SAR-CoV 2 ANTIGENIC EPITOPES' ROLE IN STIMULATING PRODUCTION OF s-IgA AND *β* -DEFENSIN IN MUCOSA OF BALB/c MICE

FINAL ASSIGNMENT

Submitted In Fulfilment of The Requirement for

Bachelor of Medicine

By :

Jayshri Davi S Nadarajah

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APPROVAL PAGE

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SAR-CoV 2 ANTIGENIC EPITOPES' ROLE IN STIMULATING PRODUCTION OF SECRETORY IgA (s-IgA) AND β -DEFENSIN IN MUCOSA OF BALB/c MICE

Jayshri Davi S Nadarajah

Abstract

In 2020, the National Health Commission of China confirmed a new viral pneumonia as a novel coronavirus, 2019-nCoV which was a global pandemic. SAR-CoV 2 recombinant viral peptide-based vaccine with mucosal immunity as first line defense are still under phase III clinical trial. This research studies via in silico the potential epitopes of several SAR-CoV 2 proteins and laboratory experiment via in vivo to observe production of secretory IgA (s-IgA) antibodies and β defensin on twenty-five BALB/c mice. Control group was given PBS p.o, group II mice orally immunized with ISCOM, group III orally immunized with spike epitope (A) conjugated ISCOM, group IV orally immunized with envelope and membrane epitope (B and C) conjugated ISCOM and group V mice are orally immunized with spike, envelope, and membrane epitope (A, B and C) conjugated ISCOM. Three boosters were given weekly once, and seven days after the third booster, the mice of all groups are sacrificed by cervical dislocation method. The results shows that epitope B and C has significantly increased s-IgA and β-defensin levels ($P<0.05$) in respiratory tract mucosa layer. This research concludes the increase humoral immune response and presence of mucosal homing capabilities from gut-associated lymphoid tissues (GALT) to nasopharyngeal-associated lymphoid tissues (NALT).

Key words : SAR-CoV 2, Epitope, Mucosal immunity, Secretory IgA (s-IgA), β-defensin

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LIST OF ABBREVIATION

µg/mL	:	Microgram per mililiter
Ig	:	Immunoglobulin
s-IgA	:	Secretory IgA
IL	:	Interleukin
Th1	:	T-helper 1
Th2	:	T-helper 2
Th17	:	T-helper 17
Treg	:	T-regulatory
NK cell	:	Natural Killer Cell
NAb	:	Neutralizing Antobodu
PBS	:	Phosphate-buffered Saline
ISCOM	:	Immune Stimulating Complex
ELISA	:	Enzyme-linked Immunosorbent Assay
GALT	:	Gut-associated Lymphoid Tissue
MALT	:	Mucosa-associated Lymphoid Tissue

CHAPTER 1

INTRODUCTION

1.1 Background

In 2019, World Health Organization (WHO) was alerted of a rapid infecting unfamiliar pneumonia outbreak by the Chinese government. This later gathered international attention in January 2020 when the National Health Commission of China confirmed the new viral pneumonia as a novel coronavirus, (2019-nCoV). Common symptoms faced by patients infected by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are pneumonia like symptoms such as fever, cough, malaise and shortness of breath. With the presence of secondary and tertiary cases, it is evident that human transmission has occurred via sneezing, coughing, respiratory droplet, aerosol and airborne, and contact tracing of primary cases were carried out to control the spread of the virus. Specific diagnostic point-of-care real-time RT-PCR tests have been enabled through genetic sequencing the 2019-nCoV based on full genome sequence data on the Global Initiative on Sharing All Influenza Data [GISAID] platform (Wang C et al., 2020).

