig.ac.jp.

The sequence of nucleotides allows scientists to know which proteins are capable of produced. Some produced proteins may effective as an antigen and can be used to develop as a vaccine. With these advancement of the technology, Italian scientist Rino Rappuoli has discovered a new way to develop a vaccine called reverse vaccinology. The definition of reverse vaccinology is “The vaccine development method that use genomic information and in silico tool analysis for the

identification of novel protein antigens that are exposed on the surface without culturing microorganism". The reason for choosing to search for only proteins that exist or emerge outside the cell is because they are easily accessible by the immune system (16, 17). This increases the likelihood of stimulating the immune system better than other proteins. The first pathogen against which the vaccine was prepared with the aid of reverse vaccinology was for serogroup B *Neisseria meningitidis*. The *in-silico* approach for designing the vaccine by reverse vaccinology came into play due to high rate of mortality due to cause of meningitides by bacteria and *N. meningitis*. The classical way for the production of the vaccine against meningitis failed due to similarity of the proteins to humans and also because of the hypervariable nature of the pathogen. The whole genome of *N. meningitidis* was analyzed and with computer aid, the specific sequences were selected that are surface protein and can act as a vaccine candidate. (18)

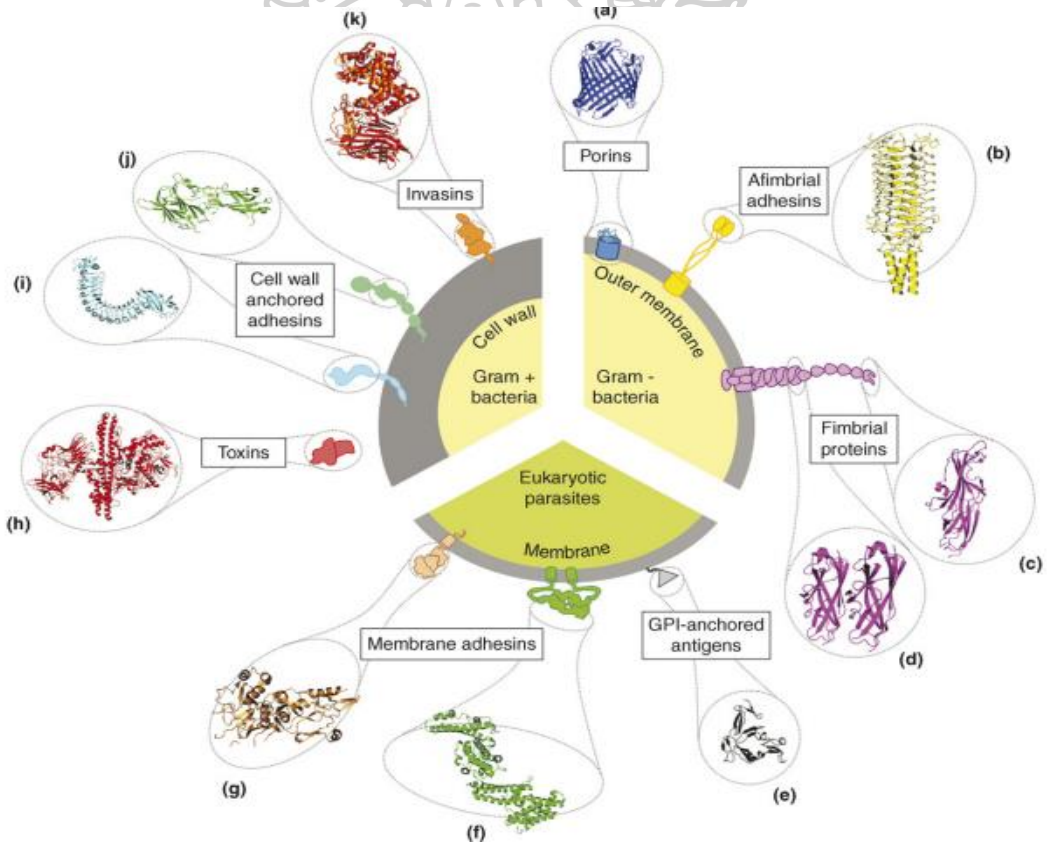


Figure 2 *Examples of surface and secretory proteins of different types of germs.(17)*

The general step in the development of vaccine by reverse vaccinology (Figure 3) starts with searching for the entire genome

sequence of the pathogens from previously mentioned databases. A computer program was then used to analyze the whole genome sequence to find out the genes involved in the production of cell surface or secreted proteins. Recombinant technology is then used to mutate the genes with the appropriate strains to produce that protein, which is then tested for their ability to stimulate the immune system by injections into laboratory animals. If any protein can stimulate the immune system of the experimental animal, it will be taken as an option to develop the vaccine further. The process of finding the right antigens using the reverse vaccinology process takes only 1-2 years, which is very short compared to at least 5-15 years to develop a subunit vaccine. (19)

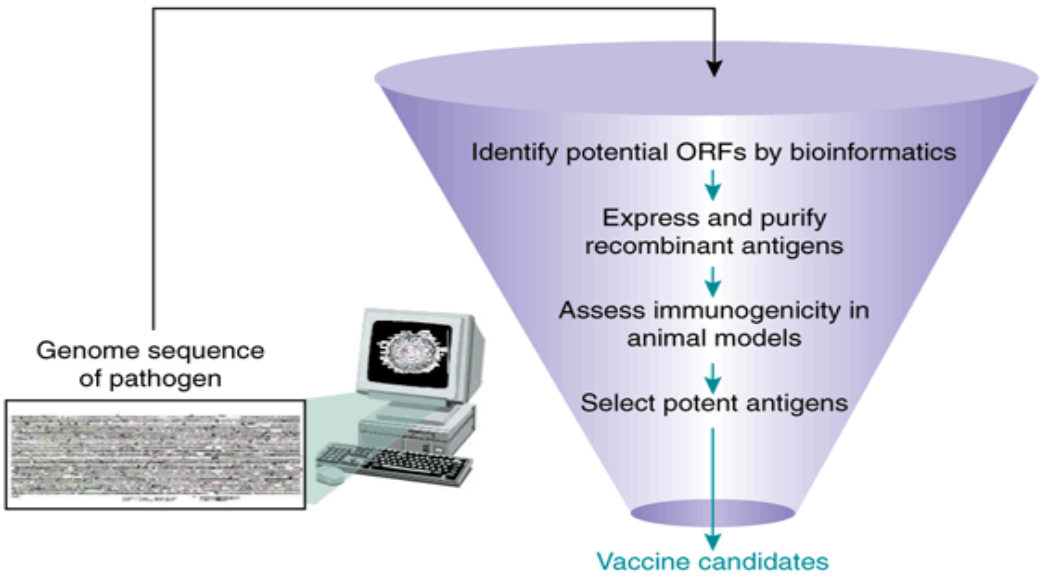


Figure 3 *The general step in the development of vaccine by reverse vaccinology.(19)*

When comparing reverse vaccinology development processes with traditional vaccine development processes such as subunit vaccines, inactivated vaccine and the live attenuated vaccine. The following advantages and disadvantages are found:

Advantages:

1) It takes less time to find the right antigens.
2) Developed vaccines have fewer side effects. Due to reverse vaccinology use only the sequences of the strains to develop the vaccine. There is no need to use other parts of the virus as a component of the vaccine.

3) Reverse vaccinology process can be used for non-cultured or highly dangerous strains.
4) A large number of new antigens can be discovered. Increasing the chance of successful vaccine development.

Disadvantage:

Reverse vaccinology cannot locate non-protein antigens, such as polysaccharide, glycolipid and lipid antigens, so that appropriate antigens may not be found for vaccine development for some strains.

2.9 Bioinformatics Tools

Bioinformatics tools used in the reverse vaccine development process are in the form of computer programs. There are both that must be installed on a personal computer or enabled for use via the internet. Currently, many bioinformatic tools were developed. The same prediction may be able to use many bioinformatic tools. Each program is different in terms of usage restrictions such as with different types of organisms (prokaryotes, eukaryotes, plants, animals, or viruses) or use different databases and analysis principles, etc. Therefore, users must search for program that reliable and suitable for use in their own work.

Examples of tools commonly used in the reverse vaccine development process include ORF Finder, protein subcellular localization prediction tools, adhesin-like proteins prediction tool and B-cell epitope prediction tools. These tools still have developed, improve, or build new tools regularly with greater accuracy. However, the predicted antigens obtained from these tools must still be examined with appropriate animal experiments to see the next actual results.

2.9.1 Open reading frame prediction Tools

Open reading frame (ORF) is a section of genes that can be genetically decoded into peptides or proteins during translation (Figure 4). Usually, such sections start with the initial codon (ATG) and ends with a stop codon (usually TAA, TAG or TGA). Examples of tools in this group, such as: GENMARK, GLIMMER, ORF Finder. Each tool differs in principle, how it is used, and the type of organism it can predict.

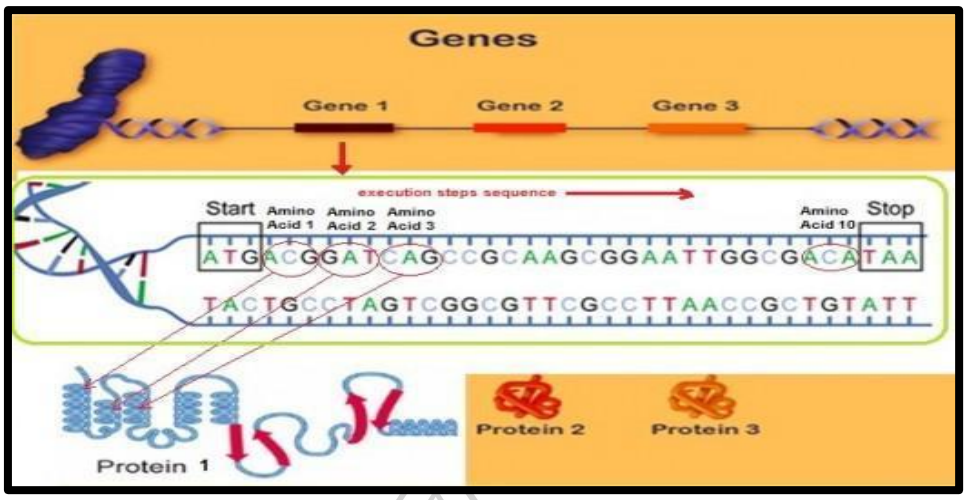


Figure 4 Example of Open Reading Frames (ORF) process.(20)

ORF Finder is an ORF prediction tool developed by the National Center for Biotechnology Information (NCBI) (20) (Figure 5-6). This tool will predict ORF positions covering all 6 reading frames. The tool's predictive results can also be passed on to Blast, a tool used to search for and compare genes from other organisms of similar sequencing. This makes it more convenient for researchers to develop vaccines.

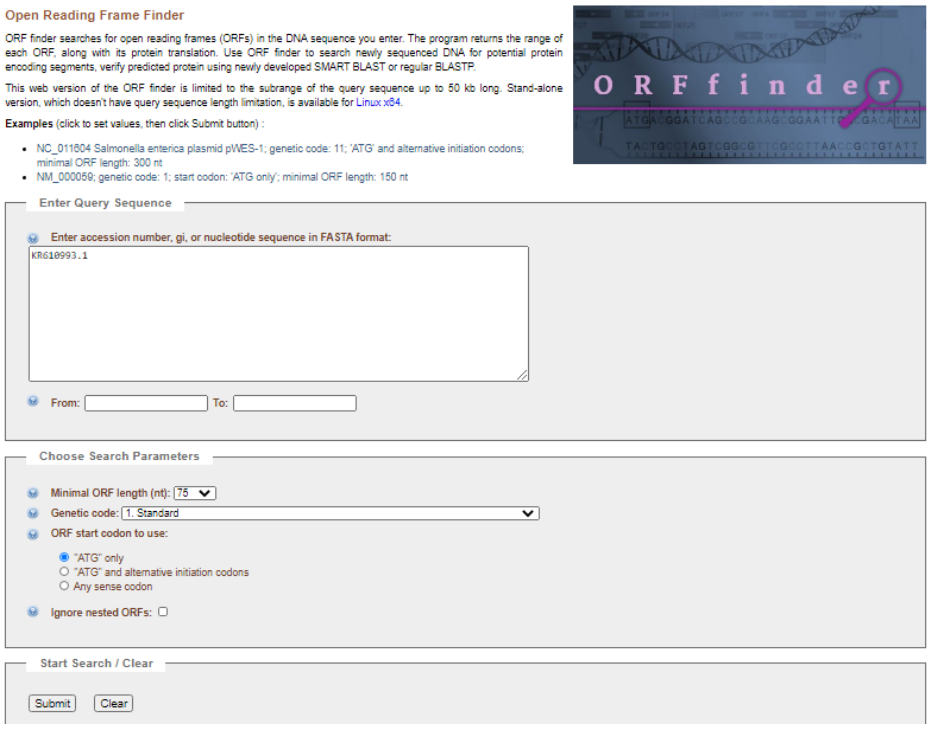


Figure 5 ORF Finder from NCBI.(20)

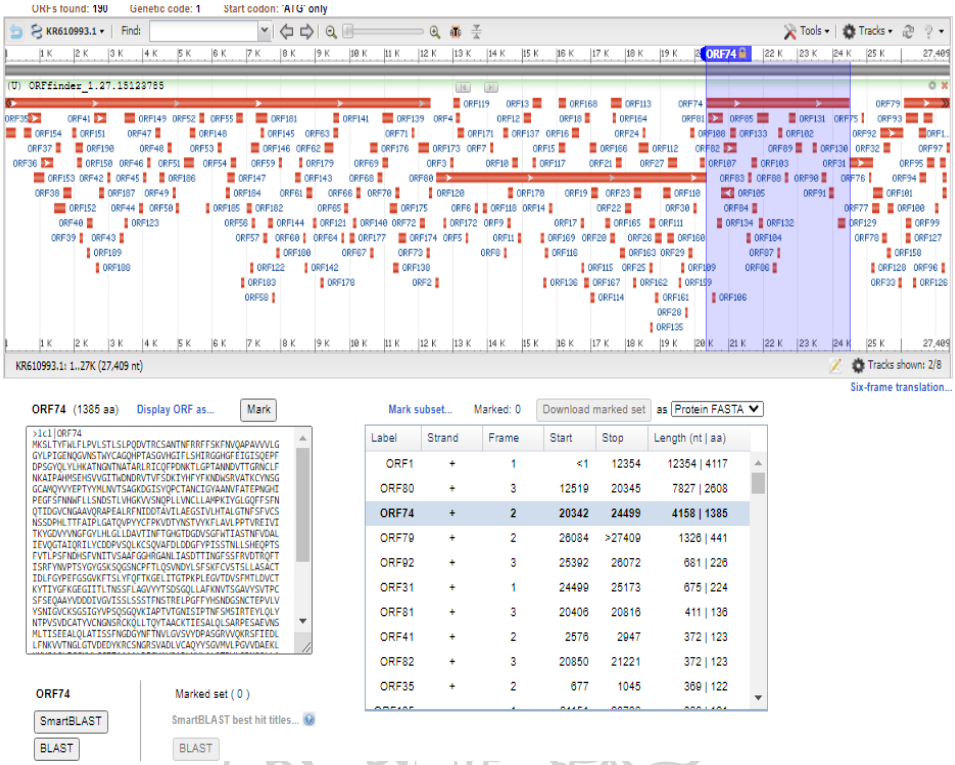


Figure 6 *Example of the predicted results obtained from the NCBI's ORF Finder.*

2.9.2 Protein subcellular localization prediction tool

In general, all bacterial proteins are synthesized in the cytoplasm, and most remain here to carry out their unique functions. Other proteins, however, contain export signals that direct them to other cellular locations such as cytoplasmic membrane, cell wall, outer membrane, or outside the cell as in Figure 7. (21)

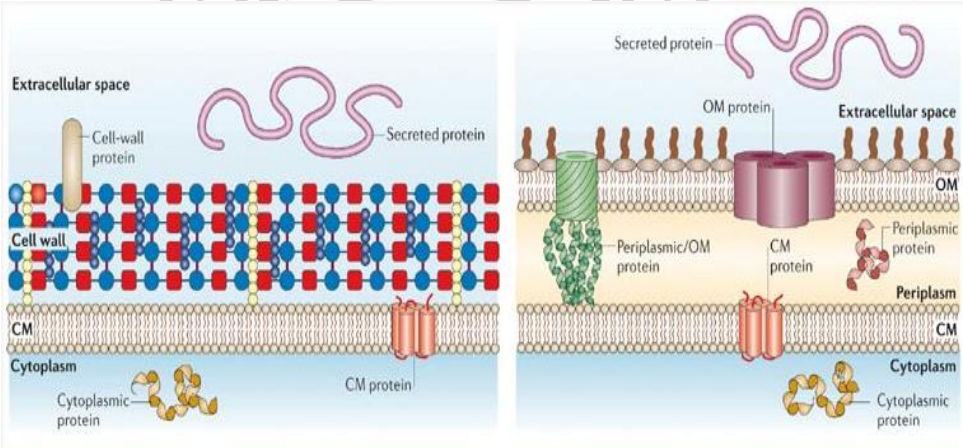


Figure 7 *Type and location of the protein is made up of gram-positive and gram-negative bacteria.(21)*

However, only proteins found on the cell surface and extracellular proteins qualify as good antigens according to the principle of reverse vaccinology. As a result, many researchers are interested in the development of tools for predicting the location of intracellular proteins. These tools differ in many areas, such as the predictive principle and types of predictable organisms. Examples of protein subcellular localization prediction tools such as PSORTb, TMHMM, SignalP (Figure 8) (22) , SCLPred and iLoc-Virus.

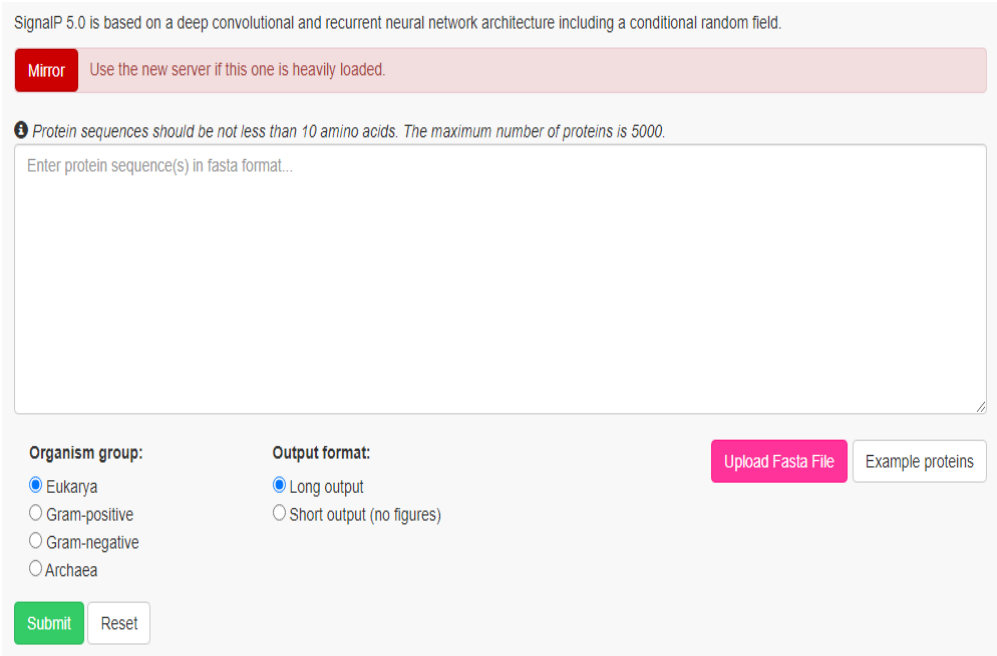


Figure 8 *SignalP web-based program.(22)*

iLoc-Virus (23, 24) (Figure 9-11) uses an amino acid sequence in the FASTA format to predict the location of proteins that are generated within the virus. Six locations can be predicted: extracellular, cell membranes, cytoplasm, endoplasmic reticulum, capsid, and nucleus. The prediction accuracy of this tools is about 78.2%. To obtain the predicted result with the expected success rate, the entire sequence of the query protein rather than its fragment should be used as an input. A sequence with less than 50 amino acid residues is generally deemed as a fragment. Then, the limitation of this tool is that it cannot predict the location of proteins made up of a sequence of fewer than 50 amino acids.

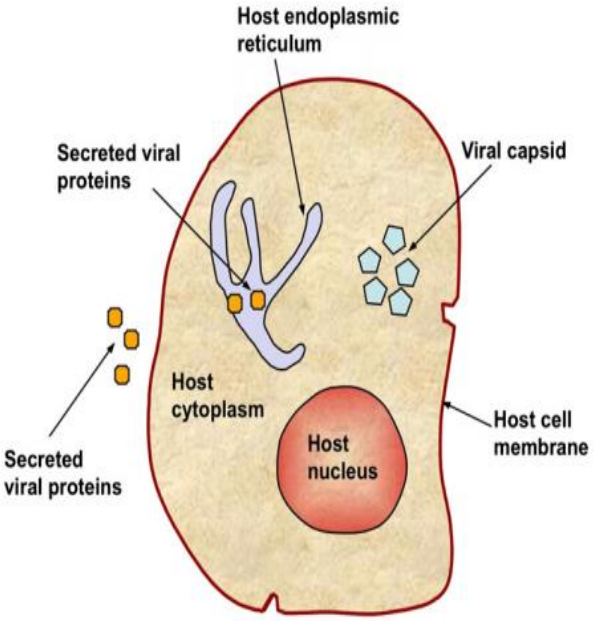


Figure 9 The six locations of viral proteins of iLoc-Virus.(23)

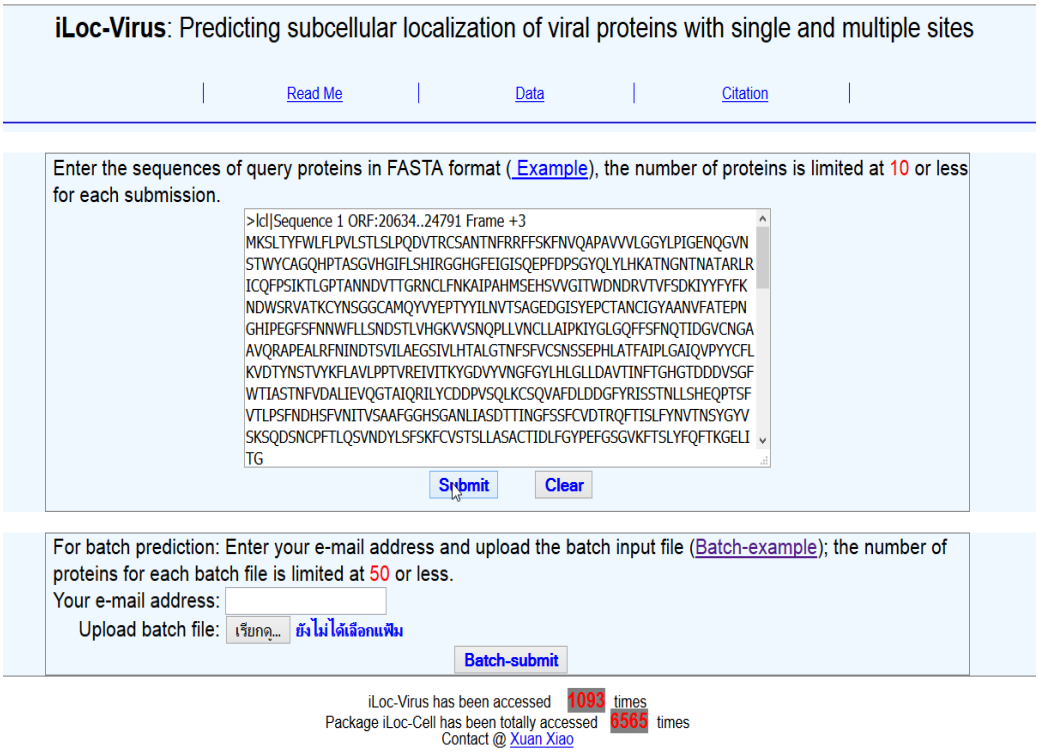


Figure 10 iLoc-Virus web-based program.(24)

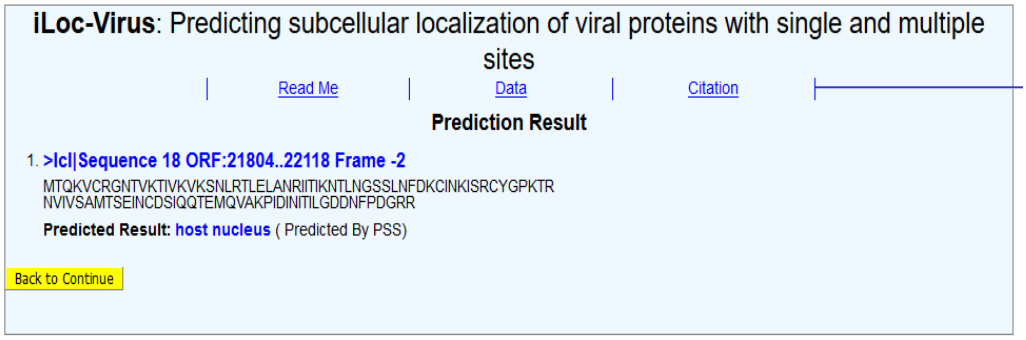


Figure 11 Example of predict results obtained from iLoc-Virus.(24)

2.9.3 Adhesin-like proteins prediction tool

Adhesin is known to stimulate the immune system as it mediates the binding of infection with receptors on the cell surface of the host, an essential process in causing disease or infection. Then, proteins that have adhesin-like property may be good vaccine antigens. The tool used to determine the likelihood that a protein that is created within a cell is adhesin or has similar properties to adhesion, namely SPAAN (25). This program can be run on computers with Linux operating systems. The result from prediction will be shown as P_{ad} value (P_{ad} = the probability of a protein being an adhesin). Most of the adhesins (96%) have $P_{ad} \geq 0.51$ whereas all the non-adhesins (100%) have P ad < 0.51 (Figure 12-13).