SARS-CoV 2, a single stranded RNA virus with spike protein beta-coronavirus origins from the Coronoviridae family in the Nidovirales order. Unlike previous coronaviruses, there are no genetic fingerprints of reverse genetic systems used in coronavirus engineering and no genetic sequences of preexisting viruses been forward engineered for SARs-CoV 2, which makes it evident that COVID-19 is a bat-derived sarbecovirus. Via novel mechanism, SARS-CoV 2 receptor-binding domain

binds to human ACE2 receptor and has high affinity to many other mammal cells (Morens DM et al., 2020). General coronavirus entry mechanism is based on cellular proteases that includes human airway trypsin-like protease (HAT), cathepsins and transmembrane protease serine 2 (TMPRSS2), which splits the spike protein and causes further penetration in host cells. The receptor-binding domain (RBD) regions in spike protein of SARS-CoV 2 maintains the van der Waals forces where 394 glutamine residue in RBD recognizes the critical lysine 31 residue on ACE2 receptors of humans and binds to it. Through endosomal pathway, a confirmation change in S protein enables fusion of viral envelope with cell membrane which then releases the RNA and proceeds to translate RNA genome into replicase polyproteins pp1a and 1ab, later cleaving into small viral proteinases in host cells. Series of subgenomic mRNAs are made by these polymerases which translates into viral protein, eventually assembled into virions with genome RNA in endoplasmic reticulum (ER) and Golgi then transported out of cell via vesicles (Shereen MA et al.,2020).

Since the first case detected in March 2020, in Indonesia, the number of confirmed COVID-19 cases has drastically increased, accounting to 939 948 confirmed cases with 26 857 deaths as of January 20, 2021. Despite the Guidelines on Standardized Procedures for Doctors' Protection in the COVID-19 Era practiced by Indonesia Medical Association (Ikatan Dokter Indonesia (IDI), it has still been a battle to contain and treat the rapidly growing numbers of new cases (World Health Organization, 2021). This puts a heavy urgency on the development if a definitive treatment and broadly protective vaccine to combat the spread of spiking new cases, but

nevertheless coronavirus evolve at a high-speed, hindering the ongoing efforts to get ahead of the pandemic (Morens DM et al., 2020).

Current vaccine development in the United States, China, Russia and United Kingdom are undergoing phase 3 large-scaled clinical trails, while lending hands to countries like Indonesia and Malaysia, where the types of vaccines developed are nucleoside-modified messenger RNA (modRNA) vaccine encoding, protein subunit vaccines and vector vaccines containing weakened live viral vector. These vaccines are exposed to the body to trigger an immune response of memory T-lymphocytes and B-lymphocytes, and when the body is exposed to the virus, these memory cells recognize and fight the virus (Centers of Disease Control and Prevention,2021). Vaccination stands as a crucial tool in combating the virus however conventional vaccine development methods are relatively time consuming, requires extensive methodologies and trails which eventually increases the cost of production (Dong R et al., 2020).

Immunoinformatic tools however have contributed to the rise of epitope-based vaccine. B-cell and T-cell epitopes of SARS-CoV 2 have been identified, where 120 potential sequences are generated based on spike (S) and nucleocapsid (N) protein (Ahmed SF et al.,2020). *In silico* epitopes used as vaccine candidates have shown that secretory IgA (s-IgA) and β-defensin in mucosa of respiratory tract are induced, therefore this theory is implied for SARS-CoV 2 virus in hope to induce humoral immunity of mucosa layer in respiratory tract via oral immunization though mucosal homing capabilities, as a first line defense mechanism since the porta of entry of SARS-CoV 2 is via the respiratory mucus layer of host (Mufida DC et al.,2018, Mufida

DC et al., 2019). Hence, *in silico* mapped antigen epitopes both spike and non-spike proteins such as Epitope Spike SARS-CoV 2= FLVLLPLVSSQCVNL (Epitope A), Epitope Envelope protein SARS-CoV 2= VNSVLLFLAFVVFLLVTLASS (Epitope B) and Epitope Membrane protein SARS-CoV 2= LYIIKLIFLWLLWPVTLACFVLAAVY (Epitope C) are generated and used study the immune response of s-IgA and β-defensin of respiratory tract mucosa layer in this experimental study.

1.2 Problem Summary

1.2.1 General Problem Summary

Will the immunization of SAR-CoV 2 antigenic protein epitopes; Epitope Spike SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B) and Epitope Membrane protein SARS-CoV 2 (Epitope C), induce humoral immune response in lungs' mucosa layer of BALB/c mice.

1.2.2 Specific Problem Summary

1.2.2.1 Will the immunization of SAR-CoV 2 antigenic protein epitopes; Epitope A,B and C, induce production of secretory Ig-A (s-IgA) in lungs' mucosa layer of BALB/c mice.

1.2.2.2 Will the immunization of SAR-CoV 2 antigenic protein epitopes; Epitope A,B and C, induce production of β-defensin in lungs' mucosa layer of BALB/c mice.

1.2.2.3 Will the immunization of SAR-CoV 2 antigenic protein epitopes; Epitope A,B and C, induce mucosal homing capabilities in lungs' mucosa layer of BALB/c mice.

1.3 Research Objective

1.3.2 General Objective

To know if orally administered SARs-Cov 2 antigenic protein epitopes A,B and C can induce humoral immune responses, especially secretory Ig-A (s-IgA) and β-defensin in mucosa layer of lungs.

1.3.3 Specific Objective

1.3.3.1 To analyze the production levels of secretory Ig-A (s-IgA) humoral immune response in lungs' mucosa of BALB/c mice orally immunized with SARS-CoV 2 antigenic protein epitopes A, B and C

1.3.3.2 To analyze the production levels of β-defensin humoral immune response, in lungs' mucosa of BALB/c mice orally immunized with SARS-CoV 2 antigenic protein epitopes A, B and C

1.3.3.3 To study the production of mucosal homing capabilities from oral immunization of SARS-CoV 2 antigenic protein epitopes A, B and C in nasopharyngeal-associated lymphoid tissues (NALT) of lungs in BALB/c mice used in this experiment.

1.4 Benefits

1.4.1 Academic Benefits

To be used as a theoretical base and as an additional knowledge in further researches of the use of SAR-CoV 2 antigenic protein epitopes in medical field.

1.4.2 Practical Benefits

To contribute materials in establishing a candidate for epitope-based COVID-19 vaccine.

CHAPTER 2
LITERATURE REVIEW

2.1 SARS-CoV 2 Virus

Coronavirus (CoV) origins from a large family virus that causing illness ranging from mild to severe symptoms. The two common types of coronaviruses that are known to cause diseases with severe symptoms are Middle East Respiratory Syndrome (MERS-CoV) and Severe Acute Respiratory Syndrome (SARS-CoV). Coronaviruses are zoonotic, hence are transmitted from animals and humans. Research suggests that SARS-CoV was transmitted from civet cats to humans and MERS-CoV from camels to humans. The novel coronavirus (2019-nCoV) is a type of virus that has never been previously identified in humans and no known animal transmission of 2019-nCoV, although some study shows that the virus originates from pangolins and bats. Clinical manifestations usually appear within 2 to 14 days after exposure. The signs and symptoms of infection are respiratory symptoms such as fever, cough, and shortness of breath, and in severe cases, pneumonia, respiratory syndrome, kidney failure, and even death. 2019-nCoV spreads via human-to-human transmission through droplets while sneezing or coughing. Currently, treatments given are based on relieving symptoms and increase endurance (Kementrian Kesehatan, 2020).

Since early 2020, humans are facing a pandemic of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). SARS-CoV-2 causes coronavirus disease, abbreviated as COVID-19. The spread of SARS-CoV-2 around the world threatened

a pandemic that is affecting billions of people. This virus appears to be a new pathogen among humans. The current vaccine initiative is still under third phase clinical trial, but no definitive drugs available for SARS-CoV-2, although many are developing rapidly, and some may be available at short notice. Recent initiatives have shown that serum from recovered humans is an option for treatment and even prevention of COVID-19, which can only be available when there are enough people who have recovered and can donate immunoglobulin containing serum (Casadevall A and Pirofski L, 2020).

It is known that, 30% of annual respiratory infections such as, rhinitis, pharyngitis, sinusitis, bronchiolitis, and pneumonia in human population are resulted by coronaviruses (Jevsnik et al., 2012). The impact is mainly associates with respiratory infections that are relatively mild and self-limiting. Infection from this virus can cause severe diseases in neonates, the elderly, and people with early comorbidities (Fehr and Perlman, 2015). Coronavirus are considered a potential threat to global public health after the emergence of SARS-CoV in 2002 (9 % case fatality rate, CFR), and MERS-CoV in 2012 (35 % case fatality rate, CFR). The initial clinical manifestations of MERS and SARS were largely similar where, influenza-like symptoms with fever, chills, dry cough, headache, malaise and dyspnea often occur early in the course of the disease (Donnelly et al., 2003). The average incubation period is estimated at 4-6 days with a range of 2 to 8 days between onset of symptoms and hospitalization, while the average time from the onset of symptoms to death in fatal cases ware 23 days (de Wit et al., 2016). Fatal outcomes were most common in those aged above 60 years (43 % CFR), while no deaths were reported in children and adolescents, however fatal illness was reported in 6.8 % of patients below 60