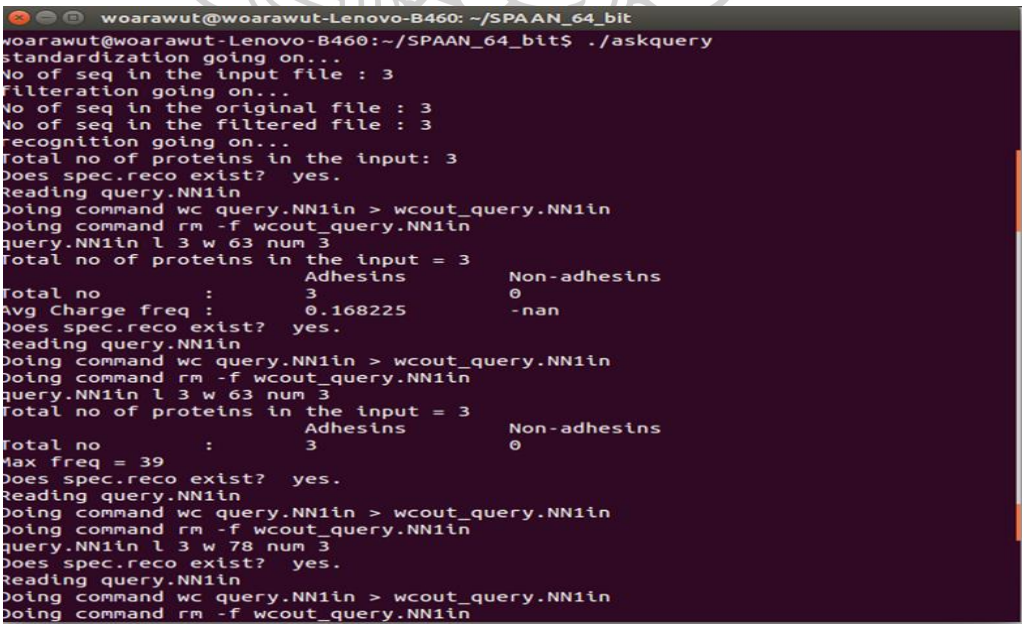


Figure 12 Processing SPANN on linux shell.

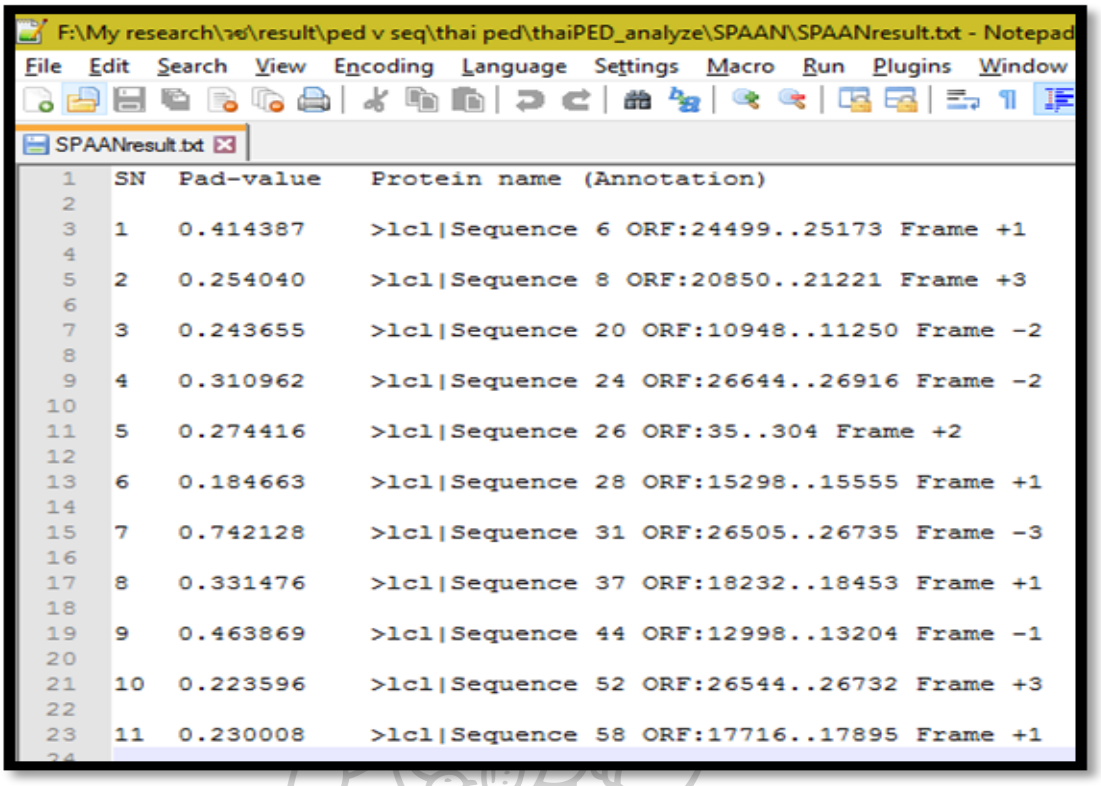


Figure 13 Examples of results predicted by SPAAN program.

2.9.4 B-cell epitope prediction tools

B cell epitope is the part of an antigen that bind to receptors lead to eliciting of humoral immune response. For that reason, epitopes are very important in the development of vaccines. Finding an epitope in the past was difficult and costly as it is a process that must be done within the laboratory. Later, with the advancement in bioinformatics, more researchers were interested in the development of epitope predictive tools, resulting in shortening search times and reducing costs.

Epitopes can be divided by binding to immune cells into two types: T cell epitope and B cell epitope. T cell epitope can bind with MHC or T cell receptor and B cell epitope can bind with B cell receptor or antibody. By the arrangement of epitopes on antigens bound to immune cells, they can be subdivided into two types: continuous epitope and discontinuous epitope (Figure 14). About 90% of T cell epitope are continuous epitopes in contrast with B cell epitope that 90% of them are discontinuous epitopes. Continuous epitope is an epitope with a linear form when it is caught with receptor, most of which is approximately 9-12 amino acids. Unlike Discontinuous epitope, the epitope binds to the receptor is widely distributed within the long chain of proteins about 15-22 amino acids,

which are curled in a three-dimensional pattern. As a result, predicting the epitope is more difficult than continuous epitope.

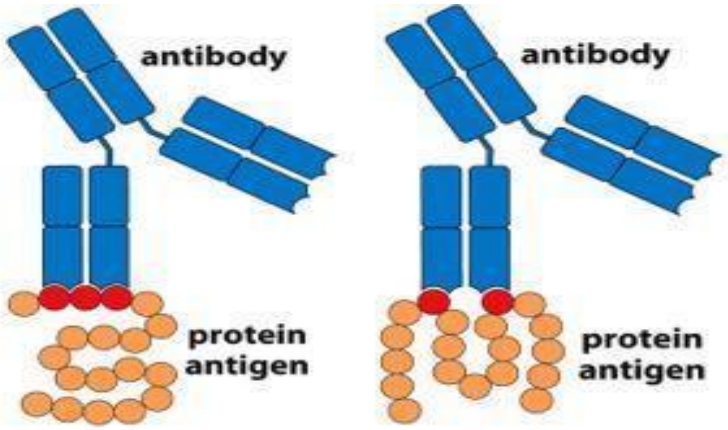


Figure 14 Continuous and Discontinuous epitope characteristic on protein antigen.(26)

Several tools have been developed to predict the epitope of B cell. Most of them can be accessed via the Internet. These tools can be divided into two groups according to the nature of the prediction data:

1.Tools that uses amino acids sequence to predict epitope.

Tools in this group use a scoring principle for each amino acid in the chain to determine whether it is part of the epitope, according to the Epitope database used by the tool. Since the tools in this group use only a chain of amino acids for their prediction, they are easy to use and can always predict epitopes. However, as a result of using only a chain of amino acids, the tool was unable to group the amino acid expected to be epitope into the same group. This makes predictions very wrong in cases where the amino acid chain used in the prediction contains more than one epitope. An example of a tool in this group is CBTOPE (26, 27). (Figure 15-16)

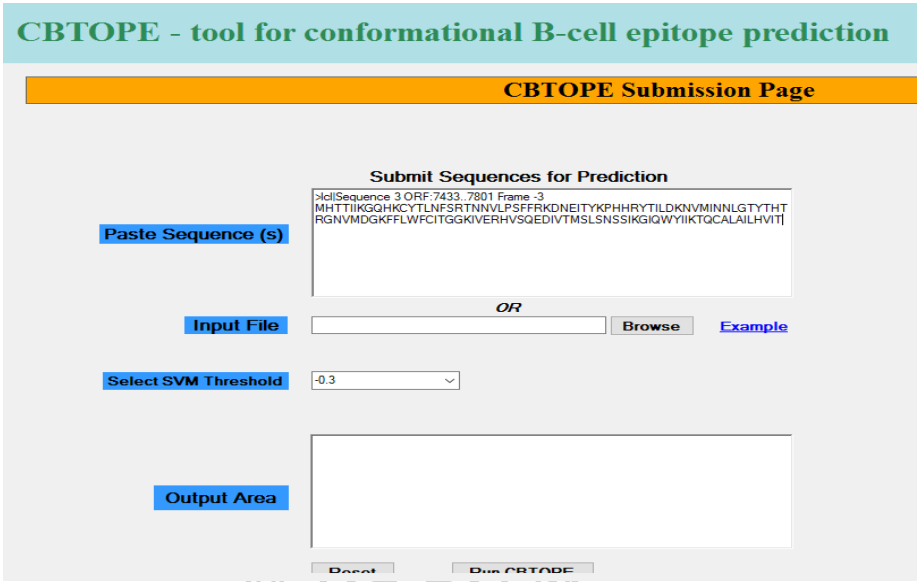


Figure 15 CBTOPE web-based program.(27)

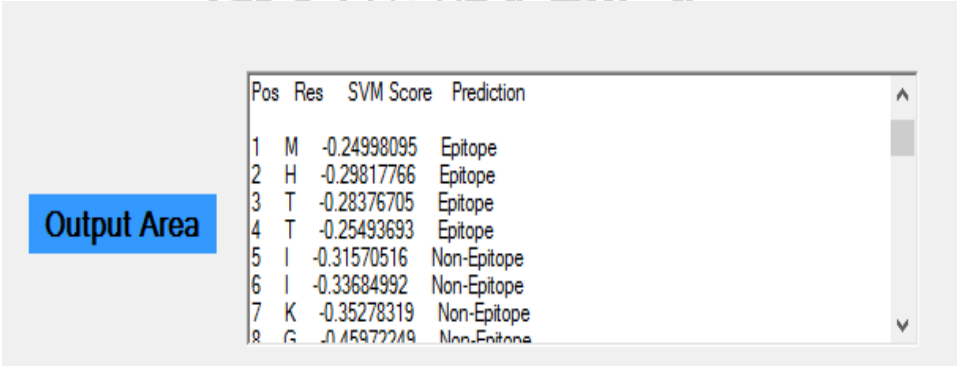


Figure 16 Example of prediction results from CBTOPE.

2. Tools that uses amino acid chain structures to predict epitope

The instruments in this group used amino acid chain structure to predict, which yields more accurate predictions of epitope than those using amino acid sequences. In addition, the results of the prediction can often be displayed in three dimensions, allowing the epitope arrangement to be clearly seen and how many epitopes in a chain of amino acids can be distinguished. However, these tools are quite limited in their use because prediction is based on the three-dimensional structure of the amino acid chain obtained by X-ray crystallography of the antigen-antibody binding process that is a costly process, resulting in a small number of existing three-dimensional structures. An example of a tool in this group is ElliPro (28) (29) (Figure 17-18).

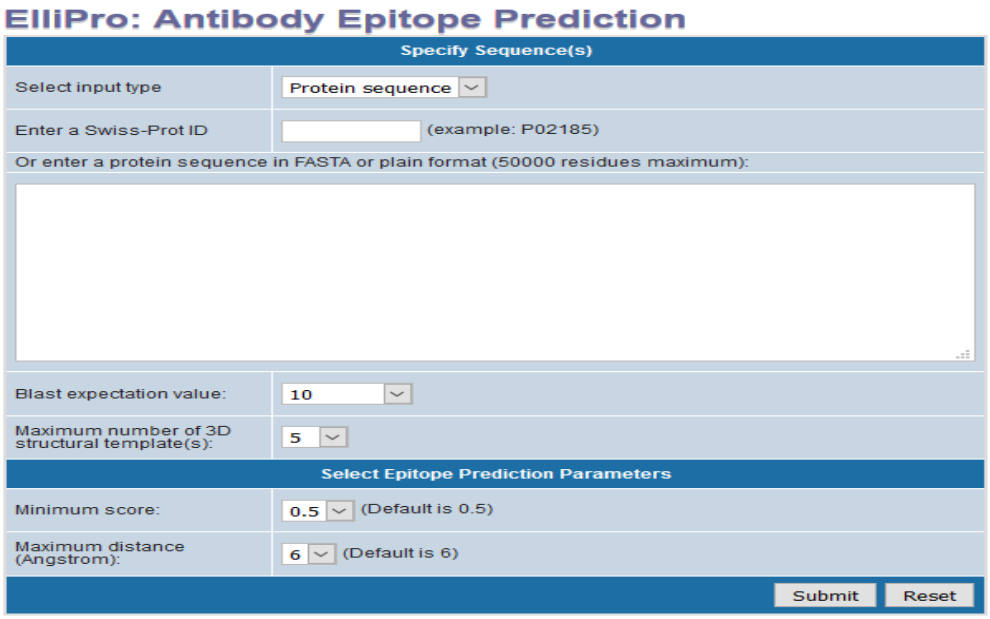


Figure 17 *ElliPro web-based program.(29)*

Predicted Discontinuous Epitope(s):

No.	Residues	Number of residues	Score	3D structure
1	_:R39, _:A40, _:S41, _:S42, _:E43, _:I44	6	0.844	View
2	_:M1, _:K2, _:D3, _:L4, _:C6	5	0.724	View
3	_:S75, _:N76, _:C77, _:L78, _:V96, _:L97	6	0.715	View
4	_:P27, _:S28, _:P29, _:A57, _:L58, _:S59, _:C60, _:N61, _:A62, _:D63, _:S64, _:M65, _:V66, _:Q85, _:T86, _:Y87, _:V88, _:A89, _:Q90, _:S91	20	0.702	View
5	_:V35, _:T50, _:S51, _:A52, _:D53, _:S54	6	0.541	View

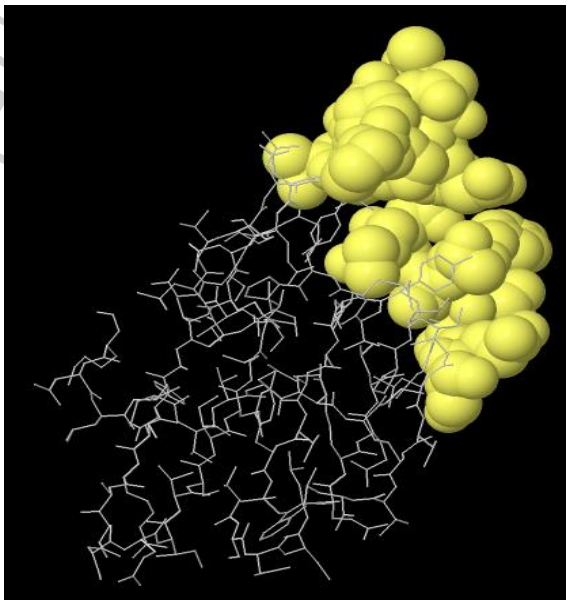


Figure 18 *Example of prediction results from ElliPro web-based program. It could display the 3-D structure of each epitope.*

2.10. Mucosal immune response

PEDV is mainly localized intestinal infections, but with transient viremia based on viral RNA detection in the serum of young piglets. The severity of both infections is greatest in newborn piglets. Thus, vaccination strategies for PED must focus on induction of mucosal immunity to protect the target intestinal enterocytes. This necessitates protective levels of mucosal immunity in neonates at birth and throughout the nursing period. Due to the impermeable placenta of sows, piglets are born agammaglobulinic and rely solely on colostrum and milk antibodies for passive immunity. This leaves the newborn piglet highly susceptible to a plethora of infectious agents. (30)

It is well documented that immunization of pregnant animals provides passive protection to suckling neonates against bacterial and viral infections (31, 32). Lactogenic immunity is described as the continuous supply of passively acquired immunoglobulins (IgG, IgM and sIgA) through the ingestion of colostrum and milk. The most abundant antibody in gut secretions, sIgA, is generated by translocation of intestinal plasma cell-produced dimeric IgA into the gut lumen via the polymeric immunoglobulin receptor on the basolateral surface of the epithelial cell (Figure 19). Once in the lumen, sIgA provides immuneprotection and contributes to intestinal homeostasis (33). After the gut-mammary-sIgA axis is initiated in the intestine by means of natural infection or oral vaccination, plasmablasts must traffic to the mammary gland to supply specific immunity via mammary secretions.

In sows, IgG is dominant in colostrum and is transudated from sow serum (34). Newborn piglets acquire colostral antibodies (mainly IgG) via nursing. These immunoglobulins are transported across the piglet's intestinal epithelium only within the first 24–48 h after birth. During the next 2–3 days, in the transition to milk, sIgA becomes dominant and persists in milk throughout lactation. Thus, IgG antibodies absorbed from sow colostrum provide piglets with serum antibodies that reflect the specificities of those in sow serum and prevent systemic infections. In contrast the IgA antibodies dominant in milk and function to provide local passive protection to the piglet intestinal tract. Its resistance to proteolytic enzymes affords sIgA a high level of stability in the gastrointestinal tract. This knowledge was critical to aid in the design of enteric vaccines to induce passive milk sIgA antibodies.

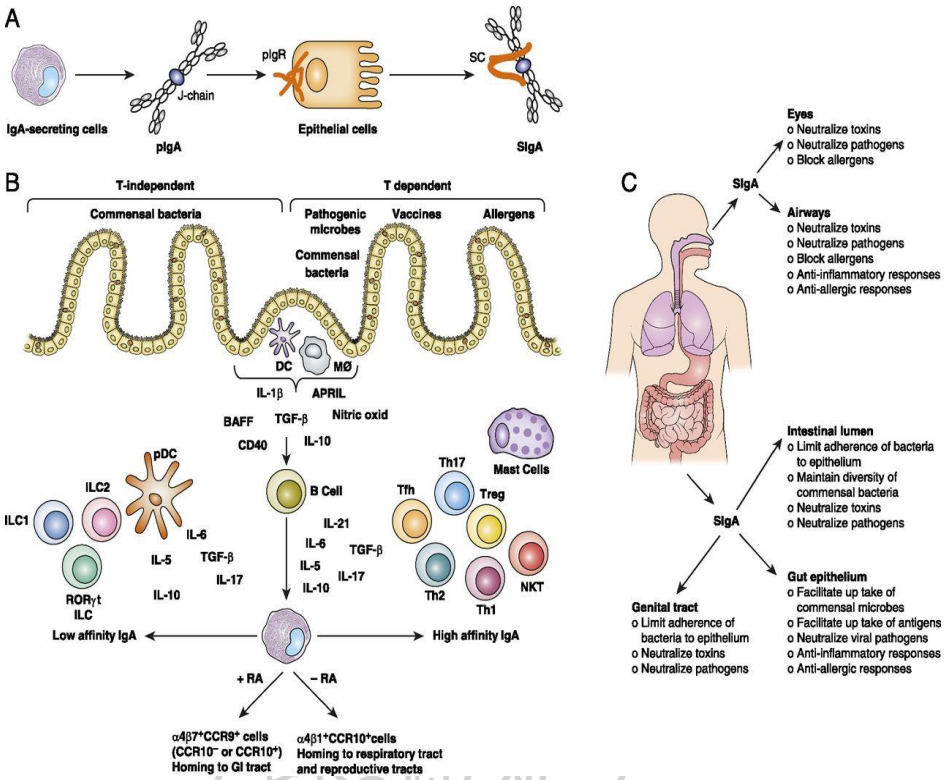


Figure 19 *Mechanism of mucosal antibodies response to antigen.(32)*

CHAPTER III
MATERIALS AND METHODS

3.1 Materials and chemicals:

Materials

3.1.1 Spectrophotometer
3.1.2 ELISA plate analyzer
3.1.3 Animal control room and cages
3.1.4 Computer notebook Dell
3.1.5 Microsoft office version 2016
3.1.6 Syringe 1cc, 5 cc, 10 cc
3.1.7 Operation set
3.1.8 Beaker 10, 50, 100, 500mL
3.1.9 Cylinders 10, 100 mL
3.1.10 Microcentrifuge tube
3.1.11 Homogenizer
3.1.12 Centrifuge machine
3.1.13 Freezer 4, -20-degree Celsius

Chemicals

3.2.1 Phosphate-buffered saline (PBS), pH7.4
3.2.2 Tween in PBS Buffer
3.2.3 Bovine serum albumin
3.2.4 Chloral hydrate solution
3.2.5 Heparin solution
3.2.6 Peptide synthesized
3.2.7 Horseradish Peroxidase-labelled goat anti-mouse IgG
3.2.8 Horseradish Peroxidase-labelled goat anti-mouse IgA
3.2.9 O-phenylenediamine tablet/solution
3.2.10 Protease inhibitors and 2% saponin

3.2 Methods

3.2.1. Genetic retrieval Thailand PEDV genome

Complete PEDV genome sequences were retrieved from NCBI (GenBank) database and published articles.

1. Search GenBank database (https://www.ncbi.nlm.nih.gov/ genbank) with the following keyword: “Porcine epidemic diarrhea

virus Thailand complete genome", "PEDV", "complete PEDV", and "PEDV Thailand".
2. Filtering criteria were focused on epidemic in Thailand and complete genome sequence as possible.
3. Download selected search results from GenBank in file FASTA format and also in associated formats.

3.2.2 Prediction expression and localization proteins

1. The selected Thailand PEDV sequence was used to predict possible proteins by NCBI's ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder). Parameters were assigned as Minimal ORF length (nt):75, Genetic code: Standard, and ORF start codon use: "ATG" only (default criteria).
2. All possible proteins predictions from ORFs finder were predicted their localization using iLoc-Virus (23) (http://www.jci-bioinfo.cn/iLoc-Virus), all results were collected and grouped.
3. Secreted proteins and cell membrane proteins localization were focused for a potential candidate vaccine.