years. Phase one of SARS infection is associated with increased viral load and early symptoms of illness like fever and malaise, meanwhile during the second phase fever, hypoxemia, and decreased viral load, is onserved, while patients commonly develop pneumonia and diagnosed radiographically and 20 % of patients develop acute respiratory distress syndrome (ARDS). Common laboratory features of SARS are lymphopenia, thrombocytopenia, disseminated intravascular coagulation, and elevated levels of lactate dehydrogenase and lactate creatine kinase. 6.7% and 84% of patients undergo acute renal impairment and proteinuria, respectively (AL-Ahmadi and Roland, 2005; Walston et al., 2008; de Wit et al., 2016).

Before the COVID-19 outbreak, the world was stirred by SARS and MERS, and with this history, it is not the first time coronavirus is making citizens panic. Having the same symptoms like flu, the coronavirus develops rapidly, resulting in severe infection and even organ failure. COVID-19 has become a global problem around the world today, and all countries including Indonesia are doing their best to overcome this outbreak. One of the most promising therapy for this current condition is Convalescent Plasma Therapy, which involves giving plasma from a recovered COVID-19 patient to a COVID-19 patient who is still suffering from the disease (Monica et al., 2020). Indonesia is still facing a great time combating coronavirus to date, as well as many other countries in the world. The number of COVID-19 cases continues to grow rapidly with some recoveries reported, yet with a great number of deaths. Measures to control and prevent are continuously being made to fight COVID-19.

Coronovirinae originates from the Coronoviridae family of Nidovirales Order, where coronavirus is an RNA virus enveloped with an RNA genome ranging in length

from 25.5 kb to approximately 32 kb. The spherical virus particles are 70 - 120 nm in diameter with four structural proteins. The viral envelope is covered with distinctive spike-shaped glycoproteins (S) as well as envelope (E) and membrane (M) proteins, at which protein S mediates attachment and entry in host cells. The helical nucleocapsid, consisting of the viral genome encapsulated by the nucleocapsid (N) protein, resides within the viral envelope. Two-thirds of the coronavirus genome consists of a replication complex (ORF1a and ORF1b) and codes for two large polyproteins, pp1a and pp1b. The replicase-transcriptase virus complex consists of 16 non-structural proteins (nsp1-16) encoded by the polyproteins pp1a and pp1ab, whereby both polyproteins can be cleaved by viral proteases PLpro (nsp3) and 3CLpro (nsp5). Non-structural proteins function in the formation of double-membrane vesicles originating from the rough endoplasmic reticulum and are also sites of viral replication and transcription (de Wit et al., 2016). Coronavirus also encodes the unique exoribonuclease (exoN) proofreading function of nsp14 which reduces the accumulation of mutations in the RNA genome, while the remainder of the genome is transcribed into a subgenomic mRNA set. Five additional proteins that are not required for replication but can play a role in pathogenesis were also encoded: ORF3, ORF4a, ORF4b, ORF5, and ORF8b. The remaining subgenomic RNA encodes for accessory proteins whose immunomodulatory properties or functions are still unknown.

Coronavirus proteins mediate the entry of the virus into host cells, where the spike S1 subunit contains receptor binding domains, binding to receptors on the host cell and determining viral tropism. Virus entry is mediated through the viral membrane and the host undergoes fusion through the S2 spike protein subunit (Li et al., 2016).

The analysis showed that the S1 domain, particularly RBD, was due to its role in determining host tropism and pathogenesis. SARS-CoV uses the ACE2 receptor to bind to host cells, including various types of respiratory epithelial cells, alveolar macrophages, and monocytes (Memish ZA et al., 2013; Qiu et al., 2018). Some cell types that do not have ACE2 expression are also permissive to SARS-CoV, suggesting that additional or co-receptors exist for SARS-CoV and can contribute to infection (Hamming et al., 2004; Gu J et al., 2005).

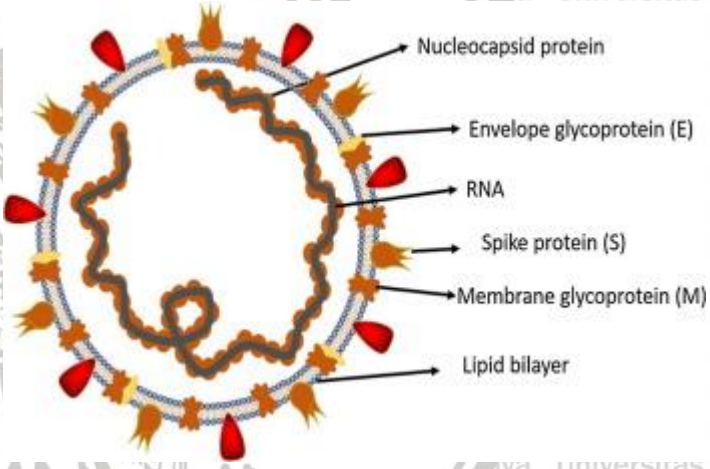

Figure 2.1 Structure of SAR-Cov 2 (Shereen MA et al., 2020)

2.2 Epitope Definition and Identification

Immune system cells do not recognize and interact with all immunogenous molecules, but lymphocytes recognize chronchus sites antigen determinants on macromolecules. A recognition mechanism is an event that binds to receptor molecules on the surface of the immune system cells which are tasked with recognizing it with parts of the immunogen molecule. Small-sized receptor molecules

on the surface of the recognition cells function to recognize, while the size of immunogens varies from very small to large. The parts bound by the receptor molecule are called antigen determinants or better known as epitopes. Epitopes can only be recognized by antibodies, so epitopes are always present on the immunogenous surface, the number of epitopes present varies from one to a few. Single epitope immunogens are known as uni-determinant, while immunogens that have more than one epitope is known as multideterminant (Subowo, 2014; Baratawidjaja and Rengganis, 2014).

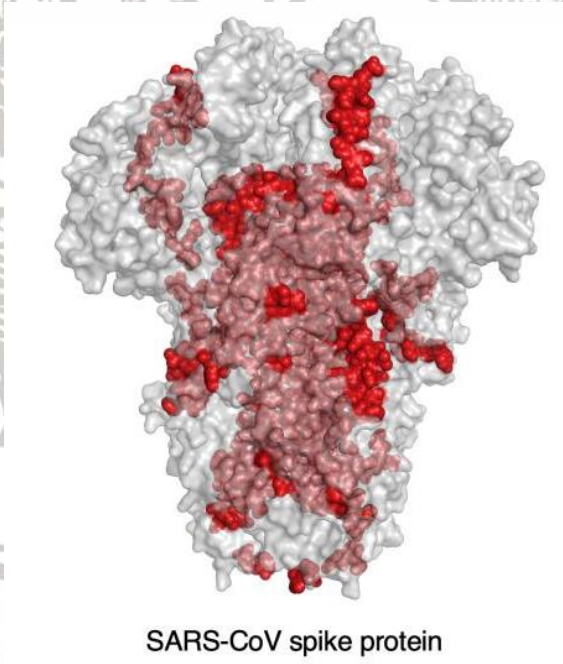

Figure 2.2 B Cell Epitopes that Map Identically to SARS-CoV-2 (Ahmeed SF et al., 2020)

Studying the composition, structure, and size of the integral (inherent) epitope of immunogen and haptenic determinants can be done with three approaches, namely: a) Cross reaction, a reaction between antibodies and other antigens which is not the cause of the immune response that produces these antibodies. The reaction results were compared with the reaction results between the original antibody and the epitope/immunogen. If the results are the same it can be concluded that the epitope identity is the same as the known antibody specificity. If the results are not the same, an antibody of another specificity is used until the same result is obtained; b) Release of the epitope from the antigen. The antigen containing the epitope to be identified is degraded (broken down) in the hope that the epitope is between the antigen fractions. Then the epitope between the antigen fractions will be identified in isolation;

c) Precipitation reaction, this reaction uses natural antigens. Synthetic antigen or synthetic hapten is reacted with antibodies that have known specificity, then by means of inhibition, the amount of precipitation that occurs is measured as well as measuring the epitope (Baratawidjaja and Rengganis, 2014).