3.2.3 Prediction of adhesin-like proteins and B cell epitope

1. All ORFs that were predicted as secreted protein and cell membrane protein will be predicted the potential for adhesin-like proteins by the SPAAN program (25). SPAAN is a Linux OS software that was installed on Dell computer, CPU core i5, Ram 8 gb. The result from prediction will be shown as P_{ad} value (The probability of a protein being an adhesion). Under neural network architecture with a multilayer feed forward topology, the most of adhesin proteins have P_{ad} value equal or greater than 0.51.
2. Following this process, predicted protein with P_{ad} value more than 0.51 was selected and further process to predicted B-cell epitopes by using seven selected bioinformatics tools: ABCpred (35), BCPred (36), COBEPro (37), EPMLR (38), FBCPred (39), LBtope (40) and SVMtrip (41). Parameters of each tool were assigned following a recommended use software.
3. Sequence from each bioinformatic tools with the top 20 predictions score were selected.
4. The region of peptide chains that were predicted as epitope by most bioinformatics tools were selected.

3.2.4. Peptides synthesis and Immunization

1. The selected-candidate peptides were focused and ordered to be synthesized by conjugated in-line with a pan HLA DR-binding epitope (PADRE) (42).
2. All peptides were synthesized from Genscript (Piscataway, NJ, USA). The 99% purity and standard analysis were done following the company. The peptide was kept in 4 °C until dissolved and used.
3. Twenty female BALB/cMlac mice, 2-month-old, were received from National Laboratory Animal Center (Mahidol University, THAILAND). They are divided into 4 groups, 1 control groups and 3 experimental groups for evaluate three selected-candidate peptides. Each group contained 5 mice per cage.
4. In each experimental group, mice were inoculated in the thigh with 0.2 mL (about 1 microgram) of synthesized peptide dissolved in sterile phosphate-buffered saline (PBS), pH 7.4 (Final concentration 5 microgram/mL).
5. In the control group (5%BSA dissolved in PBS) were inoculated.
6. The all injections were carried out 4 times at 2 weeks' intervals in each group (define as 0, 2, 4, 6 week).
7. These procedures were reviewed and approved for the animal experimental design (approval No. 001/2558) under the Animal ethic committee of Institution (Faculty of Pharmacy, Silpakorn University).

3.2.5 Harvesting of specimens by PERFEXT method

The mice were killed 2 weeks after the last injection (after 6 week). The collection of mucosal extracts was carried out by the method of perfusion–extraction (PERFEXT) (43) with slight modifications.

1. Mice were bled under anesthesia with 100 mL of 30% of chloral hydrate in PBS containing 1% heparin by intraperitoneal injection.
2. Blood was kept in a sterile microcentrifuge tube.
3. The 50mL PBS solution containing 1% heparin was injected into the mice at right-lower of the heart
4. The select organs were collected from exsanguinated mice. Following, there were jejunum, cecum, colon, ileum, spleen.
5. The peritoneum and fat were discarded. They were extensively washed in PBS to remove the extracellular fluid.

6. All organs were homogenized under 4 °C, and then pellets were kept frozen (-20 °C) in a PBS solution containing protease inhibitors and 2% saponin.
7. Intracellular molecules were extracted by freeze-thawing (from -20 °C) to 4 °C and separated from insoluble components by centrifugation 10,000g for 15 min under 4 °C.

3.2.6 Detection antibodies levels by ELISA method

Standard procedure for ELISA plate preparation (Indirect ELISA) was used. These are the procedures for running the ELISA method.

1. Immobilization of antigen (OVA-linked peptides 5 µg/ml) were coated on ELISA microtiter plates (Nunc, Roskilde, Denmark) overnight at 4 ºC with 50 µl per well. Seal the plate to prevent evaporation.
2. Remove the excess antigen and wash 3 times with 0.1% (v/v) Tween 20 in PBS (Washing solution).
3. Blocking by add 100 µl of 5% (w/v) BSA in PBS per well and incubate at 37 °C for 1 hour to reduce non-specific binding of the target protein into the well.
4. Washing the blocking buffer, and washing 3 times with a washing solution.
5. Add 50 µl of mucosal lysates (1:20 dilution) in sample well. For the dilution curve, prepare a dilution series of the sample on the same plate. 50 µl of 5% (w/v) BSA in PBS and Anit-OVA antibody were used as negative and positive control respectively. Allow it to incubate at 37°C for 1 hour.
6. Remove the samples and wash 3 times with a washing solution.
7. 50 µl of Horseradish Peroxidase-labelled goat anti-mouse IgG or IgA (1:5,000 dilution) was added. Allow it to incubate at 37°C for 1 hour.
8. Remove the samples, and wash 3 times with a washing solution.
9. Add 50 µl of freshly prepared O-phenylenediamine in peroxidase buffer solution.
10. Allow it to incubate in the dark place about 5–15 minutes.
11. Add 50 µl of 2.5 M H_2SO_4 to stop the reaction when the color is sufficiently developed.

12 Measure the absorption at 492 nm by a microplate reader.

CHAPTER IV
RESULTS

4.1 Complete Thailand PEDV genome sequences from GenBank

Thailand PEDV genome sequences were retrieved from GenBank and research articles. In GenBank, four completed Thailand PEDV genome sequences are the following GenBank number: KR610991.1, KR610992.1, KR610993.1, and KR610994.1. There is a research article (44), published in 2015, present two PEDV complete genome sequences in the Eastern Region of Thailand, KR610991.1 (EAS1) and KR610993.1 (CBR1), respectively. According recently database retrieval, it was also found EAS2 (GenBank: KR610992.1) and CBR2 (GenBank: KR610994.1). All genome sequences were selected and compared for multiple alignment genome sequences using Clustal Omega Tool in EMBL-EBI webservice (45). It was found that EAS1 and EAS2 strains, CBR1 and CBR2, CBR1 and EAS1, CBR2 and EAS2 showed 100%, 99.82%, 96.24%, 96.21% nucleotide sequence similarities (Figure 20). Moreover, CBR1 strain has nucleotide sequence of spike gene similar to spike gene of frequently founded Chinese and Thai PEDV up to 94.2% - 98.5% (13). In this study, Porcine epidemic diarrhea virus clone CBR1 (GenBank:KR610993.1), complete genome was selected for candidate Thailand PEDV epitope for reversed vaccine design.

Percent Identity Matrix - created by Clustal2.1

1: KR610991.1	100.00	100.00	96.24	96.21
2: KR610992.1	100.00	100.00	96.24	96.21
3: KR610993.1	96.24	96.24	100.00	99.82
4: KR610994.1	96.21	96.21	99.82	100.00

Figure 20 *Percent identity matrix of four PEDV genome sequences obtained from Clustal Omega Tool.*

4.2 Prediction of total open reading frame in genome sequence

By using NCBI's ORF Finder, 190 ORFS were predicted under criteria selection nucleotide's length more than 75. There are 6 ORFs were known from complete CBR1 stain and within 3 ORFs were identified protein location as surface-exposed proteins, spike protein, envelope and membrane protein, respectively (44). Then, there were 184 ORFs that unknown the localization of protein.

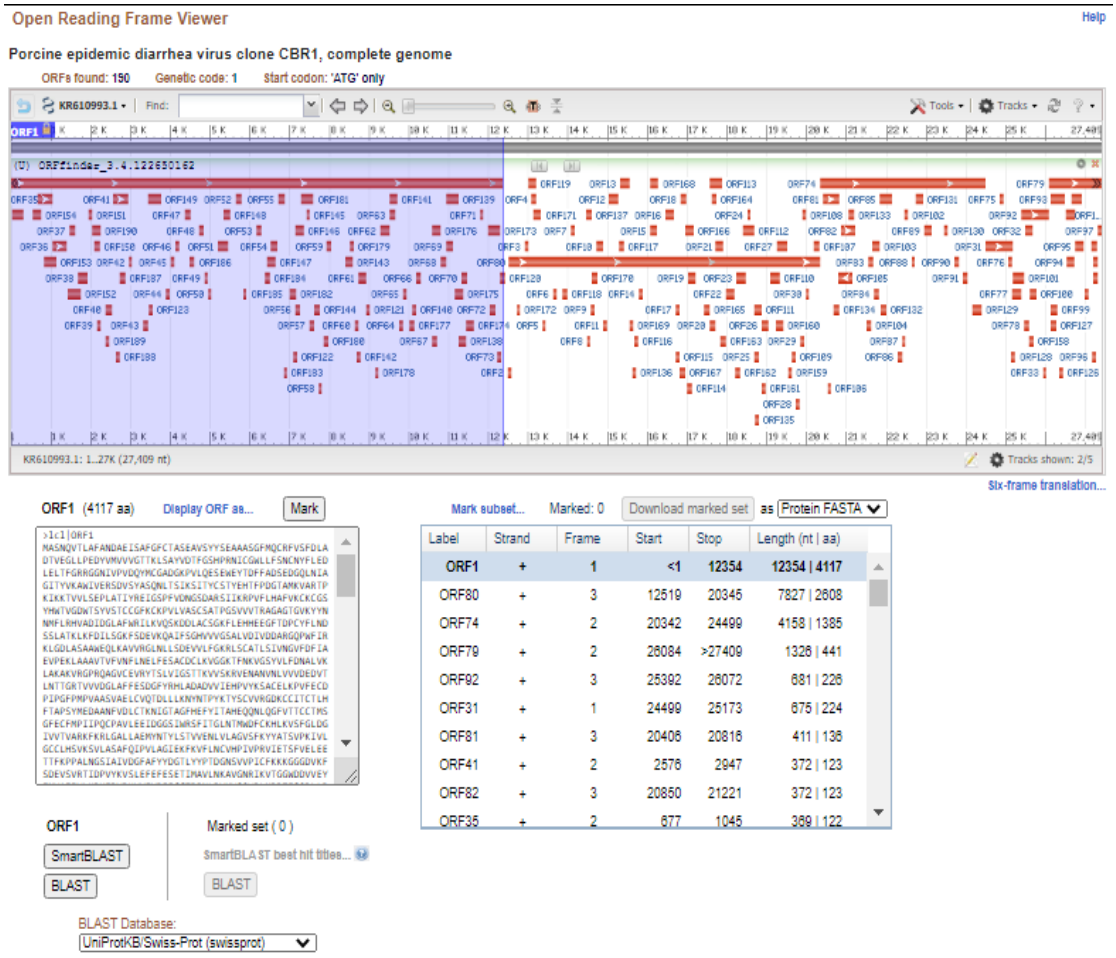


Figure 21 *Result of ORFs prediction from CBR1 strain PEDV complete genome by ORF Finder.*

Table 2 *Protein positions of Six ORFs from CBR1 strain.*

ORF Position	Position of protein
1..12309, 12309..20345	Pol1
20342..24499	Spike protein
24499..25173	Accessory protein
25154..25384	Envelope protein
25392..26072	Membrane protein
26084..27409	Nucleocapsid protein

4.3 Prediction of the cellular protein location.

To develop vaccine by reverse vaccinology method, only surface-exposed proteins and secreted proteins have high potential to become an effective antigen (46). Then, 184 ORFs from the previous step will be predicted for their cellular location. There are many tools that can predict the location of viral produced proteins such as iLoc-virus, Virus-mPloc. The iLoc-Virus was selected in this study with higher accuracy (78.2%) rather than Virus-mPloc (60.3%) (23). The principle of this tool is analysis of export signals on proteins that direct them to other cellular locations. However, the limitation of software was noted for peptide chain less than 60 amino acids may unpredictable. Total 128 ORFs were excluded, the rest 56 ORFs were predicted with iLoc-Virus program (Table 3). The prediction result obtained from program (Table 4), only 9 ORFs may be membrane proteins and 1 ORF may be secreted protein.

Table 3 Cellular location prediction result of 56 ORFs by iLoc-Virus. (PSSM = Position-Specific Scoring Matrix)

Position of protein	ORF position	protein length	Predicted by
Secreted	12998 to 13204 Frame -1	69	PSSM
Host Cytoplasm	20406..20816 Frame +3	137	PSSM
	2576..2947 Frame +2	124	Gene Ontology
	20786..21151 Frame -1	122	PSSM
	26139..26477 Frame +3	113	PSSM
	7284..7607 Frame -3	108	PSSM
	1432..1752 Frame -2	107	PSSM
	829..1134 Frame -2	102	PSSM
	18386..18676 Frame -1	97	PSSM
	25181..25465 Frame -1	95	PSSM
	9272..9520 Frame +2	83	Gene Ontology
	1682..1915 Frame +2	78	PSSM
	11151..11375 Frame -3	75	Gene Ontology
	7141..7362 Frame -2	74	Gene Ontology
	544..756 Frame -2	71	PSSM
	16453..16662 Frame +1	70	PSSM
	13125..13325 Frame -3	67	PSSM
	10596..10796 Frame -3	67	PSSM
	16074..16268 Frame -3	65	PSSM
	22944..23129 Frame +3	62	Gene Ontology
	19224..19409 Frame -3	62	Gene Ontology
	16063..16245 Frame +1	61	PSSM
	11251..11430 Frame -2	60	PSSM
Host cell membrane	20850..21221 Frame +3	124	PSSM

	10948..11250 Frame -2	101	PSSM
	26644..26916 Frame -2	91	PSSM
	35..304 Frame +2	90	PSSM
	15298..15555 Frame +1	86	PSSM
	26505..26735 Frame -3	77	Gene Ontology
	18232 to 18453 Frame +1	74	PSSM
	26544..26732 Frame +3	63	GO#
	17716..17895 Frame +1	60	PSSM
Host nucleus	677..1045 Frame +2	123	PSSM
	1046..1402 Frame +2	119	PSSM
	7284..7607 Frame -3	108	PSSM
	21825..22145 Frame +3	107	Gene Ontology
	3451..3771 Frame -2	107	PSSM
	829..1134 Frame -2	102	PSSM
	17570..17881 Frame -1	104	Gene Ontology
	11997..12293 Frame -3	99	PSSM
	5195..5467 Frame +2	91	PSSM
	19246..19503 Frame +1	86	PSSM
	19085..19315 Frame -1	77	PSSM
	15034..15261 Frame +1	76	PSSM
	24163..24387 Frame -2	75	Gene Ontology
	6436..6654 Frame -2	73	PSSM
	2037..2252 Frame -3	72	Gene Ontology
	11430..11633 Frame -3	68	Gene Ontology
	16956..17156 Frame -3	67	PSSM
	17974..18168 Frame +1	65	Gene Ontology
	17008..17199 Frame +1	64	Gene Ontology
	16063..16245 Frame +1	61	PSSM
	25604..25783 Frame -1	60	Gene Ontology
	21647..21826 Frame -1	60	Gene Ontology
	11251..11430 Frame -2	60	PSSM
	9517..9696 Frame -2	60	PSSM
	8741..8920 Frame +2	60	Gene Ontology
Viral capsid	26742..26957 Frame +3	72	PSSM
	8407..8586 Frame -2	60	PSSM

Table 4 *13 possible effective epitopes were predicted by iLoc-Virus.*

(PSSM = Position-Specific Scoring Matrix)

10 possible epitopes from CBR1 gene			
Position of protein	**ORFs position**	**Protein length**	**Predict by**
Cell membrane	35 to 304 Frame +2	90	PSSM
	10948 to 11250 Frame -2	101	PSSM
	15298 to 15555 Frame +1	86	PSSM
	17716 to 17895 Frame +1	60	PSSM
	18232 to 18453 Frame +1	74	PSSM
	20850 to 21221 Frame +3	124	PSSM

	26505 to 26735 Frame -3	77	Gene Ontology
	26544 to 26732 Frame +3	63	Gene Ontology
	26644 to 26916 Frame -2	91	PSSM
Secreted protein	12998 to 13204 Frame -1	69	PSSM
Known 3 possible epitopes from GenBank			
Position of protein	**ORFs position**	**Protein length**	**Reference protein ID**
Spike protein	20342 to 24499	1,386	AKH453338.1
Envelope protein	25154 to 25384	77	AKH45340.1
Membrane protein	25392 to 26072	227	AKH45341.1

4.4 Prediction of adhesin-like proteins.

From previous step, 10 ORFs that were predicted by iLoc-Virus and 3 ORFs that have already known as spike protein, envelope protein and membrane protein were predicted for adhesin-like property. Adhesin are often good vaccine targets because it mediates their adherence to host cell surface receptors for successful colonization. SPAAN is a Linux OS based software that can identify adhesin-like proteins. The prediction results were shown as P_{ad} value (The probability of a protein being an adhesin). Most of the adhesins (96%) have P_{ad} more than 0.51. Results of the prediction from SPAAN shown in Fig 22. Only 2 ORFs, SK1 (Spike protein) and S8 (Hypothetical protein) have P_{ad} value more than 0.51 (0.60, 0.74, respectively).

Name	ORF	P_{ad}
S1	35 to 304	0.27
S2	10948 to 11250	0.24
S3	12998 to 13204	0.46
S4	15298 to 15555	0.18
S5	17716 to 17895	0.23
S6	18232 to 18453	0.33
S7	20850 to 21221	0.25
S8*	26505..26735	0.74
S9	26544 to 26732	0.22
S10	26644 to 26916	0.31
SK1*	20342 to 24499	0.6
SK2	25154 to 25384	0.29
SK3	25392 to 26072	0.47

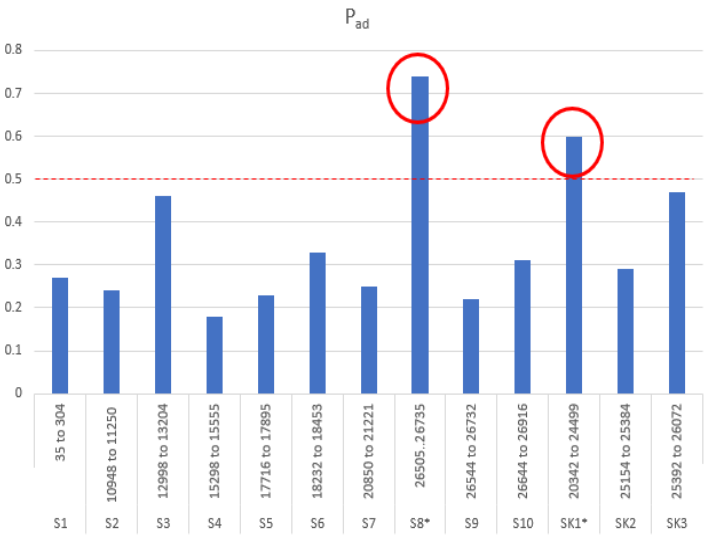


Figure 22 Prediction result from SPAAN.

4.5 B cell epitope prediction on selected sequences.

From previously, two selected ORFs, Spike protein (SK1*) and Hypothetical protein (S8*) that were predicted to be adhesin were selected for the B cell epitope prediction process. Nowadays, there are many predictive software developers, these programs differ in many ways, such as methods of computation, Reference database, selected algorithm etc. Such differences make the choice of either tool to be used as the primary tool, which may result in an incomprehensible prediction. In this research, eight B cell epitope prediction programs that were accessible via the internet at the time of research were selected, namely ABCPred, BCPREDS, BepiPred, FBCPred, COBEpro, SVMTriP, LBtope and EPMLR. The prediction results from BepiPred are in the form of scores of each amino acid on genome sequence. High score means high probabilities to be an epitope. The prediction results from other tools are in the form of scores for the amino acid sequence range that qualifies as an epitope. Table 5 to Table 12 show examples of predictive results from each tool.

Table 5 *Top 20 scores of possible epitopes predicted by ABCPred*

Hypothetical protein (S8*)		Spike protein (SK1*)	
Sequence	**Score**	**Sequence**	**Score**
TGYETCYCYYYCLLCS	0.89	GELITGTPKPLEGVTD	0.96
SEKLHDPGYFHDSVNY	0.87	PEVIPDYIDVNKTLDE	0.96
TGCCLCYYSGIWTCCC	0.85	MQYVYEPTYYMLNVTS	0.95
IWTCCCHCHDSCYVNL	0.84	SFSEQAAYVDDDIVGV	0.92
HDSVNYRGTGCCLCYY	0.79	DWSRVATKCYNSGGCA	0.92
YCYYYCLLCSEKLHDP	0.78	VREIVITKYGDVYVNG	0.91
		CGACFSGCCRGPRLQP	0.91
		ASLIGGMVLGGFTAAA	0.90
		PGVVDAEKLHMYSASL	0.90
		TIDLFGYPEFGSGVKF	0.90
		AHMSEHSVVGITWDND	0.90
		FEIGISQEPFDPSGYQ	0.89
		FCCISTGCCGCCGCCG	0.89
		ADLVCAQYYSGVMVLP	0.88
		KRSFIEDLLFNKVVTN	0.87
		GVSVYDPASGRVVQKR	0.87
		LATISSFNGDGYNFTN	0.86
		TEYLQLYNTPVSVDCA	0.86
		LQSVNDYLSFSKFCVS	0.86
		PTSYGYGSKSQGSNCP	0.86

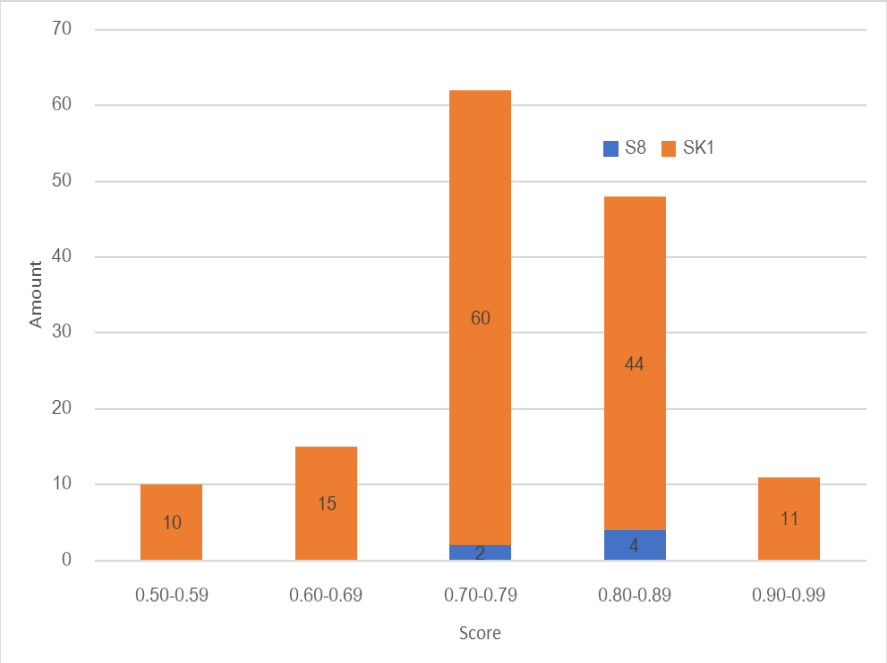


Figure 23 *Number of predicted epitopes from ABCpred (S8 vs SK1).*

Table 6 *Top 20 scores of possible epitopes predicted by BCPREDS.*

Hypothetical protein (S8*)		Spike protein (SK1*)	
Sequence	**Score**	**Sequence**	**Score**
SVNYRGTGCCLCYYSGIWTC	0.899	ECVKSQSQRYGFCGGDGEHI	0.997
HLTGFCSWTGYETCYCYYYC	0.863	PDNKTLGPTANNDVTTGRNC	0.995
		YHSNDGSNCTEPVLVYSNIG	0.994
		FQFTKGELITGTPKPLEGVT	0.986
		GENQGVNSTWYCAGQHPTAS	0.979
		FYNVPTSYGYGSKSQGSNCP	0.976
		GGCAMQYVYEPTYYMLNVTS	0.974
		PPTVREIVITKYGDVYVNGF	0.972
		ATQVPYYCFPKVDTYNSTVY	0.968
		RTEYLQLYNTPVSVDCATYV	0.962
		TKYTIYGFKGEGIITLTNSS	0.957
		NVLGVSVYDPASGRVVQKRS	0.953
		PSGYQLYLHKATNGNTNATA	0.946
		GACFSGCCRGPRLQPYEAFE	0.920
		VKIAPTVTGNISIPTNFSMS	0.917
		GTNFSFVCSNSSDPHLTTFA	0.833
		SVVGITWDNDRVTVFSDKIY	0.811
		VTINFTGHGTDGDVSGFWTI	0.772
		LTRDQLPEVIPDYIDVNKTL	0.737