B cells and T cells recognize different types of epitopes on the same antigen molecule. Lymphocytes can also interact with complex antigens at various stages of the antigen structure. B cells bind to antigens in solution, familiar epitopes that tend to be easy to find on the immunogenous surface. T cell epitopes on different proteins in the peptide are usually derived from the digestion of pathogenic proteins by an enzyme known as T-cell Receptor (TCR) in complex with Major histocompatibility complex (MHC). Macromolecules can have a wide variety of epitopes each stimulating the

production of a different, specific antibody. The paratope is a part of the epitope-binding antibody or TCR that binds the epitope to the antigen. Immune responses such as nucleic acid, protein, and carbohydrate can occur against all chemical groups (Baratawidjaja and Rengganis, 2014).

2.3 Mucosal Immune System

Immune system provides host defense against pathogens wherever these may enter or spread, with series of anatomically distinct compartments can be distinguished, first of which is specially adapted to generate a response to pathogens present in a particular set of body tissues especially in spleen and peripheral lymph nodes responding to antigen spread through blood. A second compartment of the adaptive immune system is located near the surfaces where most pathogens invade, is the mucosal immune system (mucosal associated lymphoid tissue, MALT). Two further distinct compartments are those of the body cavities; peritoneum and pleura, and the skin. The key features are that immune responses induced within one compartment are largely confined in expression to that particular compartment, and is that lymphocytes are restricted to particular compartments by expression of homing receptors that are bound by ligands, known as addressins, that are specifically expressed within the tissues of the compartment. Mucosal surfaces of the body are particularly vulnerable to infection, as they are thin and permeable barriers to the interior of the body because of their physiological activities in gas exchange of the lungs, food absorption in the gut, sensory activities of eyes, nose, mouth, and throat, and reproduction in uterus and vagina, and the necessity for permeability of the

surface lining these sites are vulnerable to infection and majority of infectious agents invade the human body through these routes (Janeway Jr CA et al., 2001).

The migration of immune cells from mucosal inductive to effector tissues is the cellular basis for the common mucosal immune system, CMIS. Thus, mucosal vaccination elicits immune responses in distant, multiple mucosal effector sites (Kiyono H et al., 2008). Mucosal inductive sites, including gut-associated lymphoreticular tissue (GALT) and nasopharyngeal-associated lymphoreticular tissue (NALT), collectively comprise a mucosa-associated lymphoreticular tissue (MALT) network for provision of a continuous source of memory B and T cells to mucosal effector sites (Brandtzaeg P, 2007). The MALT contains T-cell zones, B cell-enriched areas containing a high frequency of surface IgA-positive (s-IgA+) B cells and a subepithelial area with APCs for the initiation of specific immune responses and is covered by a follicle-associated epithelium that consists of a subset of differentiated microfold (M) epithelial cells, columnar epithelial cells and lymphoid cells, which play a central role in the initiation of mucosal immune responses. M cells take up antigens (Ags) from the lumen of the intestinal and nasal mucosa and transport them to the underlying APCs, including dendritic cells (DCs). Recent studies have now identified isolated lymphoid follicles (ILFs) in the mouse small intestine. The ILFs have been identified as a part of GALT and as such are a mucosal inductive tissue (Hamada H et al., 2002). These ILFs mainly contain B cells, DCs and M cells in the overlying epithelium. Mucosal effector sites, including the lamina propria regions of the GI, the upper respiratory (UR), and reproductive tracts, secretory glandular tissues and

intestinal intraepithelial lymphocytes, contain Ag-specific mucosal effector cells such as IgA-producing plasma cells and B and T cells.