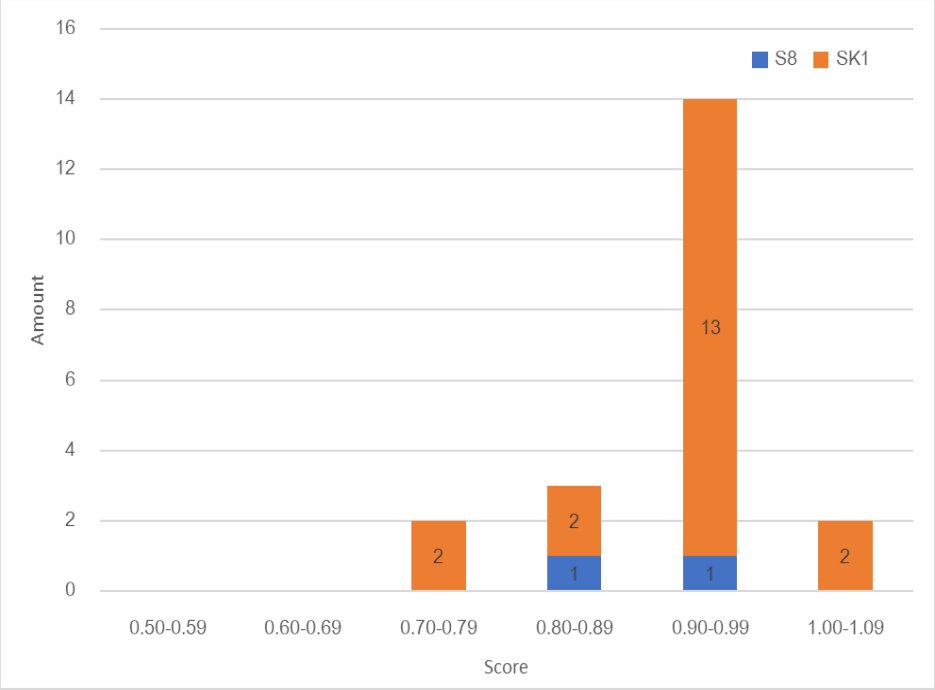


Figure 24 *Number of predicted epitopes from BCPREDS (S8 vs SK1).*

Table 7 *Top 20 scores of possible epitopes predicted by FBCPred.*

Hypothetical protein (S8*)		Spike protein (SK1*)	
Sequence	**Score**	**Sequence**	**Score**
DPGYFHDSVNYRGT	0.947	HSNDGSNCTEPVLV	1
YSGIWTCCCHCHDS	0.850	GFKGEGIITLTNSS	1
TGFCSWTGYETCYC	0.805	SQSQRYGFCGGDGE	1
		GPTANNDVTTGRNC	1
		YLHKATNGNTNATA	1
		TISSFNGDGYNFTN	1
		GVSVYDPASGRVVQ	0.999
		GGCAMQYVYEPTYY	0.999
		RCSANTNFRRFFSK	0.998
		TSAFESVKEAISQT	0.996
		LITGTPKPLEGVTD	0.996
		GYGSKSQGSNCPFT	0.996
		FCCISTGCCGCCGC	0.994
		ASDTTINGFSSFRV	0.994
		PEVIPDYIDVNKTL	0.994
		YCFPKVDTYNSTVY	0.991
		CFSGCCRGPRLQPY	0.990
		GTNFSFVCSNSSDP	0.990
		TVDEDYKRCSNGRS	0.985
		KNVTSGAVYSVTPC	0.982

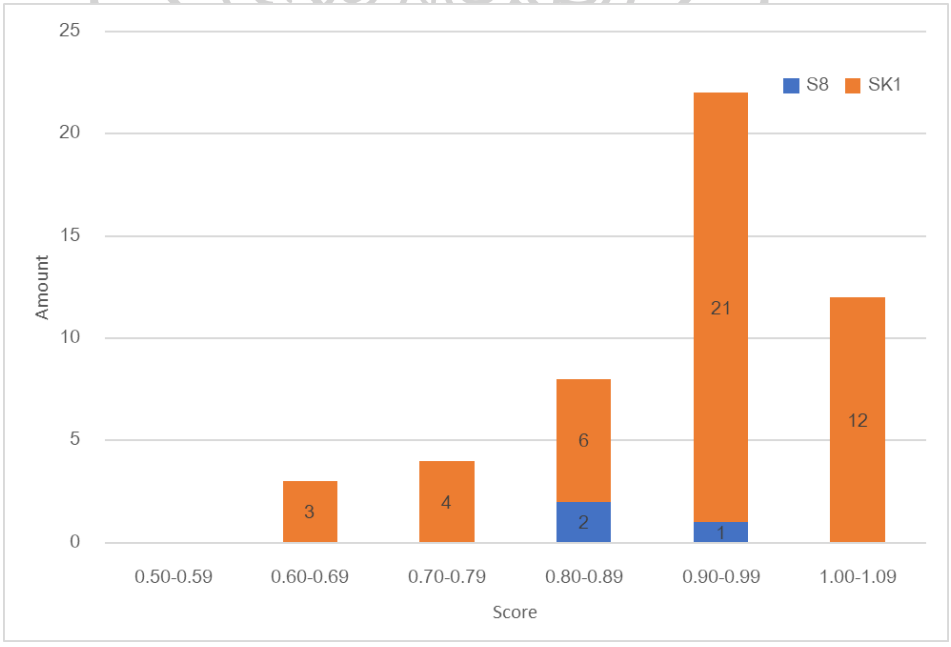


Figure 25 *Number of predicted epitopes from FBCPred (S8 vs SK1).*

Table 8 *Top 20 scores of possible epitopes predicted by COBEpro.*

Hypothetical protein (S8*)		Spike protein (SK1*)	
Sequence	**Score**	**Sequence**	**Score**
CHDSCY	0.788	GKDGISY	0.844
LHDPGY	0.677	PTVGDF	0.836
EKLHDP	0.668	LREPGL	0.833
KLHDPG	0.666	GDGYNF	0.819
SEKLHDP	0.662	SKGLNT	0.813
CHDSCYV	0.661	PYEAFE	0.811
EKLHDPG	0.649	QAPAVV	0.806
HDSCYV	0.606	PTVTGN	0.804
KLHDPGY	0.601	QPYEAFE	0.802
CHCHDSCY	0.600	PCTANC	0.795
MSFHHGH	0.593	SGQVKI	0.788
HCHDSCYV	0.590	VTTGRNC	0.786
SFHHGHL	0.586	GNSRCKQ	0.784
VNYRGTGC	0.586	GQVKIA	0.784
SEKLHDPG	0.581	NGDGYNF	0.783
CCHCHDSC	0.567	MSEHSV	0.782
LHDPGYF	0.566	TGRNCL	0.782
DSVNYRGTGC	0.566	SNGRSVA	0.780
NYRGTGC	0.566	GHRGAN	0.777
MSFHHG	0.565	GKDGISYQ	0.776

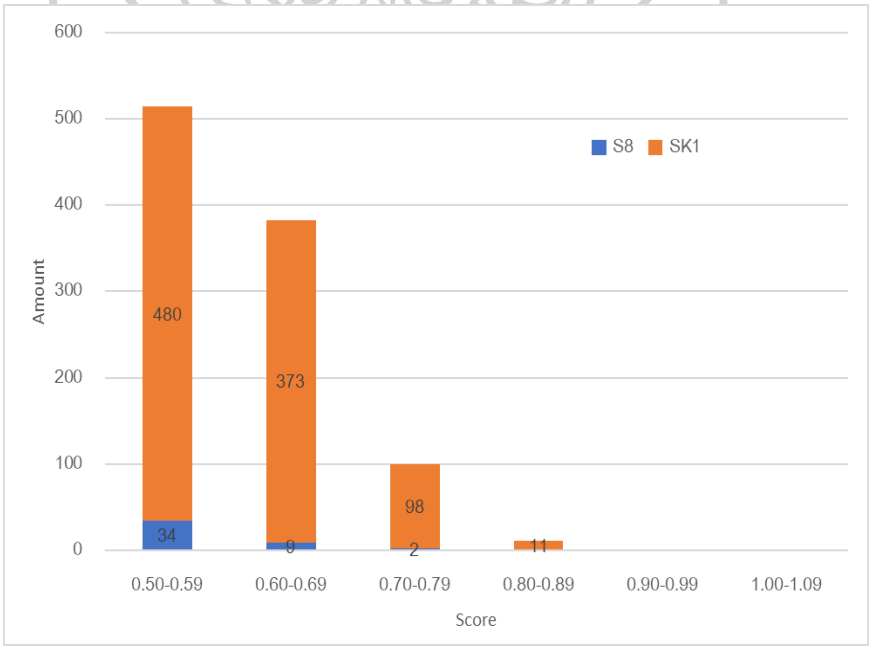


Figure 26 *Number of predicted epitopes from COBEpro (S8 vs SK1).*

Table 9 *Top 20 scores of possible epitopes predicted by SVMTriP.*

Hypothetical protein (S8*)		Spike protein (SK1*)	
Sequence	**Score**	**Sequence**	**Score**
FHHGHLTGFCSWTGYETCYC	1.0	FSGCCRGPRLQPYEAFEKVH	1.000
VNYRGTGCCLCYYSGIWTCC	0.8	LPGVVDAEKLHMYSASLIGG	0.755
		FSEQAAYVDDDIVGVISSLS	0.408
		DSGQLLAFKNVTSGAVYSVT	0.407
		VSVYDPASGRVVQKRSFIED	0.382
		PVLSTLSLPQDVTRCSANTN	0.356
		NGNSRCKQLLTQYTAACKTI	0.318
		NSAIGNITSAFESVKEAISQ	0.314
		KIYHFYFKNDWSRVATKCYN	0.287
		FVAQTLTKYTEVQASRKLAQ	0.276

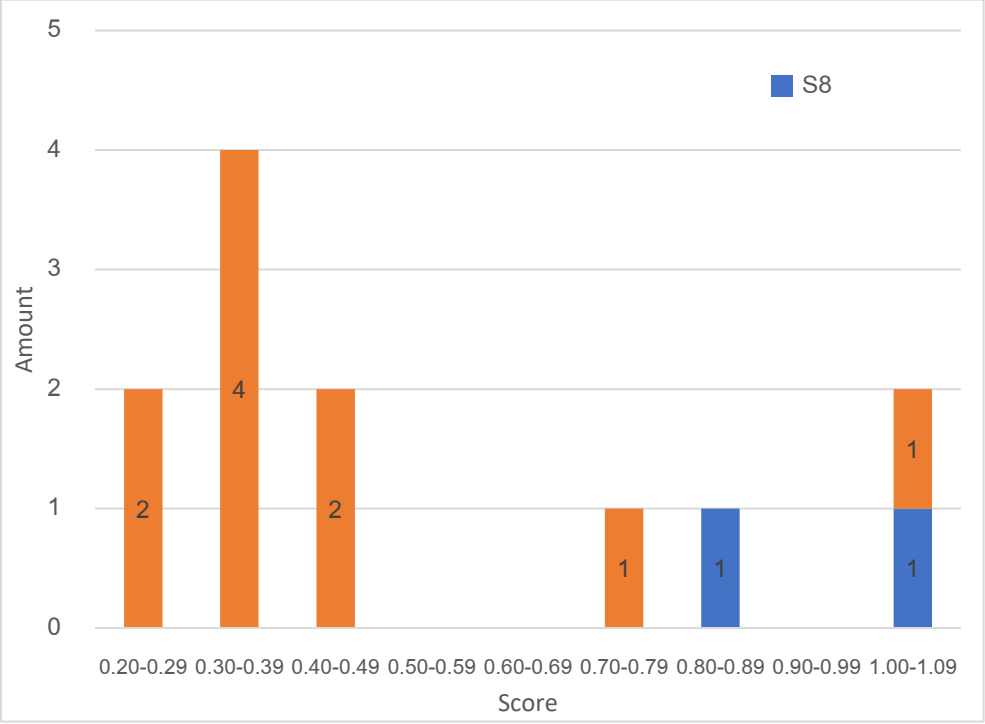


Figure 27 *Number of predicted epitopes from SVMTriP (S8 vs SK1).*

Table 10 *Top 20 scores of possible epitopes predicted by LBtope.*

Hypothetical protein (S8*)		Spike protein (SK1*)	
Sequence	**Score**	**Sequence**	**Score**
CHDSCYVNLHVKXXX	1.073	NTLVDLEWFNRVETY	1.364
FCSWTGYETCYCYYY	0.756	NSSDPHLTTFAIPLG	1.057
GFCSWTGYETCYCYY	0.692	SLIYNINNTLVDLEW	1.056
CYYYCLLCSEKLHDP	0.648	LLSHEQPTSFVTLPS	1.056
CHCHDSCYVNLHVKX	0.639	NLLSHEQPTSFVTLP	1.018
CSWTGYETCYCYYYC	0.632	QSLIYNINNTLVDLE	1.017
YYYCLLCSEKLHDPG	0.630	LIYNINNTLVDLEWF	1.000
VNYRGTGCCLCYYSG	0.589	INNTLVDLEWFNRVE	0.992
NYRGTGCCLCYYSGI	0.562	YVNLTRDQLPEVIPD	0.944
LTGFCSWTGYETCYC	0.552	SSDPHLTTFAIPLGA	0.923
SWTGYETCYCYYYCL	0.533	GGCAMQYVYEPTYYM	0.921
YYSGIWTCCCHCHDS	0.523	TYVNLTRDQLPEVIP	0.915
DSCYVNLHVKXXXXX	0.520	TNLLSHEQPTSFVTL	0.913
LHDPGYFHDSVNYRG	0.517	VVQKRSFIEDLLFNK	0.912
KLHDPGYFHDSVNYR	0.504	VVTYVNLTRDQLPEV	0.905
DPGYFHDSVNYRGTG	0.495	IYNINNTLVDLEWFN	0.905
WTGYETCYCYYYCLL	0.484	SRRMYEPRKPTVGDF	0.897
YYCLLCSEKLHDPGY	0.484	QVPYYCFPKVDTYNS	0.893
CYYSGIWTCCCHCHD	0.482	YPISSTNLLSHEQPT	0.892
XXXMSFHHGHLTGFC	0.476	YVPSQSGQVKIAPTV	0.883

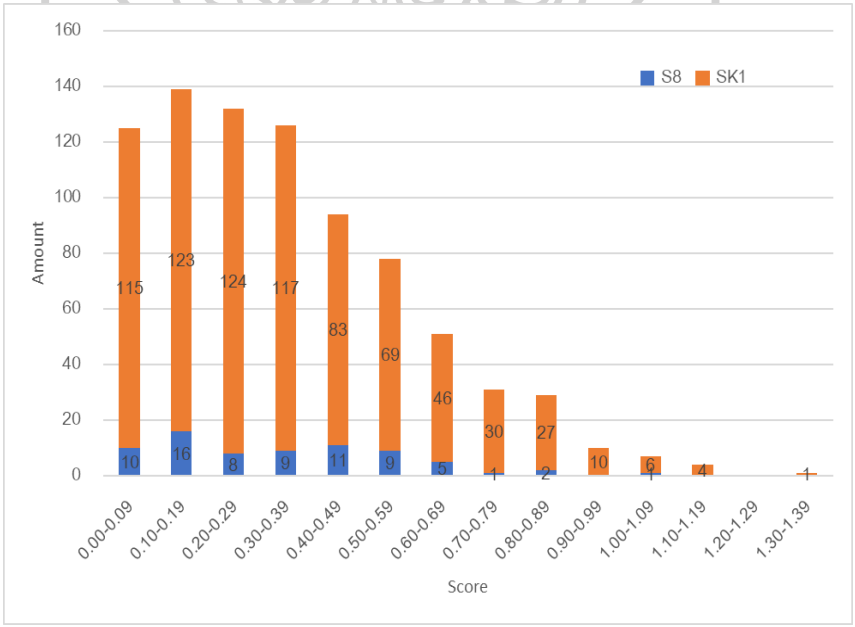


Figure 28 *Number of predicted epitopes from LBtope (S8 vs SK1).*

Table 11 *Top 20 scores of possible epitopes predicted by EPMLR.*

Hypothetical protein (S8*)		Spike protein (SK1*)	
Sequence	**Score**	**Sequence**	**Score**
FHDSVNYRGTGCCLC	1	VGITWDNDRVTVFSD	1
TGYETCYCYYYCLLC	1	GCCGACFSGCCRGPR	1
YFHDSVNYRGTGCCL	1	DGVCNGAAVQRAPEA	1
WTGYETCYCYYYCLL	1	ATEYFVSSRRMYEPR	1
TCCCHCHDSCYVNLH	-0.051	CVKSQSQRYGFCGGD	1
		TATEYFVSSRRMYEP	1
		KCYNSGGCAMQYVYE	1
		YKRCSNGRSVADLVC	1
		IDGVCNGAAVQRAPE	1
		RCSANTNFRRFFSKF	1
		VSVYDPASGRVVQKR	1
		QGVNSTWYCAGQHPT	1
		SFRVDTRQFTISRFY	1
		SHEQPTSFVTLPSFN	1
		ECVKSQSQRYGFCGG	1
		TKCYNSGGCAMQYVY	1
		GAVYSVTPCSFSEQA	1
		GVSVYDPASGRVVQK	1
		NQGVNSTWYCAGQHP	1
		CVVTYVNLTRDQLPE	1

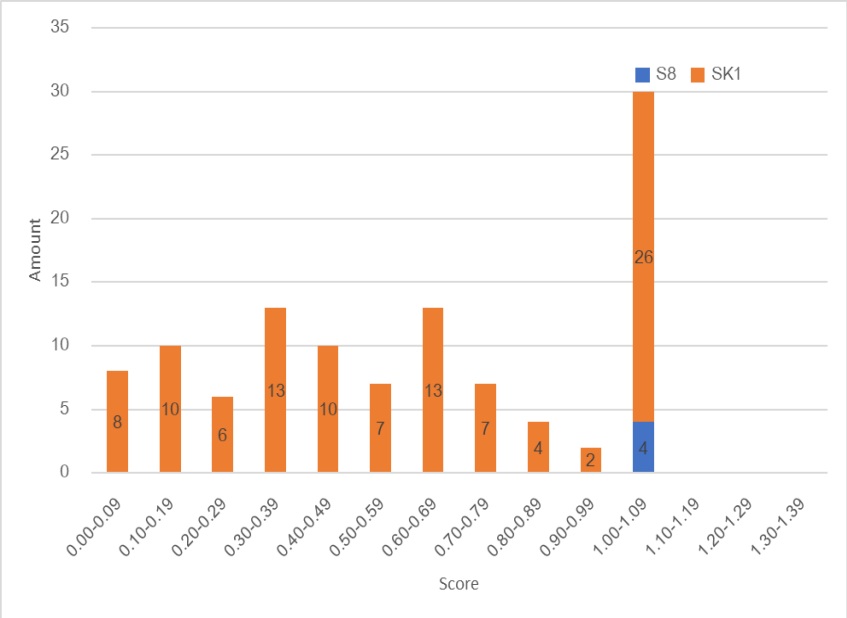


Figure 29 *Number of predicted epitopes from EPMLR (S8 vs SK1).*

Table 12 *Example of 20 possible epitopes predicted by BepiPred.*

Hypothetical protein (S8*)			Spike protein (SK1*)		
Amino acid	**Position**	**Score**	**Amino acid**	**Position**	**Score**
L	33	0.56	D	22	0.47
H	34	0.71	V	23	0.42
D	35	0.46	T	24	0.62
P	36	0.39	R	25	0.68
G	37	0.45	C	26	0.61
Y	38	0.80	S	27	0.40
F	39	0.63	P	54	0.58
H	40	0.45	I	55	0.67
N	44	0.58	G	56	0.78
Y	45	0.65	E	57	0.69
R	46	0.38	N	58	1.08
			Q	59	1.18
			G	60	1.43
			V	61	0.92
			N	62	0.60
			Y	66	0.35
			C	67	0.35
			A	68	0.37
			G	69	0.47
			Q	70	0.94

Table 13 Summary of top two scores result from seven B-cell epitope prediction tools.

Method	Hypothetical protein (S8*)		Spike protein (SK1*)	
	Sequence	Score	Sequence	Score
ABCPred	TGYETCYCYYYCLLCS	0.89	GELITGTPKPLEGVTD	0.96
	SEKLHDPGYFHDSVNY	0.87	PEVIPDYIDVNKTLDE	0.96
BCPREDS	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

The predictive B-cell epitope results from listed tools were compared and selected the region of peptide chains that were predicted as epitope by most tools. The comparing result found 6 possible epitopes from 20342-24499 (Spike protein) and 2 possible epitopes from 26505-26735 (Hypothetical protein) (Table 13). All predicted epitopes were examined in Genbank database. The BEH7 and BEH8 were reported as nucleocapsid protein that is not suitable for epitope vaccine development. The BES1-BES6 were selected and compared to 4 types commercial vaccine from China, Japan, Korea (CV777, 83P-5, DR13 and SM98), respectively. There were three novel epitopes (BES1, BES2, BES6) difference form commercial vaccine. They will be evaluated for potential in IgA and IgG stimulation in animal model.

Table 14 Potential B-cell epitope from ORF 20342-24499 and ORF 26505-26735.

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

4.6 Comparing stimulated IgA level from three epitopes by ELISA.

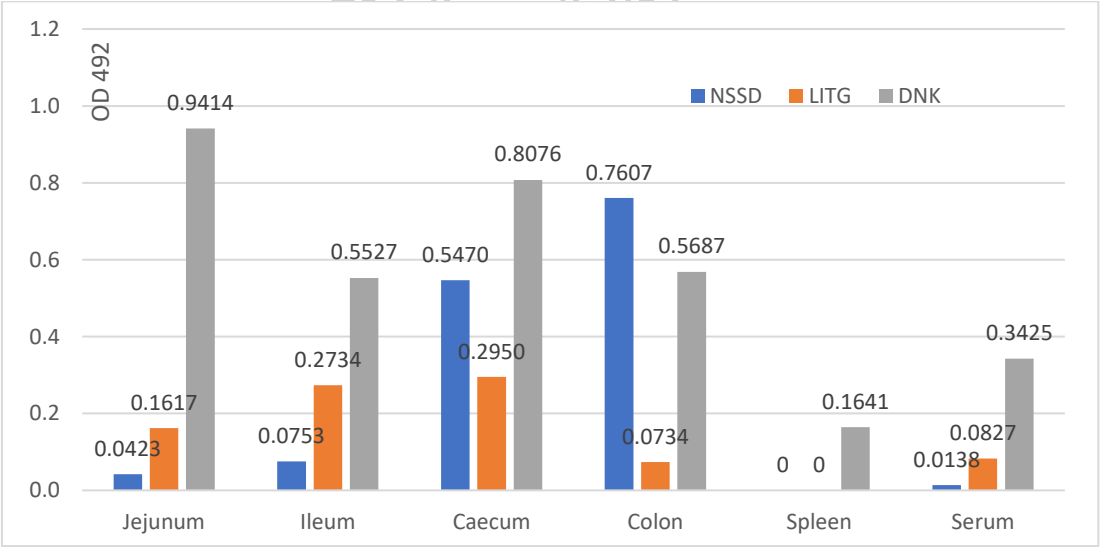


Figure 30 Comparison of the average ELISA absorbance (IgA) from each mice's organs that were stimulated with three epitopes.

Three epitopes were injected to mice. Then, IgA levels that secrete in jejunum, ileum, caecum, colon, spleen and serum were measured by ELISA. The result was shown in Figure 30. BES1 (PADRE-DNKTLGPTANNDVTT) is the best epitope that can stimulate highest IgA secreting level in almost organs except colon. Only in colon, BES6 (PADRE-NSSDPHL) epitope can stimulate the secretion of IgA better than BES1.

4.7 Comparing stimulated IgG level from three epitopes by ELISA.

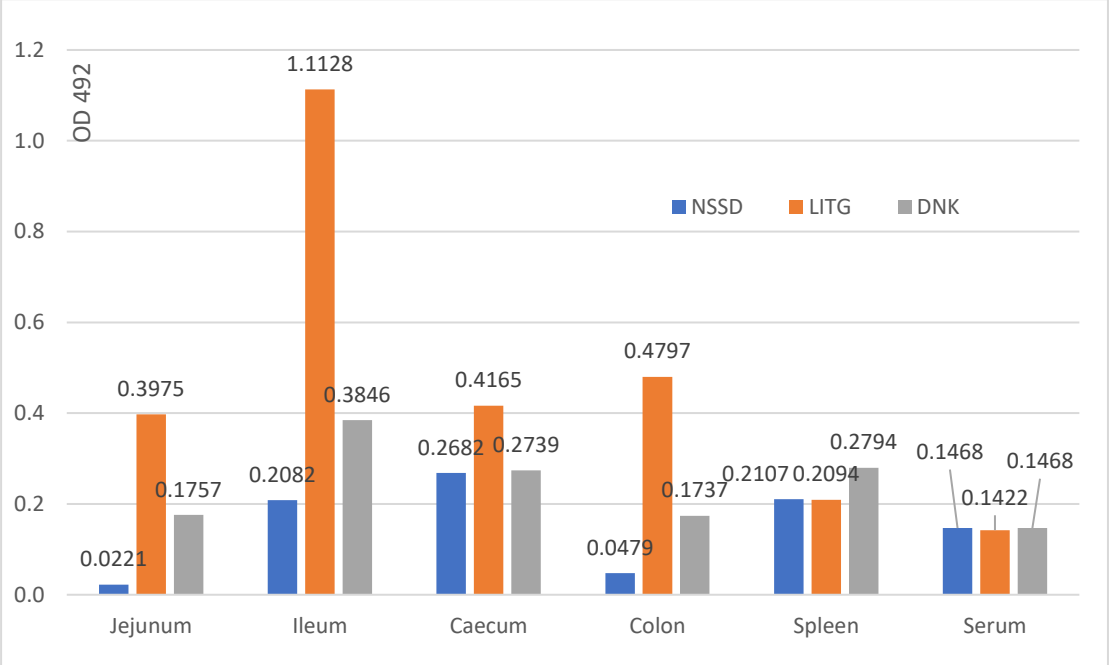


Figure 31 *Comparison of the average ELISA absorbance (IgG) from each mice's organs that were stimulated with three epitopes.*

From Figure 31, BES2 (PADRE-LITGTPKPLEGV) is the best epitope in IgG stimulation. It can stimulate highest IgG secreting level in 4 out of 6 organs (jejunum, ileum, caecum and colon). The rest organs (spleen and serum) secreted IgG in highest level by the stimulation of BES1 (PADRE-DNKTLGPTANNDVTT) epitope.

CHAPTER V
DISCUSSION

Developing a vaccine for the PEDV virus or swine diarrhea virus was still challenged, especially when outbreak reoccur in Asia, including China, Japan, Korean and Thailand. In this research, focus on searching for B Cell Epitope for PEDV because PED has a 100% high mortality rate in the piglets that is less than 1 week. But there are two major issues that it is not possible to direct vaccination of PEDV for neonatal piglets; (1) in seropositive sows, maternal antibodies may interfere with live oral vaccine-induced protection; and (2) in piglets, three weeks are needed for actual antibody production in piglets (30). Therefore, the protection with vaccines must be done by relying on the principle of 'gut-mammary-sIgA axis. Pregnant sow must be vaccinated with vaccine that can induce mucosal antibody in intestine. After that, plasmablasts will traffic to the mammary gland to supply specific immunity to suckling piglets via colostrum and milk (lactogenic immunity). Currently there are two types of PED vaccine available in the market; inactivated vaccine and live attenuated vaccine. Inactivated vaccines are more stable, easy to transport and cheaper than live attenuated vaccine. But it was less effective and often require booster shots. Live attenuated vaccines are usually very effective, and a single dose is often enough to induce long-lasting immunity. However, it cannot use in poor health condition pigs and it have chance for reversion of virus from attenuated to virulent form. There is some research that support this evidence. Wang et al. (47) found that some farms have experienced disease resulting from a PEDV isolate that was very closely related to the attenuated DR13 vaccine strain. This may be resulted from the reversion of the DR13 vaccine strain to virulence. Then PED subunit vaccine from reverse vaccinology may be the better choice than live attenuated vaccine and inactivated vaccine. While subunit vaccines are technically inactivated, they do not involve the whole pathogen (but rather a fragment of a pathogen) and are considered a distinct category of vaccines. Their response tends to be more robust than inactivated vaccines because the fragment was chosen because of its strong antigenic (immune-stimulating) effect.

The past vaccine development has many limitations, such as spending a lot of time, unable to discover the right antigen and not being able apply to non-cultivable microorganisms. To solve these limitation, Rino Rappuoli has presented the new method for developing a vaccine in reverse vaccinology. The first vaccine produced by such methods is vaccine for *Neisseria Meningitidis* Serogroup B. This vaccine development process similar with this research in two steps. First step, it started with searching for all possible ORFs by ORF Finder and Second step was checking the location of possible producing proteins by PSORTB, Signal P and TMpred. After that, proteins were expressed in *E. Coli* and test for immunogenicity in mice. However, in this research, there are two additional steps from original: the step to examine adhesin-like properties by SPANN and searching for b cell epitope by 8 tools; ABCpred (35), BCPred (36), COBEPro (37), EPMLR (38), FBCPred (39), LBtope (40), BepiPred (48) and SVMtrip (41). Increasing these two processes, this research saves time and costs rather than traditional research. But the prediction accuracy will depend on selected b cell epitope prediction tools and it may find a lesser number of possible epitopes.

Each B cell epitope prediction tools were different in database and prediction methods as show in table 15, then the prediction accuracy of each tool was different in value. ABCPred and LBtope are 66.41% and 86% respectively. While BCPred, BepiPred, COBEpro, EPMLR and SVMtrip have the following AUC value 0.758, 0.620, 0.829, 0.728 and 0.702 (49). In addition, these tools are used to develop new vaccines for many other diseases such as Covid19, nocardiosis disease, onchocerciasis. In our study, the predictive B-cell epitope results from eight tools were compared and selected the region of peptide chains that were predicted as epitope by most tools. Three novel possible peptides that could be a good candidate vaccine were found. They will further analysis for the potential to stimulate IgA and IgG in animal models.

Table 15 Prediction methods of B cell epitope prediction tools.

Predictor	Prediction methods	Institution
ABCpred	Artificial neural networks	Institute of Microbial Technology, India.
BCPREDS	Support Vector Machine	Artificial Intelligence Research Laboratory, College of Information Sciences and Technology, Penn State University, USA
COBEpro	Support Vector Machine	Dep. of Computer Science and Institute for Genomics and Bioinformatics, University of California, USA.
EPMLR	Multiple Linear Regression	The Key Laboratory of Bioinformatics, Tsinghua University, China.
FBCPred	Support Vector Machine	Artificial Intelligence Research Laboratory,College of Information Sciences and Technology, Penn State University, USA
LBtope	Support Vector Machine & Physicochemical propensity scales & Amino Acid Pairs	Department of Computational Biology, Indraprastha Institute of Information Technology, India
BepiPred	Hidden Markov Model & Parker hydrophilicity scale	DTU Health Tech, Denmark.
SVMTriP	Support Vector Machine	University of Nebraska, USA.

Normally, peptide antigen produces a relatively weak immune response, and thus requires the use of immunostimulants (adjuvants) for optimal efficacy. Then in this study, PADRE, a universal synthetic 13 amino acid peptide that has ability to activate CD4+ T cells (50) was synthesized in line with three novel possible peptides. The mucosal antibody response found in mice after 4-times injection of three selected peptides (BES1 (PADRE-DNKTLGPTANNDVTT), BES2 (PADRE-LITGTPKPLEGV) and BES6 (PADRE-NSSDPHL)) with 2-weeks intervals period. The level of IgA and IgG that were stimulated from that 3 select peptides were different in six positions. BES 1 can stimulate the highest IgA secreting level in almost all organs except the colon. But when examining the stimulation level of IgG, it found that BES2 can stimulate the highest level of IgG secreting in 4 out of 6 organs (jejunum, ileum, caecum and colon). Therefore, in developing a PED vaccine, BES1 and BES2 that can stimulate high levels of IgA and IgG in mice should be taken to further test for the immune stimulation in the pig.

There are two major routes of vaccine transmission to pig; oral and intramuscular inoculation. Song et al. (51) compares route of transmission between oral and intramuscular vaccination of vero cell attenuated PEDV DR13 strain. They found that piglets from the oral inoculated sows' group have the lowest mortality rate at 13 % compared with 60% and 100% in IM inoculated and control group, respectively. This research concludes that oral inoculation induces higher IgA concentrations in colostrum than intramuscular inoculation. However, intramuscular inoculation is the suitable way for pigs. Because it can ensure that all pigs get an accurate dose for mucosal immunity activation. Decroix et al. (43) found that an intramuscular administration of amino acid PADRE-ELDKWA sequence induced a mucosal immune response to a conserved epitope of human immunodeficiency virus in mice. Then intramuscular vaccination may be used in the further test for the immune stimulation of BES1 and BES2 in pig or future PED vaccine.

CHAPTER VI
CONCLUSION

Porcine epidemic diarrhea virus (PEDV) causes acute diarrhea, vomiting, dehydration and high mortality in neonatal piglets. The disease was reported in the European and Asian over the last 30 years. High biosecurity to control PED should be practiced prior to expected epidemics. Concurrently, pregnant sows are immunized by either feedback or vaccination. However, the current feedback or vaccination protocols are frequently inefficient or unsafe, due to: 1) no standardized protocol for feedback; 2) poor capacities of current vaccines (live or killed) to induce lactogenic immunity; 3) antigenic differences of vaccine vs. Thailand epidemic strains; and 4) live vaccines may revert to virulent PEDV or recombine with field PEDV strains to generate new strains after they are applied in the field. Therefore, new vaccines are required for prevention and control of PED.

According to the advance in immunological and information technology, the new vaccine development method “reverse vaccinology” was proposed by Rino Rappuoli. This method can shorten vaccine development time and may discover new antigen. In this research, reverse vaccinology principle was used to search for new B cell epitopes of PED that can stimulate IgG and IgA level in mice. The process starts from finding complete genome of the Thailand PEDV from GENBANK and analyzed the genome by using bioinformatics tools into 4 major steps. Firstly, identification Open Reading Frame (ORF) by NCBI's ORF Finder. Then, iLoc-Virus was used to predict protein subcellular localization. Protein that are exposed on the surface were selected and further analyze for adhesin-like protein by using SPANN. Finally, B cell epitope were identified by several b cell epitope prediction tools, ABCpred (35), BCPred (36), COBEPro (37), EPMLR (38), FBCPred (39), LBtope (40) and SVMtrip (41). Three novel predicted epitopes, BES1(DNKTLGPTANNDVTT), BES2 (LITGTPKPPLEGV) and BES6 (NSSDPHL) were found. After that, they were synthesized, injected in mice and measured mucosal antibodies response. By using ELISA technique, we found that BES1 could stimulate highest IgA secreting level in almost organs except colon. While highest IgG secreting level in 4 out of 6 organs (jejunum, ileum, caecum and colon) was stimulated by BES2.

The combination of two candidated epitope should be further test for immune stimulation in pig. New effective PED vaccine to control the widely spread PED in Thailand may be found from this research.

APPENDIX

Appendix 1 Result of ORFs prediction from CBR1 strain PEDV complete genome by ORF Finder.

ORF No.	Position	
	Start	Stop
1	<1	12354
2	12478	12570
3	12919	13005
4	13087	13206
5	13354	13431
6	13624	13710
7	14068	14172
8	14467	14550
9	14563	14667
10	14713	14877
11	14878	14970
12	15034	15261
13	15298	15555
14	15775	15879
15	16063	16245
16	16453	16662
17	16705	16809
18	16831	16959
19	17008	17199
20	17554	17694
21	17716	17895
22	17974	18168
23	18232	18453
24	18505	18582
25	18685	18792
26	18871	19041
27	19246	19503
28	19729	19815
29	19831	19938
30	19978	20055
31	24499	25173
32	25495	25671
33	25930	26019
34	35	304
35	677	1045
36	1046	1402
37	1472	1621

ORF No.	Position	
	Start	Stop
38	1682	1915
39	2159	2245
40	2372	2545
41	2576	2947
42	2969	3049
43	3320	3433
44	3893	3994
45	4007	4105
46	4121	4219
47	4361	4507
48	4718	4846
49	4865	4948
50	4964	5041
51	5195	5467
52	5642	5782
53	6164	6286
54	6539	6703
55	6716	6892
56	7151	7252
57	7493	7609
58	7739	7816
59	7937	8041
60	8642	8728
61	8741	8920
62	9272	9520
63	9521	9670
64	9761	9841
65	9845	9952
66	10187	10297
67	10592	10693
68	10799	10942
69	10943	11113
70	11321	11455
71	11798	11875
72	12035	12172
73	12233	12313
74	20342	24499
75	24851	24934
76	25070	25147
77	25154	25384
78	25454	25588
79	26084	27409

ORF No.	Position	
	Start	Stop
80	12519	20345
81	20406	20816
82	20850	21221
83	21555	21665
84	21684	21821
85	21825	22145
86	22281	22394
87	22404	22481
88	22632	22712
89	22944	23129
90	23700	23834
91	23916	24038
92	25392	26072
93	26139	26477
94	26544	26732
95	26742	26957
96	27171	27275
97	27324	>27407
98	27325	27188
99	26305	26129
100	25783	25604
101	25465	25181
102	22552	22448
103	21826	21647
104	21628	21512
105	21151	20786
106	20608	20528
107	20305	20162
108	19987	19889
109	19717	19625
110	19315	19085
111	18859	18683
112	18676	18386
113	17881	17570
114	17110	16994
115	16798	16718
116	15751	15659
117	15406	15296
118	13981	13871
119	13204	12998
120	12403	12293
121	9019	8933
122	7198	7106

ORF No.	Position	
	Start	Stop
123	3559	3467
124	27288	27139
125	26916	26644
126	26445	26344
127	26265	26137
128	25239	25144
129	24387	24163
130	23550	23437
131	22932	22738
132	21993	21868
133	21201	21049
134	20802	20662
135	18789	18712
136	15714	15625
137	14598	14443
138	11430	11251
139	11250	10948
140	10188	10099
141	9696	9517
142	8775	8695
143	8586	8407
144	7767	7627
145	7509	7423
146	7362	7141
147	6654	6436
148	5514	5347
149	3771	3451
150	2223	2098
151	2052	1945
152	1752	1432
153	1134	829
154	756	544
155	27311	27204
156	27077	26985
157	26735	26505
158	25712	25578
159	19625	19542
160	19409	19224
161	18965	18864
162	18314	18213
163	17975	17835
164	17717	17622
165	17555	17412

ORF No.	Position	
	Start	Stop
166	17156	16956
167	16937	16800
168	16268	16074
169	15638	15540
170	14732	14598
171	13325	13125
172	12797	12717
173	12293	11997
174	11633	11430
175	11375	11151
176	10796	10596
177	10130	10008
178	9233	9153
179	8615	8529
180	7970	7869
181	7607	7284
182	7157	7005
183	6938	6852
184	6509	6390
185	5921	5841
186	4616	4482
187	2864	2736
188	2726	2631
189	2468	2370
190	2252	2037

Appendix 2 Score of possible epitopes predicted from spike protein (SK1*) by ABCPred

Rank	Sequence	Start position	Score
1	GELITGTPKPLEGVTD	625	0.96
1	PEVIPDYIDVNKTLDE	1238	0.96
2	MQYVYEPTYYMLNVTS	204	0.95
3	SFSEQAAYVDDDIVGV	701	0.92
3	DWSRVATKCYNSGGCA	188	0.92
4	VREIVITKYGDVYVNG	395	0.91
4	CGACFSGCCRGPRLQP	1360	0.91
5	ASLIGGMVLGGFTAAA	955	0.90
5	PGVVDAEKLHMYSASL	942	0.90
5	TIDLFGYPEFGSGVKF	600	0.90
5	AHMSEHSVVGITWDND	156	0.90
6	FEIGISQEPFDPSGYQ	91	0.89
6	FCCISTGCCGCCGCCG	1346	0.89
7	ADLVCAQYYSGVMVLP	927	0.88
8	KRSFIEDLLFNKVVTN	893	0.87
8	GVSVYDPASGRVVQKR	879	0.87
9	LATISSFNGDGYNFTN	861	0.86
9	TEYLQLYNTPVSVDCA	794	0.86
9	LQSVNDYLSFSKFCVS	576	0.86
9	PTSYGYGSKSQGSNCP	558	0.86
9	RILYCDDPVSQLKCSQ	460	0.86
9	IEVQGTAIQRILYCDD	451	0.86
9	CGCCGCCGACFSGCCR	1354	0.86
9	PVLSTLSLPQDVTRCS	12	0.86
10	TFAIPLGATQVPYYCF	359	0.85
10	AAVQRAPEALRFNIDD	310	0.85
11	PFDPSGYQLYLHKATN	99	0.84
11	SYAVQARLNYLALQTD	975	0.84
11	TPVSVDCATYVCNGNS	802	0.84
11	GQVKIAPTVTGNISIP	770	0.84
11	KFLAVLPPTVREIVIT	386	0.84
11	LLSNDSTLVHGKVVSN	261	0.84
12	VGVISSLSSSTFNSTR	714	0.83
12	GQHPTASGVHGIFLSH	69	0.83
12	SGVKFTSLYFQFTKGE	611	0.83
12	ENQGVNSTWYCAGQHP	57	0.83
13	CKTIESALQLSARPES	830	0.82
13	SKSQGSNCPFTLQSVN	565	0.82

Rank	Sequence	Start position	Score
13	NGHIPEGFSFNNWFLL	247	0.82
13	VVGITWDNDRVTVFSD	163	0.82
13	MYEPRKPTVGDFVQIE	1208	0.82
14	VTNGLGTVDEDYKRCS	906	0.81
14	HIRGGHGFEIGISQEP	84	0.81
14	SFLAGVYYTSDSGQLL	669	0.81
14	LEGVTDVSFMTLDVCT	635	0.81
14	TGHGTDGDVSGFWTIA	427	0.81
14	MPKIYGLGQFFSFNQT	287	0.81
14	TLGPTANNDVTTGRNC	133	0.81
14	DEIALTLREPGLVLFT	1176	0.81
14	VIAIAGLCVNDEIALT	1166	0.81
14	SSSIDDIYSRLDILSA	1064	0.81
15	TSFVTLPSFNDHSFVN	499	0.80
15	DVTRCSANTNFRRFFS	22	0.80
15	YGFCGGDGEHIFSLVQ	1130	0.80
15	AQTLTKYTEVQASRKL	1099	0.80
16	TVDEDYKRCSNGRSVA	912	0.79
16	DGYNFTNVLGVSVYDP	870	0.79
16	HGIFLSHIRGGHGFEI	78	0.79
16	NFSFVCSNSSDPHLTT	344	0.79
16	TGPSLSLNVFNATYLN	1262	0.79
16	LHTVLVPGDFVNVIAI	1154	0.79
16	ESVKEAISQTSKGLNT	1015	0.79
17	SGSIGYVPSQSGQVKI	759	0.78
17	AVYSVTPCSFSEQAAY	693	0.78
17	NITVSAAFGGHRGANL	514	0.78
17	DVSGFWTIASTNFVDA	434	0.78
17	AVTINFTGHGTDGDVS	421	0.78
17	NCIGYAANVFATEPNG	233	0.78
18	DVCTKYTIYGFKGEGI	647	0.77
18	TSLLASACTIDLFGYP	592	0.77
18	DGISYQPCTANCIGYA	223	0.77
18	GPRLQPYEAFEKVHVQ	1370	0.77
18	NSAIGNITSAFESVKE	1004	0.77
19	DTTINGFSSFRVDTRQ	533	0.76
19	TVFSDKIYHFYFKNDW	174	0.76
19	QDTATEYFVSSRRMYE	1195	0.76
20	SGVMVLPGVVDAEKLH	936	0.75
20	FSMSIRTEYLQLYNTP	788	0.75
20	ANLIASDTTINGFSSF	527	0.75

Rank	Sequence	Start position	Score
20	IVLHTALGTNFSFVCS	335	0.75
20	IVLHTALGTNFSFVCS	335	0.75
20	KCYNSGGCAMQYVYEP	195	0.75
20	VDLEWFNRVETYIKWP	1311	0.75
20	HRSESLRNTTEELQSL	1287	0.75
20	ARLRICQFPDNKTLGP	121	0.75
20	TKVQEVVNSQGAALTQ	1036	0.75
20	SQTSKGLNTVAHALTK	1022	0.75
21	GFTAAAALPFSYAVQA	965	0.74
21	LSARPESAEVNSMLTI	839	0.74
21	PGFFYHSNDGSNCTEP	732	0.74
21	AFDLDDGFYPISSTNL	477	0.74
21	TGEIADLKHRSESLRN	1279	0.74
21	VKSQSQRYGFCGGDGE	1123	0.74
21	TEVQASRKLAQQKVNE	1106	0.74
21	YQLYLHKATNGNTNAT	105	0.74
22	SRCKQLLTQYTAACKT	817	0.73
22	GNISIPTNFSMSIRTE	780	0.73
22	VLGGYLPIGENQGVNS	48	0.73
22	CLFNKAIPAHMSEHSV	148	0.73
22	FVQIESCVVTYVNLTR	1219	0.73
22	TGRLSALNAFVAQTLT	1088	0.73
22	DVQVDRLITGRLSALN	1080	0.73
23	KRCSNGRSVADLVCAQ	918	0.72
23	DTRQFTISRFYNVPTS	545	0.72
23	SQLKCSQVAFDLDDGF	469	0.72
24	NCTEPVLVYSNIGVCK	743	0.71
24	KGEGIITLTNSSFLAG	658	0.71
24	SKFCVSTSLLASACTI	586	0.71
24	FYPISSTNLLSHEQPT	484	0.71
25	ATYVCNGNSRCKQLLT	809	0.70
25	YTSDSGQLLAFKNVTS	676	0.70
25	YYCFPKVDTYNSTVYK	371	0.70
25	RFNIDDTAVILAEGSI	320	0.70
25	VETYIKWPWWVWLIIF	1319	0.70
25	NTTEELQSLIYNINNT	1294	0.70
25	EILASLPNRTGPSLSL	1253	0.70
26	FNQTIDGVCNGAAVQR	299	0.68
26	MLNVTSAGKDGISYQP	214	0.68
27	LSHEQPTSFVTLPSFN	493	0.67

Rank	Sequence	Start position	Score
28	SGRVVQKRSFIEDLLF	887	0.65
28	QLLAFKNVTSGAVYSV	682	0.65
28	FSLVQAAPQGLLFLHT	1141	0.65
28	QGAALTQLTVQLQHNF	1045	0.65
28	MKSLTYFWLFLPVLST	1	0.65
29	DTYNSTVYKFLAVLPP	378	0.64
29	VVSNQPLLVNCLLAMP	273	0.64
30	TQYTAACKTIESALQL	824	0.63
30	RKLAQQKVNECVKSQS	1112	0.63
31	YVNGFGYLHLGLLDAV	407	0.62
32	YLALQTDVLQRNQQLL	984	0.61
32	NGNTNATARLRICQFP	114	0.61
33	DVTTGRNCLFNKAIPA	141	0.59
34	LNTVAHALTKVQEVVN	1028	0.58
35	QLLAESFNSAIGNITS	997	0.57
36	RLDILSADVQVDRLIT	1073	0.55
37	MLTISEEALQLATISS	851	0.54
38	SAEVNSMLTISEEALQ	845	0.53
38	NVQAPAVVVLGGYLPI	40	0.53
38	TNFRRFFSKFNVQAPA	30	0.53
38	QLTVQLQHNFQAISSS	1051	0.53
39	TIASTNFVDALIEVQG	440	0.51

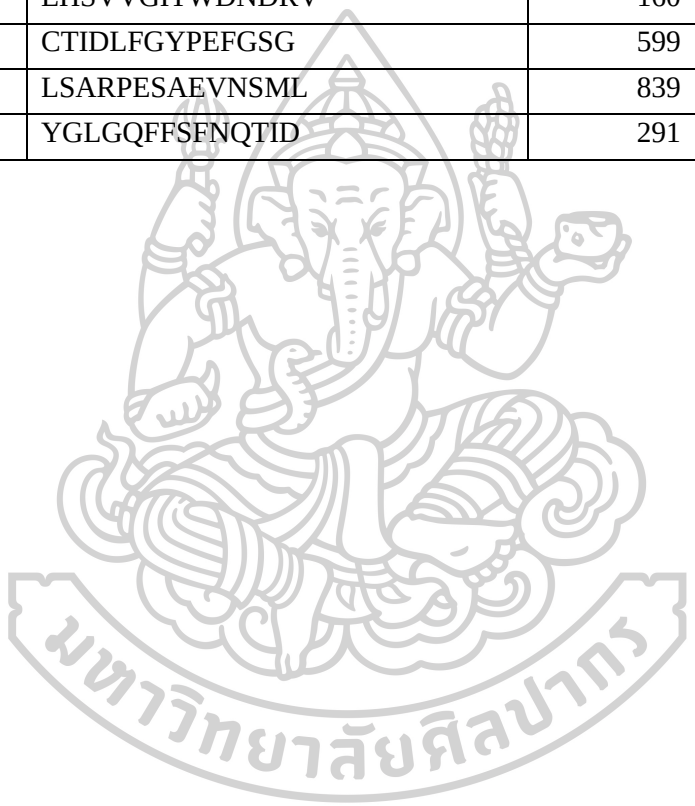
Appendix 3 Score of possible epitopes predicted from hypothetical protein (S8*) by ABCPred.

Rank	Sequence	Start position	Score
1	TGYETCYCYYYCLLCS	15	0.89
2	SEKLHDPGYFHDSVNY	30	0.87
3	TGCCLCYYSGIWTCCC	48	0.85
4	IWTCCCHCHDSCYVNL	58	0.84
5	HDSVNYRGTGCCLCYY	40	0.79
6	YCYYYCLLCSEKLHDP	21	0.78

Appendix 4 Score of possible epitopes predicted from spike protein (SK1*) by FBCPred.

Rank	Sequence	Start position	Score
1	HSNDGSNCTEPVLV	737	1.000
2	GFKGEGIITLTNSS	656	1.000
3	SQSQRYGFCGGDGE	1125	1.000
4	GPTANNDVTTGRNC	135	1.000
5	YLHKATNGNTNATA	108	1.000
6	TISSFNGDGYNFTN	863	1.000
7	GVSVYDPASGRVVQ	879	0.999
8	GGCAMQYVYEPTYY	200	0.999
9	RCSANTNFRRFFSK	25	0.998
10	TSAFESVKEAISQT	1011	0.996
11	LITGTPKPLEGVTD	627	0.996
12	GYGSKSQGSNCPFT	562	0.996
13	FCCISTGCCGCCGC	1346	0.994
14	ASDTTINGFSSFRV	531	0.994
15	PEVIPDYIDVNKTL	1238	0.994
16	YCFPKVDTYNSTVY	372	0.991
17	CFSGCCRGPRLQPY	1363	0.990
18	GTNFSFVCSNSSDP	342	0.990
19	TVDEDYKRCSNGRS	912	0.985
20	KNVTSGAVYSVTPC	687	0.982
21	GFWTIASTNFVDAL	437	0.976
22	SDKIYHFYFKNDWS	177	0.973
23	NVTSAGKDGISYQP	216	0.965
24	DLVCAQYYSGVMVL	928	0.963
25	CKSGSIGYVPSQSG	757	0.958
26	PTSFVTLPSFNDHS	498	0.953
27	VLPPTVREIVITKY	390	0.949
28	SHIRGGHGFEIGIS	83	0.937
29	YNINNTLVDLEWFN	1304	0.935
30	ASLPNRTGPSLSLN	1256	0.922
31	PVSQLKCSQVAFDL	467	0.917
32	TWYCAGQHPTASGV	64	0.910
33	SSSTFNSTRELPGF	721	0.908
34	YLQLYNTPVSVDCA	796	0.880

Rank	Sequence	Start position	Score
35	IAPTVTGNISIPTN	774	0.846
36	TGEIADLKHRSESL	1279	0.843
37	SRRMYEPRKPTVGD	1205	0.831
38	HGKVVSNQPLLVNC	270	0.808
39	VTINFTGHGTDGDV	422	0.798
40	VETYIKWPWWVWLI	1319	0.780
41	LAGVYYTSDSGQLL	671	0.766
42	FATEPNGHIPEGFS	242	0.753
43	EHSVVGITWDNDRV	160	0.714
44	CTIDLFGYPEFGSG	599	0.689
45	LSARPESAEVNSML	839	0.688
46	YGLGQFFSFNQTID	291	0.687



Appendix 5 Score of possible epitopes predicted from spike protein (SK1*) by COBEpro.

Rank	Sequence	Start position	Score
1	GKDGISY	220	0.844
2	PTVGDF	1213	0.836
3	LREPGL	1181	0.833
4	GDGYNF	868	0.819
5	SKGLNT	1024	0.813
6	PYEAFE	1374	0.811
7	QAPAVV	41	0.806
8	PTVTGN	775	0.804
9	QPYEAFE	1373	0.802
10	PCTANC	228	0.795
11	SGQVKI	768	0.788
12	VTTGRNC	141	0.786
13	GNSRCKQ	814	0.784
14	GQVKIA	769	0.784
15	NGDGYNF	867	0.783
16	MSEHSV	157	0.782
17	TGRNCL	143	0.782
18	SNGRSVA	920	0.780
19	GHRGAN	522	0.777
20	TSKGLN	1023	0.774
21	KPTVGDF	1212	0.773
22	TVTGNI	776	0.770
23	GGHRGAN	521	0.769
24	RPESAE	841	0.768
25	ELPGFF	729	0.767
26	PPTVRE	391	0.766
27	YEAFEK	1375	0.765
28	AGKDGIS	219	0.764
29	KDGISYQ	221	0.761
30	HMSEHS	156	0.759
31	KIAPTV	772	0.759
32	FDPSGYQ	99	0.759
33	NGNSRCK	813	0.758
34	NGRSVA	921	0.753

Rank	Sequence	Start position	Score
35	GHRGANL	522	0.753
36	IAPTVT	773	0.751
37	PASGRVVQ	884	0.751
38	QSGQVK	767	0.749
39	QLATIS	859	0.748
40	DPSGYQ	100	0.748
41	NVQAPAV	39	0.744
42	SFNDHSF	505	0.740
43	TTGRNCLF	142	0.734
44	FNKAIP	149	0.734
45	APTVTGN	774	0.732
46	RCSNGRS	918	0.732
47	VKIAPTV	771	0.732
48	QPCTANC	227	0.732
49	GGHRGANL	521	0.732
50	CSNGRSV	919	0.730
51	PKIYGL	287	0.729
52	SFNGDG	865	0.728
53	TATEYF	1196	0.728
54	AAFGGHR	518	0.722
55	TLREPG	1180	0.721
56	FGGHRGAN	520	0.721
57	CNGNSRC	812	0.719
58	GENQGVNS	55	0.718
59	QPYEAFEK	1373	0.717
60	DTATEY	1195	0.716
61	LQLATIS	858	0.716
62	VTTGRNCL	141	0.715
63	QVKIAP	770	0.714
64	RCSNGR	918	0.713
65	VTGNIS	777	0.712
66	LATISS	860	0.710
67	VPGDFV	1158	0.707
68	RELPGFF	728	0.707
69	KDGIS	221	0.707
70	PASGRVVQK	884	0.706
71	IPTNFS	783	0.705

Rank	Sequence	Start position	Score
72	DVTTGRN	140	0.704
73	ASGRVVQK	885	0.704
74	HRGANL	523	0.702
75	IAPTVTG	773	0.700
76	SQSGQVK	766	0.699
77	TVTGNIS	776	0.699
78	RKPTVGDF	1211	0.697
79	GTPKPL	629	0.697
80	GRNCLF	144	0.696
81	KHRSESLR	1285	0.696
82	PSFNDHSF	504	0.694
83	NGRSVAD	921	0.693
84	FDPSGY	99	0.693
85	FNDHSF	506	0.693
86	KPTVGD	1212	0.693
87	SGQVKIAP	768	0.693
88	LQPYEAF	1372	0.691
89	RLQPYEA	1371	0.690
90	EQPTSF	495	0.690
91	NSRCKQL	815	0.689
92	ENQGVNS	56	0.688
93	NQGVNST	57	0.688
94	QTSKGLN	1022	0.688
95	QSGQVKIA	767	0.687
96	VLPPTVR	389	0.686
97	KFNVQAP	37	0.686
98	LPNRTG	1257	0.685
99	GNSRCKQL	814	0.685
100	AFGGHRGA	519	0.685
101	GVTDVSF	636	0.685
102	NVQAPAVV	39	0.685
103	AHMSEHS	155	0.685
104	ISTGCC	1348	0.685
105	QFPDNK	126	0.684
106	GDGYNFT	868	0.684
107	VCKSGS	755	0.683
108	APTVTGNI	774	0.682

Rank	Sequence	Start position	Score
109	AGKDGISYQ	219	0.682
110	KIAPTVTG	772	0.681
111	ARPESA	840	0.680
112	LFNKAI	148	0.680
113	IGENQGV	54	0.679
114	ESAEVNS	843	0.679
115	NDEIAL	1174	0.678
116	TVGDFV	1214	0.678
117	PESAEV	842	0.677
118	FNKAIPA	149	0.677
119	SARPESA	839	0.676
120	QGVNST	58	0.674
121	EPFDPSG	97	0.673
122	GQVKI	769	0.673
123	YEAFE	1375	0.673
124	VNLTRDQ	1229	0.672
125	LPGFFY	730	0.671
126	LKHRSESL	1284	0.671
127	PTNFSM	784	0.671
128	CRGPRL	1367	0.670
129	QLATISS	859	0.669
130	DGVCNGA	303	0.668
131	KNVTSG	686	0.667
132	FNVQAPAV	38	0.667
133	LTRDQLP	1231	0.667
134	SKFNVQA	36	0.666
135	QAPAV	41	0.666
136	PPTVR	391	0.666
137	SLPNRT	1256	0.666
138	TVTGN	776	0.665
139	GNTNATA	114	0.664
140	NLTRDQ	1230	0.664
141	SSFNGD	864	0.663
142	LSARPES	838	0.663
143	YNTPVS	799	0.663
144	TIDGVC	301	0.662
145	DGYNFT	869	0.662

Rank	Sequence	Start position	Score
146	GKDGISYQP	220	0.661
147	NGDGYNFT	867	0.660
148	NGNTNAT	113	0.660
149	TGRNCLFN	143	0.659
150	PGDFVN	1159	0.659
151	NGNSRCKQ	813	0.658
152	AEGSIV	330	0.658
153	ATISSF	861	0.657
154	TINGFS	534	0.657
155	FNGDGYNF	866	0.657
156	CKSGSI	756	0.656
157	LREPGLV	1181	0.655
158	SLPQDV	17	0.654
159	DLKHRSES	1283	0.654
160	TNGNTNAT	112	0.651
161	VDDDIV	708	0.651
162	MSEHS	157	0.651
163	PTVTGNIS	775	0.650
164	SNGRSV	920	0.650
165	YVDDDI	707	0.650
166	TTGRNC	142	0.648
167	PSQSGQVK	765	0.648
168	ADLKHRSES	1282	0.646
169	FKNVTSG	685	0.646
170	DPSGYQL	100	0.645
171	NTPVSV	800	0.644
172	GVCNGA	304	0.644
173	KRCSNG	917	0.644
174	CISTGC	1347	0.643
175	NTNATA	115	0.640
176	TPKPLE	630	0.639
177	FSKFNVQ	35	0.638
178	QAAPQGL	1144	0.638
179	PYEAFEKV	1374	0.637
180	LPQDVT	18	0.637
181	LVQAAPQ	1142	0.636
182	VQAAPQGL	1143	0.635

Rank	Sequence	Start position	Score
183	TGRNCLFN	143	0.659
184	PGDFVN	1159	0.659
185	NGNSRCKQ	813	0.658
186	AEGSIV	330	0.658
187	PFDPSGYQ	98	0.635
188	LQSVNDYL	575	0.634
189	EGVTDV	635	0.634
190	EAFEK	1376	0.634
191	PQDVTR	19	0.634
192	QLSARP	837	0.634
193	PRLQPY	1370	0.634
194	PKPLEGV	631	0.633
195	DGISYQ	222	0.632
196	ELPGFFY	729	0.632
197	PNRTGP	1258	0.631
198	YQPCTAN	226	0.631
199	ASLPNR	1255	0.631
200	GRVVQKR	887	0.631
201	NVTSGAV	687	0.630
202	VTDVSF	637	0.629
203	RGPRLQ	1368	0.629
204	RPESAEV	841	0.629
205	VCNGNS	811	0.629
206	KSQSQR	1123	0.628
207	TEPNGH	243	0.626
208	VTSGAVY	688	0.626
209	SGRVVQKR	886	0.626
210	PEGFSFNNW	250	0.626
211	TRDQLPE	1232	0.625
212	PSQSGQV	765	0.624
213	EIADLKHR	1280	0.624
214	YDPASGRV	882	0.623
215	HEQPTS	494	0.623
216	TSKGLNTV	1023	0.623
217	PRKPTVGDF	1210	0.622
218	TRELPGFF	727	0.622
219	KIYGLG	288	0.621

Rank	Sequence	Start position	Score
220	PNRTGPS	1258	0.620
221	PFDPSGYQ	98	0.635
222	LQSVNDYL	575	0.634
223	EGVTDV	635	0.634
224	EAFEK	1376	0.634
225	PQDVTR	19	0.634
226	QLSARP	837	0.634
227	PRLQPY	1370	0.634
228	PKPLEGV	631	0.633
229	DGISYQ	222	0.632
230	ELPGFFY	729	0.632
231	PNRTGP	1258	0.631
232	YQPCTAN	226	0.631
233	ASLPNR	1255	0.631
234	GRVVQKR	887	0.631
235	NVTSGAV	687	0.630
236	VTDVSF	637	0.629
237	RGPRLQ	1368	0.629
238	RPESAEV	841	0.629
239	VCNGNS	811	0.629
240	KSQSQR	1123	0.628
241	TEPNGH	243	0.626
242	VTSGAVY	688	0.626
243	SGRVVQKR	886	0.626
244	PEGFSFNNW	250	0.626
245	TRDQLPE	1232	0.625
246	PSQSGQV	765	0.624
247	EIADLKHR	1280	0.624
248	YDPASGRV	882	0.623
249	HEQPTS	494	0.623
250	TSKGLNTV	1023	0.623
251	PRKPTVGDF	1210	0.622
252	TRELPGFF	727	0.622
253	KIYGLG	288	0.621
254	PNRTGPS	1258	0.620
255	HRSESLRN	1286	0.620
256	DPASGRVV	883	0.618

Rank	Sequence	Start position	Score
257	SQTSKGLN	1021	0.618
258	VNDEIAL	1173	0.617
259	TTINGFS	533	0.617
260	NQTIDGV	299	0.617
261	VKSQSQR	1122	0.616
262	LPPTVRE	390	0.616
263	SFNDHSFV	505	0.614
264	GPRLQP	1369	0.614
265	PLEGVTD	633	0.613
266	YKRCSN	916	0.613
267	QTIDGV	300	0.611
268	IDGVCNGA	302	0.611
269	KPLEGVT	632	0.610
270	FNKVVT	901	0.609
271	NRTGPS	1259	0.608
272	PTVRE	392	0.608
273	TFNSTRE	723	0.607
274	GEIADLKH	1279	0.607
275	NSTRELP	725	0.605
276	QDVTRCS	20	0.605
277	LPSFNDHSF	503	0.605
278	STRELPG	726	0.605
279	PSGYQL	101	0.604
280	QGSNCPFT	567	0.604
281	SQSQRYG	1124	0.604
282	FNSTREL	724	0.604
283	SAGKDGISY	218	0.603
284	RVVQKR	888	0.603
285	REPGLV	1182	0.602
286	FATEPNG	241	0.602
287	IADLKHRSE	1281	0.602
288	LSLPQD	16	0.601
289	TSGAVY	689	0.599
290	CCRGPR	1366	0.599
291	ISQTSKG	1020	0.597
292	STFNSTR	722	0.597
293	SAAFGGHR	517	0.597

Rank	Sequence	Start position	Score
294	FPDNKTL	127	0.596
295	LASLPN	1254	0.596
296	PTVGDFVQ	1213	0.596
297	TGNISI	778	0.595
298	SNCPFTLQS	569	0.594
299	RDQLPE	1233	0.593
300	EPNGHI	244	0.593
301	SSTFNST	721	0.592
302	LEGVTD	634	0.591
303	NCPFTLQS	570	0.591
304	PAHMSEHS	154	0.591
305	QSQRYGF	1125	0.590
306	PCTANCI	228	0.590
307	SQGSNCPF	566	0.590
308	MPKIYG	286	0.590
309	RSESLRNT	1287	0.589
310	VTSAGKDG	216	0.588
311	TGEIADLK	1278	0.588
312	PIGENQGV	53	0.588
313	GSNCPFTLQS	568	0.586
314	ATNGNTNAT	111	0.585
315	NKAIPA	150	0.585
316	PFTLQSV	572	0.585
317	IPEGFSFNNW	249	0.584
318	ISSFNGD	863	0.584
319	DTTINGF	532	0.583
320	LAEGSIV	329	0.583
321	LLSHEQP	491	0.583
322	KSQGSNC	565	0.582
323	CPFTLQSV	571	0.582
324	DVTRCS	21	0.580
325	NDVTTGRN	139	0.578
326	GHRGANLI	522	0.578
327	PDNKTL	128	0.578
328	TISSFN	862	0.578
329	TLTNSS	663	0.577
330	VPSQSGQV	764	0.575

Rank	Sequence	Start position	Score
331	TGTPKPL	628	0.574
332	SKSQGSN	564	0.573
333	ITLTNSS	662	0.573
334	SYQPCTAN	225	0.573
335	LHKATNG	108	0.571
336	QDTATE	1194	0.571
337	SQRYGF	1126	0.571
338	NKVVTN	902	0.571
339	LTLREPG	1179	0.570
340	YVCNGNS	810	0.570
341	NCPFTLQSV	570	0.569
342	GCCRGPR	1365	0.568
343	LQLSAR	836	0.568
344	HIPEGFSFNNW	248	0.568
345	SDTTING	531	0.566
346	ASDTTIN	530	0.565
347	LPEVIP	1236	0.564
348	GFCGGDGEH	1130	0.563
349	SEHSV	158	0.561
350	VTRCSA	22	0.560
351	VVDAEK	943	0.559
352	SAEVNSM	844	0.559
353	APAVV	42	0.559
354	LVPGDF	1157	0.558
355	LSHEQPT	492	0.558
356	SHEQPTS	493	0.558
357	VQAPAVVV	40	0.558
358	YGFCGGDGEH	1129	0.556
359	EGFSFNNW	251	0.556
360	HKATNGN	109	0.556
361	RYGFCGGDGEH	1128	0.556
362	GDFVNV	1160	0.555
363	QLPEVI	1235	0.554
364	DQLPEV	1234	0.553
365	SKGLNTVA	1024	0.553
366	VAFDLDDG	475	0.552
367	SSSIDDI	1063	0.552

Rank	Sequence	Start position	Score
368	FCGGDGEH	1131	0.552
369	CLFNKA	147	0.552
370	TPVSVDC	801	0.552
371	AFEKVHV	1377	0.551
372	LSNDST	261	0.550
373	FEKVHVQ	1378	0.550
374	CGGDGEHI	1132	0.550
375	LTGEIADLK	1277	0.550
376	QSVNDYL	576	0.549
377	LYNTPV	798	0.549
378	AFDLDDG	476	0.548
379	EPRKPTVGDFV	1209	0.546
380	VDAEK	944	0.546
381	CQFPDN	125	0.545
382	CDDPVSQL	463	0.542
383	VYDPASGRVV	881	0.542
384	ALQLATIS	857	0.542
385	QPTSF	496	0.541
386	CCISTGC	1346	0.540
387	AYVDDDI	706	0.540
388	RTGPSLSL	1260	0.539
389	TSAGKDG	217	0.539
390	HMSEHSVV	156	0.538
391	VFATEPNGH	240	0.538
392	LGGYLPIGENQG	48	0.537
393	QEPFDPSG	96	0.536
394	FTLQSV	573	0.536
395	DDPVSQL	464	0.535
396	KATNGNTNAT	110	0.535
397	SIPTNF	782	0.535

Appendix 6 Score of possible epitopes predicted from hypothetical protein (S8*) by COBEpro.

Rank	Sequence	Start position	Score
1	CHDSCY	64	0.844
2	LHDPGY	32	0.836
3	EKLHDP	30	0.833
4	KLHDPG	31	0.819
5	SEKLHDP	29	0.813
6	HDSCYV	65	0.811
7	CHCHDSCY	62	0.806
8	MSFHHGH	0	0.804
9	VNYRGTGC	42	0.802
10	CCHCHDSC	61	0.795
11	DSVNYRGTGC	40	0.788
12	NYRGTGC	43	0.786
13	SVNYRGTGC	41	0.784
14	HDPGYF	33	0.784
15	FHHGHL	2	0.783
16	HDSVNYRGTGC	39	0.782
17	CCCHCHDSC	60	0.782
18	LLCSEKLHDPGYFHDSVN	26	0.780
19	LCSEKLHDPGYFHDSVNY	27	0.777
20	CLLCSEKLHDPGYFHDSV	25	0.774
21	CSEKLHDPGYFHDSVNYR	28	0.773

Appendix 7 Score of possible epitopes predicted from spike protein (SK1*) by LBtope.

Rank	Sequence	Score
1	NTLVDLEWFNRVETY	1.36
2	NSSDPHLTTFAIPLG	1.06
3	SLIYNINNTLVDLEW	1.06
4	LLSHEQPTSFVTLPS	1.06
5	NLLSHEQPTSFVTLP	1.02
6	QSLIYNINNTLVDLE	1.02
7	LIYNINNTLVDLEWF	1.00
8	INNTLVDLEWFNRVE	0.99
9	YVNLTRDQLPEVIPD	0.94
10	SSDPHLTTFAIPLGA	0.92
11	GGCAMQYVYEPTYYM	0.92
12	TYVNLTRDQLPEVIP	0.92
13	TNLLSHEQPTSFVTL	0.91
14	VVQKRSFIEDLLFNK	0.91
15	VVTYVNLTRDQLPEV	0.91
16	IYNINNTLVDLEWFN	0.91
17	SRRMYEPRKPTVGDF	0.90
18	QVPYYCFPKVDTYNS	0.89
19	YPISSTNLLSHEQPT	0.89
20	YVPSQSGQVKIAPTV	0.88
21	EQPTSFVTLPSFNDH	0.88
22	DLFGYPEFGSGVKFT	0.88
23	SNSSDPHLTTFAIPL	0.87
24	HEQPTSFVTLPSFND	0.87
25	RVVQKRSFIEDLLFN	0.87
26	TTGRNCLFNKAIPAH	0.87
27	VTYVNLTRDQLPEVI	0.86
28	VNLTRDQLPEVIPDY	0.86
29	FAIPLGATQVPYYCF	0.86
30	SDPHLTTFAIPLGAT	0.86
31	PSQSGQVKIAPTVTG	0.85
32	LSHEQPTSFVTLPSF	0.85
33	VPSQSGQVKIAPTVT	0.84
34	QKRSFIEDLLFNKVV	0.83

Rank	Sequence	Score
35	LQSLIYNINNTLVDL	0.83
36	SGGCAMQYVYEPTYY	0.83
37	TLPSFNDHSFVNITV	0.83
38	LVDLEWFNRVETYIK	0.82
39	GVNSTWYCAGQHPTA	0.81
40	SSRRMYEPRKPTVGD	0.81
41	PKPLEGVTDVSFMTL	0.80
42	VQKRSFIEDLLFNKV	0.80
43	TFAIPLGATQVPYYC	0.80
44	NKTLGPTANNDVTTG	0.80
45	SHEQPTSFVTLPSFN	0.79
46	VNSTWYCAGQHPTAS	0.79
47	TTFAIPLGATQVPYY	0.79
48	ASGRVVQKRSFIEDL	0.79
49	QPTSFVTLPSFNDHS	0.78
50	GRVVQKRSFIEDLLF	0.77
51	KGELITGTPKPLEGV	0.77
52	KRSFIEDLLFNKVVT	0.76
53	PYYCFPKVDTYNSTV	0.76
54	GYVPSQSGQVKIAPT	0.75
55	FVSSRRMYEPRKPTV	0.75
56	VSSRRMYEPRKPTVG	0.75
57	FNKVVTNGLGTVDED	0.75
58	RRMYEPRKPTVGDFV	0.75
59	VPYYCFPKVDTYNST	0.75
60	GVCKSGSIGYVPSQS	0.74
61	CTIDLFGYPEFGSGV	0.73
62	STNLLSHEQPTSFVT	0.73
63	CFSGCCRGPRLQPYE	0.73
64	AIPLGATQVPYYCFP	0.72
65	QGVNSTWYCAGQHPT	0.72
66	GELITGTPKPLEGVT	0.72
67	RSFIEDLLFNKVVTN	0.72
68	IDLFGYPEFGSGVKF	0.72
69	GCAMQYVYEPTYYML	0.72
70	QPYEAFEKVHVQXXX	0.71
71	STWYCAGQHPTASGV	0.71

Rank	Sequence	Score
72	TIDLFGYPEFGSGVK	0.71
73	SGRVVQKRSFIEDLL	0.70
74	FSGCCRGPRLQPYEA	0.70
75	NLTRDQLPEVIPDYI	0.69
76	VTLPSFNDHSFVNIT	0.69
77	DNKTLGPTANNDVTT	0.69
78	NKTLDEILASLPNRT	0.69
79	EPNGHIPEGFSFNNW	0.68
80	TKGELITGTPKPLEG	0.68
81	KPLEGVTDVSFMTLD	0.68
82	TRDQLPEVIPDYIDV	0.68
83	TLGPTANNDVTTGRN	0.67
84	NSTWYCAGQHPTASG	0.67
85	HSVVGITWDNDRVTV	0.67
86	FTKGELITGTPKPLE	0.67
87	PDNKTLGPTANNDVT	0.66
88	CKSGSIGYVPSQSGQ	0.66
89	VTTGRNCLFNKAIPA	0.66
90	TPKPLEGVTDVSFMT	0.66
91	TGRNCLFNKAIPAHM	0.66
92	GSIGYVPSQSGQVKI	0.66
93	SFMTLDVCTKYTIYG	0.65
94	SGCCRGPRLQPYEAF	0.65
95	TLDEILASLPNRTGP	0.65
96	IPLGATQVPYYCFPK	0.65
97	NKVVTNGLGTVDEDY	0.65
98	GITWDNDRVTVFSDK	0.64
99	GCCRGPRLQPYEAFE	0.64
100	VGITWDNDRVTVFSD	0.64
101	CVVTYVNLTRDQLPE	0.64
102	IEDLLFNKVVTNGLG	0.64
103	CRGPRLQPYEAFEKV	0.63
104	PEVIPDYIDVNKTLD	0.63
105	SSTNLLSHEQPTSFV	0.63
106	SQSGQVKIAPTVTGN	0.62
107	DVTTGRNCLFNKAIP	0.62
108	PSFNDHSFVNITVSA	0.62

Rank	Sequence	Score
109	IESCVVTYVNLTRDQ	0.62
110	PASGRVVQKRSFIED	0.62
111	YEPRKPTVGDFVQIE	0.62
112	SGSIGYVPSQSGQVK	0.61
113	FMTLDVCTKYTIYGF	0.61
114	LFGYPEFGSGVKFTS	0.61
115	PYEAFEKVHVQXXXX	0.61
116	FYPISSTNLLSHEQP	0.61
117	ITWDNDRVTVFSDKI	0.60
118	TQVPYYCFPKVDTYN	0.60
119	FVDALIEVQGTAIQR	0.60
120	DHSFVNITVSAAFGG	0.60
121	WDNDRVTVFSDKIYH	0.60
122	NDHSFVNITVSAAFG	0.59
123	QTLTKYTEVQASRKL	0.59
124	CTEPVLVYSNIGVCK	0.59
125	ELQSLIYNINNTLVD	0.59
126	NQGVNSTWYCAGQHP	0.58
127	VAQTLTKYTEVQASR	0.58
128	LPEVIPDYIDVNKTL	0.58
129	EPTYYMLNVTSAGKD	0.57
130	QYVYEPTYYMLNVTS	0.57
131	CCRGPRLQPYEAFEK	0.57
132	LPSFNDHSFVNITVS	0.57
133	DQLPEVIPDYIDVNK	0.57
134	VDLEWFNRVETYIKW	0.56
135	ELITGTPKPLEGVTD	0.56
136	IGVCKSGSIGYVPSQ	0.56
137	VVTNGLGTVDEDYKR	0.56
138	AAVQRAPEALRFNID	0.56
139	NDVTTGRNCLFNKAI	0.56
140	TLTKYTEVQASRKLA	0.56
141	FNDHSFVNITVSAAF	0.56
142	DNDRVTVFSDKIYHF	0.56
143	YEPTYYMLNVTSAGK	0.55
144	LTRDQLPEVIPDYID	0.55
145	ACTIDLFGYPEFGSG	0.55

Rank	Sequence	Score
146	PLGATQVPYYCFPKV	0.55
147	SFNNWFLLSNDSTLV	0.55
148	RMYEPRKPTVGDFVQ	0.55
149	VLVYSNIGVCKSGSI	0.55
150	PLEGVTDVSFMTLDV	0.55
151	TLVHGKVVSNQPLLV	0.55
152	YHSNDGSNCTEPVLV	0.55
153	KTLDEILASLPNRTG	0.55
154	KSGSIGYVPSQSGQV	0.55
155	QFTKGELITGTPKPL	0.55
156	GAAVQRAPEALRFNI	0.54
157	AQQKVNECVKSQSQR	0.54
158	YVYEPTYYMLNVTSA	0.54
159	SFNDHSFVNITVSAA	0.54
160	YFVSSRRMYEPRKPT	0.54
161	NFVDALIEVQGTAIQ	0.54
162	RDQLPEVIPDYIDVN	0.54
163	DPVSQLKCSQVAFDL	0.54
164	EDLLFNKVVTNGLGT	0.54
165	LREPGLVLFTHELQD	0.53
166	LDEILASLPNRTGPS	0.53
167	LGPTANNDVTTGRNC	0.53
168	FVTLPSFNDHSFVNI	0.53
169	VNKTLDEILASLPNR	0.53
170	MYEPRKPTVGDFVQI	0.53
171	EHSVVGITWDNDRVT	0.53
172	KTLGPTANNDVTTGR	0.53
173	ARLRICQFPDNKTLG	0.53
174	SACTIDLFGYPEFGS	0.53
175	AQTLTKYTEVQASRK	0.52
176	TEPNGHIPEGFSFNN	0.52
177	ISSTNLLSHEQPTSF	0.52
178	QQKVNECVKSQSQRY	0.52
179	SQEPFDPSGYQLYLH	0.51
180	CAMQYVYEPTYYMLN	0.51
181	KVVTNGLGTVDEDYK	0.51
182	AMQYVYEPTYYMLNV	0.51

Rank	Sequence	Score
183	AHALTKVQEVVNSQG	0.51
184	EVIPDYIDVNKTLDE	0.51
185	EPGLVLFTHELQDTA	0.50
186	YSVTPCSFSEQAAYV	0.50
187	GKDGISYQPCTANCI	0.50
188	YHFYFKNDWSRVATK	0.50

Appendix 8 Score of possible epitopes predicted from spike protein (SK1*) by EPMLR

Rank	Sequence	Score
1	VGITWDNDRVTVFSD	1.00
2	VVGITWDNDRVTVFS	1.00
3	GCCGACFSGCCRGPR	1.00
4	CGCCGACFSGCCRGP	1.00
5	DGVCNGAAVQRAPEA	1.00
6	ATEYFVSSRRMYEPR	1.00
7	CVKSQSQRYGFCGGD	1.00
8	TATEYFVSSRRMYEP	1.00
9	KCYNSGGCAMQYVYE	1.00
10	YKRCSNGRSVADLVC	1.00
11	IDGVCNGAAVQRAPE	1.00
12	RCSANTNFRRFFSKF	1.00
13	VSVYDPASGRVVQKR	1.00
14	QGVNSTWYCAGQHPT	1.00
15	SFRVDTRQFTISRFY	1.00
16	SHEQPTSFVTLPSFN	1.00
17	ECVKSQSQRYGFCGG	1.00
18	TKCYNSGGCAMQYVY	1.00
19	GAVYSVTPCSFSEQA	1.00
20	GVSVYDPASGRVVQK	1.00
21	NQGVNSTWYCAGQHP	1.00
22	CVVTYVNLTRDQLPE	1.00
23	SSFRVDTRQFTISRF	1.00
24	LSHEQPTSFVTLPSF	1.00
25	SCVVTYVNLTRDQLP	1.00
26	GVHGIFLSHIRGGHG	1.00
27	ATNGNTNATARLRIC	0.93
28	TRCSANTNFRRFFSK	0.90
29	LPFSYAVQARLNYLA	0.88
30	GFEIGISQEPFDPSG	0.87
31	TSAGKDGISYQPCTA	0.86
32	VDLEWFNRVETYIKW	0.84
33	GPTANNDVTTGRNCL	0.79
34	ALPFSYAVQARLNYL	0.78

Rank	Sequence	Score
35	LVDLEWFNRVETYIK	0.78
36	ANVFATEPNGHIPEG	0.74
37	VLVPGDFVNVIAIAG	0.72
38	QAPAVVVLGGYLPIG	0.72
39	TRELPGFFYHSNDGS	0.70
40	ADVQVDRLITGRLSA	0.69
41	YTAACKTIESALQLS	0.67
42	PTNFSMSIRTEYLQL	0.66
43	TVLVPGDFVNVIAIA	0.66
44	GTPKPLEGVTDVSFM	0.66
45	STRELPGFFYHSNDG	0.65
46	LGPTANNDVTTGRNC	0.64
47	AIPLGATQVPYYCFP	0.64
48	KATNGNTNATARLRI	0.64
49	AANVFATEPNGHIPE	0.63
50	ASACTIDLFGYPEFG	0.62
51	SADVQVDRLITGRLS	0.62
52	DYKRCSNGRSVADLV	0.61
53	YSGVMVLPGVVDAEK	0.59
54	ASLPNRTGPSLSLNV	0.55
55	VQAPAVVVLGGYLPI	0.55
56	TGTPKPLEGVTDVSF	0.54
57	SGQVKIAPTVTGNIS	0.52
58	NCLLAMPKIYGLGQF	0.52
59	QSGQVKIAPTVTGNI	0.50

Appendix 9 Score of possible epitopes predicted from spike protein (SK1*) by BepiPred.

Amino acid	Position	Score
D	22	0.47
V	23	0.42
T	24	0.62
R	25	0.68
C	26	0.61
S	27	0.40
P	54	0.58
I	55	0.67
G	56	0.78
E	57	0.69
N	58	1.08
Q	59	1.18
G	60	1.43
V	61	0.92
N	62	0.60
Y	66	0.35
C	67	0.35
A	68	0.37
G	69	0.47
Q	70	0.94
H	71	1.22
P	72	1.38
T	73	1.35
A	74	1.27
S	75	0.99
G	76	0.48
S	96	0.56
Q	97	0.81
E	98	1.25
P	99	1.46
F	100	1.94
D	101	1.71
P	102	1.56
S	103	0.96

Amino acid	Position	Score
G	104	0.61
T	113	0.71
N	114	1.28
G	115	1.40
N	116	1.48
T	117	1.42
N	118	1.26
A	119	0.70
T	120	0.35
D	130	0.58
N	131	0.95
K	132	1.04
T	133	1.50
L	134	1.53
G	135	1.45
P	136	1.30
T	137	1.43
A	138	1.27
N	139	1.53
N	140	1.44
D	141	1.48
V	142	1.25
T	143	1.12
T	144	0.73
W	168	0.40
D	169	0.43
N	170	0.37
D	171	0.39
S	190	0.41
R	191	0.37
Y	197	0.50
N	198	0.50
S	199	0.43
S	219	0.58
A	220	1.09
G	221	0.93
K	222	1.20

Amino acid	Position	Score
D	223	1.09
G	224	1.08
I	225	1.05
S	226	0.93
Y	227	0.87
Q	228	0.52
P	229	0.42
C	230	0.52
F	242	0.43
A	243	0.66
T	244	0.88
E	245	0.92
P	246	0.98
N	247	1.36
G	248	1.60
H	249	1.64
I	250	1.11
P	251	0.98
E	252	0.43
G	253	0.36
V	274	0.42
D	304	0.36
A	311	0.59
V	312	0.76
Q	313	0.90
R	314	0.87
A	315	0.53
P	316	0.45
S	350	0.35
N	351	0.45
S	352	0.95
S	353	1.02
D	354	1.12
P	355	1.08
H	356	0.69
L	357	0.49
T	367	0.47

Amino acid	Position	Score
V	369	0.41
V	377	0.36
D	378	0.52
T	379	0.84
Y	380	0.76
N	381	0.46
S	382	0.36
T	427	0.36
G	428	0.68
H	429	1.15
G	430	1.35
T	431	1.60
D	432	1.62
G	433	1.57
D	434	1.27
V	435	0.79
S	436	0.61
V	468	0.58
S	469	0.57
F	484	0.39
Y	485	0.74
P	486	0.58
I	487	0.45
H	495	0.43
E	496	0.55
Q	497	0.59
P	498	0.69
T	499	0.73
S	500	0.55
F	507	0.42
G	522	0.60
G	523	0.47
H	524	0.60
T	534	0.41
T	535	0.49
I	536	0.63
N	537	0.53

Amino acid	Position	Score
P	558	0.45
T	559	0.71
S	560	1.06
Y	561	1.11
G	562	1.33
Y	563	1.40
G	564	1.42
S	565	1.36
K	566	1.51
S	567	1.60
Q	568	1.61
G	569	1.46
S	570	1.00
N	571	0.92
C	572	0.44
E	608	0.68
F	609	0.83
G	610	0.79
S	611	0.49
G	612	0.38
L	627	0.58
I	628	0.77
T	629	0.89
G	630	0.98
T	631	0.77
P	632	1.23
K	633	1.53
P	634	1.47
L	635	1.35
E	636	1.26
G	637	0.85
V	638	0.61
Y	676	0.53
T	677	0.77
S	678	0.80
D	679	0.65
S	680	0.36

Amino acid	Position	Score
V	694	0.45
Y	695	0.37
C	700	0.43
S	701	0.57
F	702	0.63
S	703	0.50
E	704	0.39
A	706	0.38
A	707	0.85
Y	708	0.90
V	709	0.56
S	723	0.44
T	724	0.53
F	725	0.79
N	726	0.87
S	727	0.61
T	728	0.56
R	729	0.54
E	730	0.55
S	738	0.39
N	739	0.72
D	740	1.03
G	741	1.38
S	742	1.58
N	743	1.50
C	744	1.18
T	745	0.61
S	761	0.37
I	762	0.51
G	763	0.69
Y	764	0.87
V	765	0.96
P	766	0.94
S	767	1.24
Q	768	1.07
S	769	1.13
G	770	0.97

Amino acid	Position	Score
Q	771	0.95
V	772	0.78
K	773	0.67
I	774	0.50
A	775	0.48
P	776	0.42
T	777	0.70
V	778	0.45
T	779	0.75
G	780	0.51
N	781	0.57
I	782	0.50
S	783	0.69
V	804	0.50
S	805	0.56
V	806	0.37
S	817	0.38
A	841	0.61
R	842	0.94
P	843	1.08
E	844	1.31
S	845	1.31
A	846	1.25
E	847	0.87
V	848	0.35
S	866	0.43
F	867	0.71
N	868	0.65
G	869	0.95
D	870	0.65
G	871	0.63
Y	872	0.82
N	873	0.51
Y	883	0.81
D	884	0.90
P	885	1.11
A	886	0.90

Amino acid	Position	Score
S	887	0.88
G	888	0.88
R	889	0.70
V	890	0.58
V	891	0.40
L	910	0.50
G	911	0.81
T	912	0.99
V	913	0.86
D	914	0.75
E	915	0.90
D	916	0.76
Y	917	0.78
K	918	0.90
R	919	0.83
C	920	0.74
S	921	0.62
N	922	0.57
G	923	0.51
R	924	0.62
E	1015	0.36
E	1019	0.46
A	1020	0.52
I	1021	0.65
S	1022	0.82
Q	1023	0.82
T	1024	0.53
S	1025	0.63
K	1026	0.83
G	1027	0.60
L	1028	0.46
V	1041	0.38
V	1042	0.54
N	1043	0.64
S	1044	0.59
S	1065	0.37
E	1107	0.57

Amino acid	Position	Score
V	1108	0.55
Q	1109	0.55
K	1113	0.36
V	1123	0.36
K	1124	0.56
S	1125	0.60
Q	1126	0.49
S	1127	0.53
Q	1128	0.41
R	1129	0.38
F	1132	0.55
C	1133	0.64
G	1134	0.71
G	1135	0.76
D	1136	0.52
G	1137	0.42
A	1146	0.35
Q	1195	0.64
D	1196	0.76
T	1197	0.68
M	1208	0.62
Y	1209	0.88
E	1210	0.89
P	1211	1.07
R	1212	1.14
K	1213	1.41
P	1214	1.56
T	1215	1.18
V	1216	0.86
G	1217	0.58
D	1235	0.38
Q	1236	0.38
L	1237	0.56
P	1238	0.54
E	1239	0.68
V	1240	0.55
P	1242	0.36

Amino acid	Position	Score
D	1246	0.40
P	1259	0.64
N	1260	1.06
R	1261	1.44
T	1262	1.13
G	1263	1.30
P	1264	0.87
S	1265	0.69
H	1287	0.35
E	1290	0.43
S	1291	0.53
L	1292	0.76
R	1293	0.84
N	1294	0.84
T	1295	0.51
T	1296	0.46
E	1297	0.66
G	1370	0.37
P	1371	0.50
R	1372	0.67
L	1373	0.94
Q	1374	0.88
P	1375	0.54
Y	1376	0.54
E	1377	0.40

Appendix 10 Optimal levels of Ig A antibodies Abs reacting with NSSDPHL (BES6) synthesized in-line with the Pan DR epitope (PADRE).

		Jejunum					
	Positive Control	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Negative control
1st Test	2.0611	0.4821	0.2589	0.3602	0.9871	0.3312	0.1469
2ndTest	2.2626	0.3074	0.8652	0.4388	0.6798	0.3431	0.7349
Average	2.1619	0.3948	0.5621	0.3995	0.8335	0.3372	0.4409

		Ileum					
	Positive Control	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Negative control
1st Test	2.0611	0.3059	0.3934	0.3829	0.5201	0.6913	0.1469
2ndTest	2.2626	0.3490	0.2713	0.6400	0.6493	1.5952	0.7349
Average	2.1619	0.3275	0.3324	0.5115	0.5847	1.1433	0.4409

		Caecum					
	Positive Control	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Negative control
1st Test	2.5815	1.7228	1.2254	1.6422	0.4603	0.8435	0.1083
2ndTest	2.3751	0.5003	0.6017	1.0019	0.1479	0.9987	0.7503
Average	2.4783	1.1116	0.9136	1.3221	0.3041	0.9211	0.4293

		Colon					
	Positive Control	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Negative control
1st Test	2.5815	1.9330	1.1127	2.1982	1.0117	1.072	0.1083
2ndTest	2.3751	0.2115	0.1667	1.0002	1.1238	1.823	0.7503
Average	2.4783	1.0723	0.6397	1.5992	1.0678	1.4475	0.4293

		Spleen					
	Positive Control	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Negative control
1st Test	2.9366	0.1199	0.4071	0.2218	0.1484	0.1576	0.6805
2ndTest	1.8389	0.1017	0.1055	0.1674	0.1415	0.1348	0.0637
Average	2.3878	0.1108	0.2563	0.1946	0.1450	0.1462	0.3721

		Serum					
	Positive Control	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Negative control
1st Test	2.6640	0.0571	0.12	0.055	0.5783	0.4129	0.0691
2ndTest	1.7954	0.0625	0.0583	0.0621	0.1352	0.1173	0.0815
Average	2.2297	0.0598	0.0892	0.0586	0.3568	0.2651	0.0753

Organ	**Mouse 1**	**Mouse 2**	**Mouse 3**	**Mouse 4**	**Mouse 5**	**20% Trimmed Mean**
Jejunum	0.0000	0.1270	0.0000	0.3984	0.0000	0.0423
Ileum	0.0000	0.0000	0.0764	0.1496	0.7082	0.0753
Caecum	0.6765	0.4785	0.8870	0.0000	0.4860	0.5470
Colon	0.6372	0.2046	1.1641	0.6327	1.0124	0.7607
Spleen	0.0000	0.0326	0.0000	0.0000	0.0000	0.0000
Serum	0.0000	0.0000	0.0000	0.1331	0.0414	0.0138

Appendix 11 Optimal levels of Ig A antibodies Abs reacting with DNKTLGPTANNDVTT (BES1) synthesized in-line with the Pan DR epitope (PADRE).

		Jejunum					
	Positive Control	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Negative control
1st Test	1.7481	0.4619	0.8176	1.4807	0.7048	1.7573	0.0779
2ndTest	2.1543	0.4432	1.9681	1.0855	0.6474	0.7514	0.1055
Average	1.9512	0.4526	1.3929	1.2831	0.6761	1.2544	0.0917

		Ileum					
	Positive Control	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Negative control
1st Test	1.7481	0.7315	0.6602	1.6052	0.4427	0.3292	0.0779
2ndTest	2.1543	0.8888	1.0602	0.9693	0.3115	0.1829	0.1055
Average	1.9512	0.8102	0.8602	1.2873	0.3771	0.2561	0.0917

		Caecum					
	Positive Control	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Negative control
1st Test	1.9060	1.6133	1.338	1.8527	0.9206	0.2789	0.1146
2ndTest	2.6542	0.8579	0.587	2.4295	0.3078	0.7858	0.2212
Average	2.2801	1.2356	0.9625	2.1411	0.6142	0.5324	0.1679

		Colon					
	Positive Control	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Negative control
1st Test	1.9060	0.5808	2.3116	0.2312	0.4839	0.395	0.1146
2ndTest	2.6542	1.6418	2.6763	1.0275	0.2257	0.1204	0.2212
Average	2.2801	1.1113	2.4940	0.6294	0.3548	0.2577	0.1679

		Spleen					
	Positive Control	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Negative control
1st Test	1.7929	0.153	0.6794	0.3946	0.488	0.1773	0.0762
2ndTest	1.3174	0.1175	0.107	0.1387	0.1669	0.2416	0.0997
Average	1.5552	0.1353	0.3932	0.2667	0.3275	0.2095	0.0880

		Serum					
	Positive Control	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Negative control
1st Test	1.9108	0.4223	0.0968	0.0761	0.0739	0.6988	0.0604
2nd Test	1.4652	1.3326	0.9591	0.1196	0.6916	0.1574	0.1786
Average	1.6880	0.8775	0.5280	0.0979	0.3828	0.4281	0.1195

Organ	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	20% Trimmed Mean
Jejunum	0.3228	1.2631	1.1533	0.5463	1.1246	0.9414
Ileum	0.6804	0.7304	1.1575	0.2473	0.1263	0.5527
Caecum	1.1058	0.8327	2.0113	0.4844	0.4026	0.8076
Colon	0.9815	2.3642	0.4996	0.2250	0.1279	0.5687
Spleen	0.0315	0.2895	0.1629	0.2237	0.1057	0.1641
Serum	0.7737	0.4242	0.0000	0.2790	0.3244	0.3425

Appendix 12 Optimal levels of Ig A antibodies Abs reacting with LITGTPKPPLEGV (BES2) synthesized in-line with the Pan DR epitope (PADRE).

		Jejunum					
	Positive Control	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Negative control
1st Test	0.0547	0.2163	0.0985	0.2658	0.2785	0.2137	0.1652
2ndTest	2.1850	0.3278	0.2922	0.344	0.8014	0.2565	0.1124
Average	1.1199	0.2721	0.1954	0.3049	0.5400	0.2351	0.1388

		Ileum					
	Positive Control	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Negative control
1st Test	0.0547	0.3189	0.2096	0.5248	0.3158	0.2741	0.1652
2ndTest	2.1850	0.2984	0.4804	0.5946	0.4882	0.5262	0.1124
Average	1.1199	0.3087	0.3450	0.5597	0.4020	0.4002	0.1388

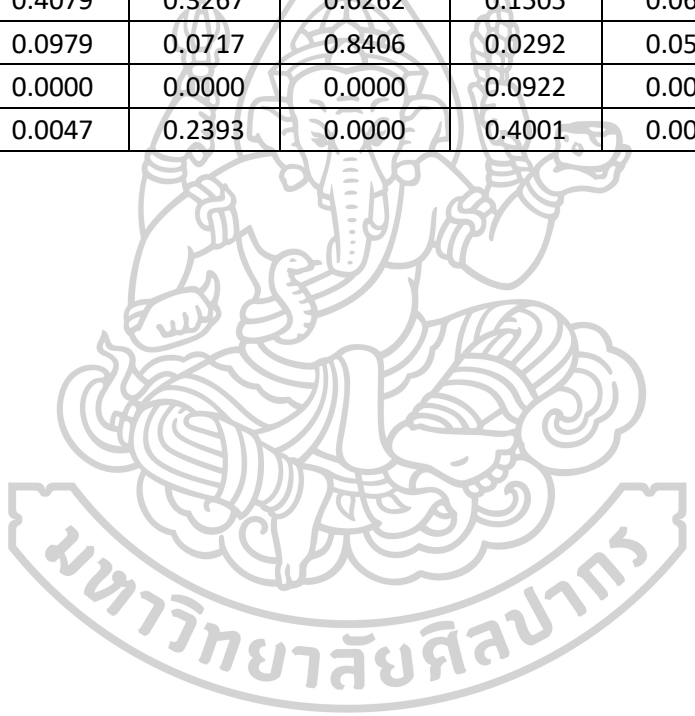
		Caecum					
	Positive Control	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Negative control
1st Test	0.0781	0.4567	0.3346	0.6688	0.203	0.1306	0.0681
2ndTest	1.9280	0.5769	0.5366	0.8015	0.3158	0.2198	0.0901
Average	1.0031	0.5168	0.4356	0.7352	0.2594	0.1752	0.0791

		Colon					
	Positive Control	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Negative control
1st Test	0.0781	0.1493	0.1231	1.4283	0.0732	0.1159	0.0681
2ndTest	1.9280	0.2644	0.2382	0.4708	0.2031	0.2032	0.0901
Average	1.0031	0.2069	0.1807	0.9496	0.1382	0.1596	0.0791

		Spleen					
	Positive Control	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Negative control
1st Test	0.0523	0.0805	0.0574	0.0721	0.0951	0.0845	0.0929
2ndTest	0.5632	0.1312	0.1867	0.1761	0.3663	0.1569	0.2656
Average	0.3078	0.1059	0.1221	0.1241	0.2307	0.1207	0.1793

		Serum					
	Positive Control	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Negative control
1st Test	0.0537	0.0471	0.6612	0.0491	0.0561	0.0901	0.0781
2nd Test	0.6121	0.2394	0.0943	0.1267	1.0211	0.1952	0.1174
Average	0.3329	0.1433	0.3778	0.0879	0.5386	0.1427	0.0978

Organ	**Mouse 1**	**Mouse 2**	**Mouse 3**	**Mouse 4**	**Mouse 5**	**20% Trimmed Mean**
Jejunum	0.1631	0.0864	0.1960	0.4310	0.1262	0.1617
Ileum	0.1997	0.2361	0.4508	0.2931	0.2912	0.2734
Caecum	0.4079	0.3267	0.6262	0.1505	0.0663	0.2950
Colon	0.0979	0.0717	0.8406	0.0292	0.0506	0.0734
Spleen	0.0000	0.0000	0.0000	0.0922	0.0000	0.0000
Serum	0.0047	0.2393	0.0000	0.4001	0.0041	0.0827



Appendix 13 Evidence for joining international conference and communication Email with speaker and participant.

The International Conference and Exhibition on
Pharmaceutical Sciences and Technology 2021 (PST 2021)
23-24 June 2021 in Bangkok, Thailand

PST BANGKOK 2021 THAILAND

CERTIFICATE OF ATTENDANCE

This is to certify that

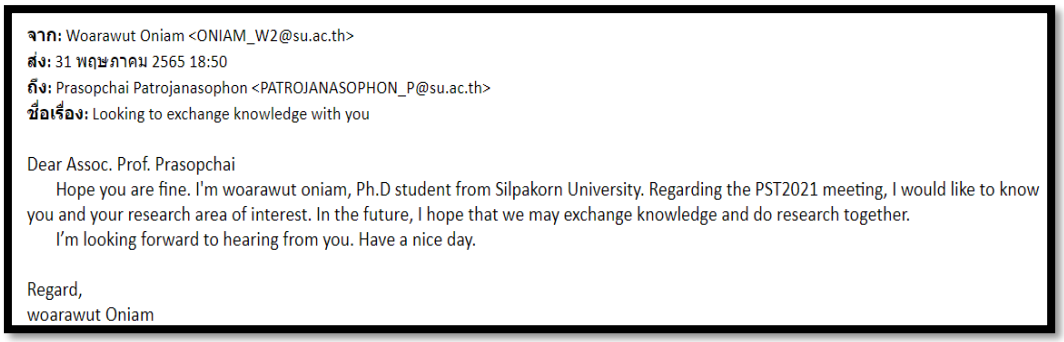
Mr.Woarawut Oniam

has participated in The International Conference and Exhibition on
Pharmaceutical Sciences and Technology 2021
"New Frontier in Pharmaceutical Sciences and Industrial Pharmacy"

Assist. Prof. Dr. Wimon Phuntuwate
Conference Chairman

Rungnapa Amarinthnukrowh
Conference Chairman

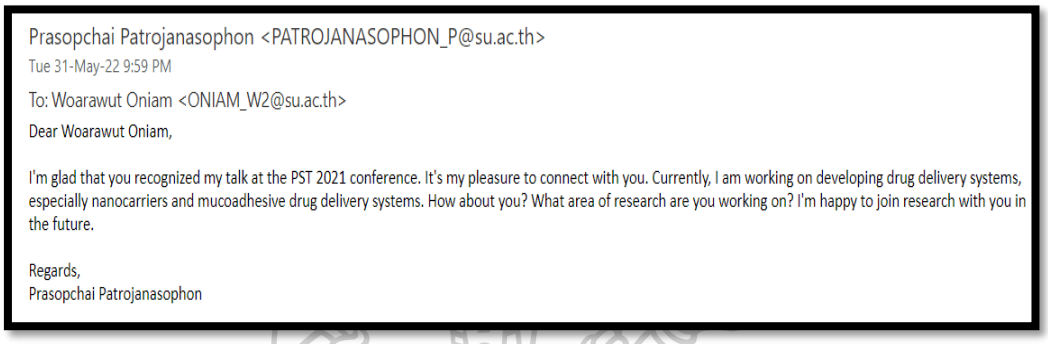
My certificate of attendance



จาก: Woarawut Oniam <ONIAM_W2@su.ac.th>
ส่ง: 31 พฤษภาคม 2565 18:50
ถึง: Prasopchai Patrojanasophon <PATROJANASOPHON_P@su.ac.th>
ชื่อเรื่อง: Looking to exchange knowledge with you

Dear Assoc. Prof. Prasopchai
Hope you are fine. I'm woarawut oniam, Ph.D student from Silpakorn University. Regarding the PST2021 meeting, I would like to know you and your research area of interest. In the future, I hope that we may exchange knowledge and do research together.
I'm looking forward to hearing from you. Have a nice day.

Regard,
woarawut Oniam

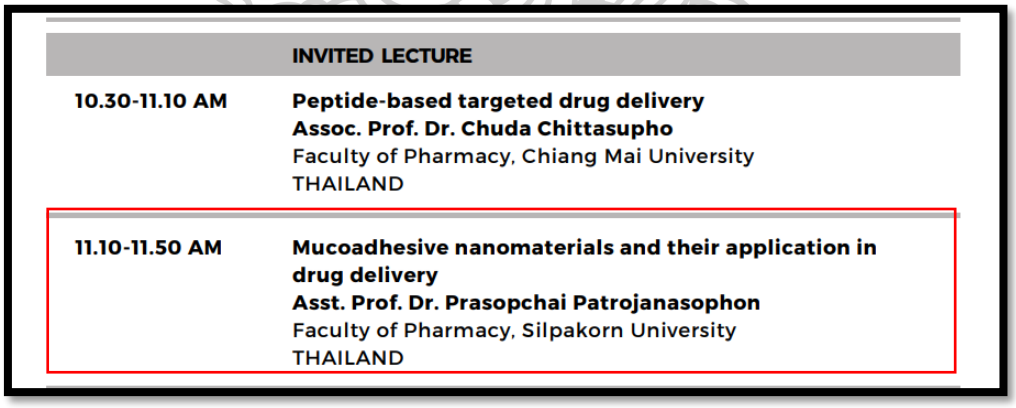


Prasopchai Patrojanasophon <PATROJANASOPHON_P@su.ac.th>
Tue 31-May-22 9:59 PM
To: Woarawut Oniam <ONIAM_W2@su.ac.th>
Dear Woarawut Oniam,

I'm glad that you recognized my talk at the PST 2021 conference. It's my pleasure to connect with you. Currently, I am working on developing drug delivery systems, especially nanocarriers and mucoadhesive drug delivery systems. How about you? What area of research are you working on? I'm happy to join research with you in the future.

Regards,
Prasopchai Patrojanasophon

Communication email with Assoc.Prof.Dr. Prasopchai Patrojanasophon (Speaker)



	INVITED LECTURE
10.30-11.10 AM	**Peptide-based targeted drug delivery** **Assoc. Prof. Dr. Chuda Chittasupho** Faculty of Pharmacy, Chiang Mai University THAILAND
11.10-11.50 AM	**Mucoadhesive nanomaterials and their application in drug delivery** **Asst. Prof. Dr. Prasopchai Patrojanasophon** Faculty of Pharmacy, Silpakorn University THAILAND

Part of PST 2021's Program book.

จาก: Woarawut Oniam <ONIAM_W2@su.ac.th>
ส่ง: Tuesday, May 31, 2022 6:48:53 PM
ถึง: Burin T.Sriwong <TSRIWONG_B@su.ac.th>
ชื่อเรื่อง: Looking to exchange knowledge with you

Dear Assist.Prof.Dr.Burin
Hope you are fine. I'm woarawut oniam, Ph.D student from Silpakorn University. Regarding the PST2021 meeting, I would like to know you and your research area of interest. In the future, I hope that we may exchange knowledge and do research together.

I'm looking forward to hearing from you. Have a nice day.

Regard,
woarawut Oniam

From: Burin T.Sriwong <TSRIWONG_B@su.ac.th>
Sent: Tuesday, May 31, 2022 7:02:48 PM
To: Woarawut Oniam <ONIAM_W2@su.ac.th>
Subject: Re: Looking to exchange knowledge with you

Dear K. Woarawut:

I am very pleased to know you. Hope you have a goodtime and learn a lot from the PST 2021 Conference. For my research interest. I am interested in Pharmaceutical Marketing area and also Quality Management. If you are interested in my research, you may search my full name from google. there are quite many publications of mine will come up.
I am looking forward to hearing back from you soon.

Best Regards

Communication email with Assist.Prof.Dr. Burin T.Sriwong

The International Conference and Exhibition on Pharmaceutical Sciences and Technology 2021 (PST 2021)

23-24 June 2021 in Bangkok, Thailand

PST BANGKOK 2021 THAILAND

CERTIFICATE OF ATTENDANCE

This is to certify that

Assist. Prof. Dr.Burin T. Sriwong

has participated in The International Conference and Exhibition on Pharmaceutical Sciences and Technology 2021 "New Frontier in Pharmaceutical Sciences and Industrial Pharmacy"

Wimon Phuntuwate
Assist. Prof. Dr. Wimon Phuntuwate
Conference Chairman

Rungnapa Amarinthnukrowh
Rungnapa Amarinthnukrowh
Conference Chairman

Certificate of Attendance of Assist.Prof.Dr. Burin T.Sriwong

จาก: Woarawut Oniam <ONIAM_W2@su.ac.th>
ส่ง: 30 พฤษภาคม 2565 23:46
ถึง: Karunrat Tewthanom <TEWTHANOM_K@su.ac.th>
ชื่อเรื่อง: Looking to exchange knowledge with you

Dear Assist Prof. Karunrat

Hope you are fine. I'm woarawut oniam, Ph.D student from Silpakorn University. Regarding the PST2021 meeting, I would like to know you and your research area of interest. In the future, I hope that we may exchange knowledge and do research together.
I'm looking forward to hearing from you. Have a nice day.

Regard,
woarawut Oniam

Karunrat Tewthanom <TEWTHANOM_K@su.ac.th>
Tue 31-May-22 8:42 PM
To: Woarawut Oniam <ONIAM_W2@su.ac.th>
Dear Ajarn Woarawut

Thank you very much for your greeting email. I am an academic staff at department of pharmacy, Silpakorn University. My research mostly about clinical pharmacy or pharmaceutical care practice. Your specialized research that I searchimg is about health informatics. I think it is also a good opportunity to integrate both knpwledges togather for improving the health care services quality. Glad to hear from you and exchange Scientific knowledges again in future.

Regards

Karunrat

Assist Prof. Karunrat Tewthanom PhD
Department of Pharmacy, Silpakorn University
Email: tewthanom_k@su.ac.th
Orchid ID: https://orcid.org/0000-0001-8496-3848
Phone: 6634253840 ext 208265

Communication email with Assist.Prof.Dr. Karunrat Tewthanom

The International Conference and Exhibition on Pharmaceutical Sciences and Technology 2021 (PST 2021)

23-24 June 2021 in Bangkok, Thailand

PST BANGKOK 2021 THAILAND

presented

CERTIFICATION OF POSTER PRESENTATION

to

Karunrat Tewthanom

For the poster presentation entitled

Genetic Screening to Avoid Adverse Drug Reaction (ADR) of Some Medical Use: A Report from The Queen Savang Vadhana Memorial Hospital

Suwanna Vorarat
Assoc. Prof. Dr. Suwanna Vorarat
Conference Chairman

Duangratana Shuwisitkul
Dr. Duangratana Shuwisitkul
Chair of Scientific Committee

Certificate of Attendance of Assist.Prof.Dr. Karunrat Tewthanom

REFERENCES

1. Song D. Porcine epidemic diarrhea: a review of current epidemiology and available vaccines. Clin Exp Vaccine Res. 2015;4:166-76.

2. Lv C, Xiao Y, Li X, Tian K. Porcine epidemic diarrhea virus: current insights. Virus Adaptation and Treatment. 2016(8):1-12.

3. Song D. Porcine epidemic diarrhoea virus: a comprehensive review of molecular epidemiology, diagnosis, and vaccine. Virus Genes. 2012;44:167-75.

4. Puranaveja S, Poolperm P, Lertwatcharasarakul P, Kesdaengsakonwut S, Boonsoongnern A, Urairong K, et al. Chinese-like Strain of Porcine Epidemic Diarrhea Virus, Thailand. Emerging Infectious Disease. 2009;15(7):1112-5.

5. Chang SH, Bae JL, Kang TJ, Kim J, Chung GH, Lim CW, et al. Identification of the epitope region capable of inducing neutralizing antibodies against the porcine epidemic diarrhea virus. Mol Cells. 2002;14(2):295-9.

6. Wang C, Yan F, Zheng X, Wang H, Jin H, Wang C, et al. Porcine epidemic diarrhea virus virus-like particles produced in insect cells induce specific immune responses in mice. Virus Genes. 2017;53(4):548-54.

7. Lee C. Porcine epidemic diarrhea virus: An emerging and re-emerging epizootic swine virus. Virol J. 2015;12:193.

8. Laude H, Gelfi J, Lavenant L, Charley B. Single amino acid changes in the viral glycoprotein M affect induction of alpha interferon by the coronavirus transmissible gastroenteritis virus. J Virol. 1992;66(2):743-9.

9. Zou D, Xu J, Duan X, Xu X, Li P, Cheng L, et al. Porcine epidemic diarrhea virus ORF3 protein causes endoplasmic reticulum stress to facilitate autophagy. Vet Microbiol. 2019;235:209-19.

10. Jung K, Saif LJ, Wang Q. Porcine epidemic diarrhea virus (PEDV): An update on etiology, transmission, pathogenesis, and prevention and control. Virus Res. 2020;286:198045.

11. Yamsakun P. **Start the year with the damage caused by PED** [cited 2015 Jan 11]. Available from: http://www.vet.cmu.ac.th/webmed/doctortui/data /ped.pdf.

12. INFECTION WITH PORCINE EPIDEMIC DIARRHOEA VIRUS: world organisation for animal health; September 2014 [cited 2018 Jun 22]. Available from:

https://www.oie.int/fileadmin/Home/fr/Media_Center/docs/pdf/factsheet_PEDV.pdf.

13. Temeeyasen G, Srijangwad A, Tripipat T, Tipsombatboon P, Piriyapongsa J, Phoolcharoen W, et al. Genetic diversity of ORF3 and spike genes of porcine epidemic diarrhea virus in Thailand. Infection, Genetics and Evolution. 2014;21:205-13.

14. Vaccines: Porcine Epidemic Diarrhea: The center for food security & public health; [cited 2015 Jan 29]. Available from: http://www.cfsph.iastate.edu/Vaccines/disease_list.php?disease=porcine-epidemic-diarrhea&lang=en.

15. Keretho S, Keinduangjun J, Piamsa-nga P. Introduction Bio-Mirror of AsiaPacific Advanced Network's biological sequence databanks. Nectec Technical J. 2000;11(8):119-26.

16. Monterrubio-Lopez GP, Gonzalez YMJA, Ribas-Aparicio RM. Identification of Novel Potential Vaccine Candidates against Tuberculosis Based on Reverse Vaccinology. Biomed Res Int. 2015;2015:483150.

17. Vivona S, Gardy JL, Ramachandran S, Brinkman FS, Raghava GP, Flower DR, et al. Computer-aided biotechnology: from immuno-informatics to reverse vaccinology. Trends Biotechnol. 2008;26(4):190-200.

18. Serruto D, Adu-Bobie J, Capecchi B, Rappuoli R, Pizza M, Masignani V. Biotechnology and vaccines: application of functional genomics to Neisseria meningitidis and other bacterial pathogens. J Biotechnol. 2004;113(1-3):15-32.

19. Capecchi B, Serruto D, Adu-Bobie J, Rappuoli R, Pizza M. The genome revolution in vaccine research. Curr Issues Mol Biol. 2004;6(1):17-27.

20. Open Reading Frame Finder: National center for biotechnology information; [cited 2015 Feb 9]. Available from: https://www.ncbi.nlm.nih.gov/orffinder.

21. Gardy JL, Brinkman FS. Methods for predicting bacterial protein subcellular localization. Nat Rev Microbiol. 2006;4(10):741-51.

22. SignalP-5.0: DTU Health Tech; [cited 2018 Jun 29]. Available from: http://www.cbs.dtu.dk/services/SignalP/.

23. Xiao X, Wu Z, Chou K. iLoc-Virus A multi-label learning classifier for identifying the subcellular localization of virus proteins with both single and multiple sites. Journal of Theoretical Biology. 2011;284(1):42-51.

24. Xiao X. iLoc-Virus [cited 2016 Sep 12]. Available from: http://www.jci-

bioinfo.cn/iLoc-Virus.

25. Sachdeva G, Kumar K, Jain P, Ramachandran S. SPAAN a software program for prediction of adhesins and adhesin-like proteins using neural networks. Bioinformatics. 2005;21:483-91.

26. Ansari HR, Raghava GP. Identification of conformational B-cell Epitopes in an antigen from its primary sequence. Immunome Res. 2010;6:6.

27. CBTOPE-Conformational B-cell epitope prediction Dr. GPS Raghava Lab; [cited 2018 Sep 15]. Available from: http://crdd.osdd.net/raghava/cbtope/submit.php.

28. Ponomarenko J, Bui HH, Li W, Fusseder N, Bourne PE, Sette A, et al. ElliPro: a new structure-based tool for the prediction of antibody epitopes. BMC Bioinformatics. 2008;9:514.

29. ElliPro: Antibody Epitope Prediction: National Institute of Allergy and Infectious Diseases; [cited 2018 Jul 4]. Available from: http://tools.iedb.org/ellipro/.

30. Langel SN, Paim FC, Lager KM, Vlasova AN, Saif LJ. Lactogenic immunity and vaccines for porcine epidemic diarrhea virus (PEDV): Historical and current concepts. Virus Research. 2016;226:93-107.

31. Bohl EH, Gupta RK, Olquin MV, Saif LJ. Antibody responses in serum, colostrum, and milk of swine after infection or vaccination with transmissible gastroenteritis virus. Infect Immun. 1972;6(3):289-301.

32. Bohl EH, Saif LJ. Passive immunity in transmissible gastroenteritis of swine: immunoglobulin characteristics of antibodies in milk after inoculating virus by different routes. Infect Immun. 1975;11(1):23-32.

33. Mantis NJ, Rol N, Corthesy B. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. Mucosal Immunol. 2011;4(6):603-11.

34. Klobasa F, Butler JE, Werhahn E, Habe F. Maternal-neonatal immunoregulation in swine. II. Influence of multiparity on de novo immunoglobulin synthesis by piglets. Vet Immunol Immunopathol. 1986;11(2):149-59.

35. Saha S, Raghava GPS. Prediction of continuous B-cell epitopes in an antigen using recurrent neural network. proteins. 2006;65(1):40-8.

36. El-Manzalawy Y, Dobbs D, Honavar V. Predicting linear B-cell epitopes using string kernels. J Mol Recognit. 2008;21(4):243-55.

37. Sweredoski MJ, Baldi P. COBEpro: a novel system for predicting continuous B-cell epitopes. Protein Eng Des Sel. 2009;22(3):113-20.

38. Lian Y, Ge M, Pan XM. EPMLR: sequence-based linear B-cell epitope prediction method using multiple linear regression. BMC Bioinformatics. 2014;15:414.

39. El-Manzalawy Y, Dobbs D, Honavar V. Predicting flexible length linear B-cell epitopes. Comput Syst Bioinformatics Conf. 2008;7:121-32.

40. Singh H, Ansari HR, Raghava GP. Improved method for linear B-cell epitope prediction using antigen's primary sequence. PLoS One. 2013;8(5):e62216.

41. Yao B, Zhang L, Liang S, Zhang C. SVMTriP: a method to predict antigenic epitopes using support vector machine to integrate tri-peptide similarity and propensity. PLoS One. 2012;7(9):e45152.

42. Alexander J, del Guercio MF, Maewal A, Qiao L, Fikes J, Chesnut RW, et al. Linear PADRE T helper epitope and carbohydrate B cell epitope conjugates induce specific high titer IgG antibody responses. J Immunol. 2000;164(3):1625-33.

43. Decroix N, Quan CP, Pamonsinlapatham P, Bouvet JP. Mucosal immunity induced by intramuscular administration of free peptides in-line with PADRE: IgA antibodies to the ELDKWA epitope of HIV gp41. Scand J Immunol. 2002;56(1):59-65.

44. Cheun-Arom T, Temeeyasen G, Srijangwad A, Tripipat T, Sangmalee S, Vui DT, et al. Complete Genome Sequences of Two Genetically Distinct Variants of Porcine Epidemic Diarrhea Virus in the Eastern Region of Thailand. Genome Announc. 2015;3(3).

45. Madeira F, Park YM, Lee J, Buso N, Gur T, Madhusoodanan N, et al. The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Res. 2019;47(W1):W636-W41.

46. Rappuoli R. Reverse vaccinology, a genome-based approach to vaccine development. Vaccine. 2001;19(17-19):2688-91.

47. Wang XM, Niu BB, Yan H, Gao DS, Yang X, Chen L, et al. Genetic properties of endemic Chinese porcine epidemic diarrhea virus strains isolated since 2010. Arch Virol. 2013;158(12):2487-94.

48. Larsen JE, Lund O, Nielsen M. Improved method for predicting linear B-cell epitopes. Immunome Res. 2006;2:2.

49. Hwang W, Lei W, Katritsis NM, MacMahon M, Chapman K, Han N. Current and prospective computational approaches and challenges for developing COVID-19

vaccines. Adv Drug Deliv Rev. 2021;172:249-74.

50. Ghaffari-Nazari H, Tavakkol-Afshari J, Jaafari MR, Tahaghoghi-Hajghorbani S, Masoumi E, Jalali SA. Improving Multi-Epitope Long Peptide Vaccine Potency by Using a Strategy that Enhances CD_{4+} T Help in BALB/c Mice. PLoS One. 2015;10(11):e0142563.

51. Song DS, Oh JS, Kang BK, Yang JS, Moon HJ, Yoo HS, et al. Oral efficacy of Vero cell attenuated porcine epidemic diarrhea virus DR_{13} strain. Res Vet Sci. 2007;82(1):134-40.

มหาวิทยาลัยศิลปากร

VITA

NAME	woarawut oniam
PLACE OF BIRTH	Bangkok
INSTITUTIONS ATTENDED	M.Sc. (Microbiology), Mahidol University, Thailand 2003 B.Sc. (Pharmacy) – Chiangmai University, Thailand 1998
HOME ADDRESS	17/24 Ladprao 43 Samsennok Huaykwang Bangkok