Secretory (S)-IgA antibody (Ab) is a major player in the mucosal immune system and is locally produced in effector tissues (Brandtzaeg P et al., 2007). M cells that are specialized epithelial cells for antigen uptake, transfer antigens via transcytosis to APCs located in pockets within M cell clusters (Schulz o and Pabst O, 2013). Dendritic cells that come in contact with antigens transcytosed through M cells enter the interfollicular T cell zone to activate naive T cells, causing effector T cells move to the B cell follicles of germinal centers (GCs) and secrete cytokines capable of promoting IgA class-switch recombination (Benmark M et al., 2012). In mucosal immune effector sites such as the lamina propria of the gut and the upper respiratory tract, IgA+ plasma cells terminally differentiate to release secretory IgA (s-IgA), the most important immune effector molecule in the mucosa. S-IgA is transported across mucosal epithelial cells via a polymeric Ig receptor (pIgR). Besides that, s-IgA is a major immune effector at mucosal surfaces that acts via three mechanisms: antigen excretion, immune exclusion, and intracellular antigen neutralization (Strugnell RA and Wijburg OL, 2010). Antigen excretion by s-IgA features the binding of s-IgA to pathogen-derived antigens, thus inhibiting pathogen–epithelial cell contact. S-IgA exerts immune exclusion by eliminating antigens via secretion of an IgA–antigen complex, and invading pathogens can also be eliminated by complex formation with IgA-joining (J) chain-pIgR. S-IgA inhibits the binding of pathogens and/or pathogenic antigens to specific receptors by neutralizing and eventually removing the pathogenic

antigens.

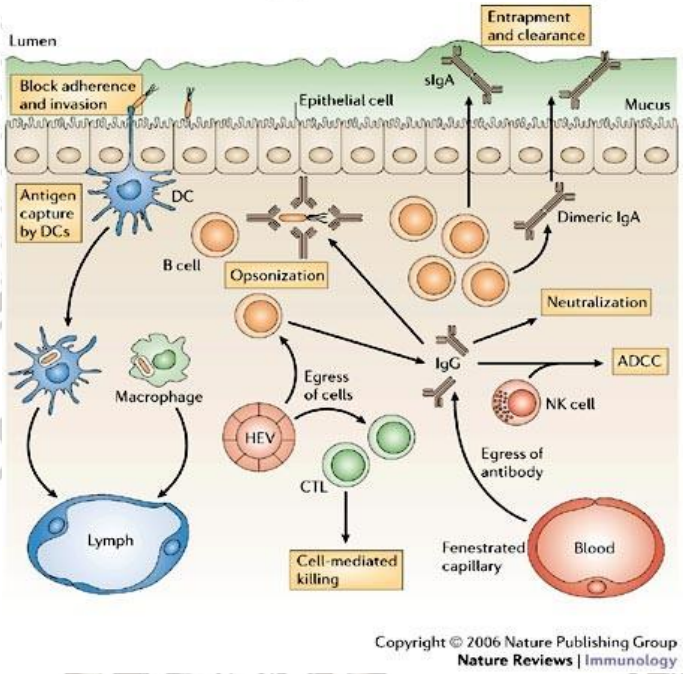

Figure 2.3 Mechanisms of Immune Protection at Mucosal Surfaces (Neutra MR and Kozlowski PA, 2006)

The presence of Ag-specific S-IgA Abs at mucosal effector sites other than the inductive sites where initial Ag sampling occurred is definitive evidence for the CMIS. To this end, immunization of GALT or NALT effectively elicits Ag-specific mucosal IgA Ab responses in diverse mucosal effector tissues with some notable differences. Indeed, activated T cells in Peyer's patches (PPs) preferentially express α4β7 and CCR9 as gut-homing receptors for their migration into the intestinal lamina propria (Campbell DJ et al., 2002). Mucosal addressin cell adhesion molecule-1 (MAdCAM-1), the ligand for α4β7, mediates T-cell recruitment into the intestinal endothelium (Butcher EC et al., 1999). In addition to mucosal T-cell homing, retinoic

acid-producing DCs in PPs regulate T cell-independent IgA class switching and gut-homing receptor expression on B cells (Mora JR et al., 2006). These findings clearly show that the CMIS exhibits distinct sites for induction and regulation of S-IgA Ab responses in mucosal effector tissues.

On the other hand, beta defensin peptides have been described and are expressed not only in the gastrointestinal tract, but also the lung, eye and skin. These peptides are synthesized as preproproteins and processed intracellularly to mature. Human b-defensin (HBD)1 is widely and constitutively expressed in epithelial cells throughout the gastrointestinal tract (Mahida YR and Chunloffe RN, 1999). Inline line to this, IL-17 and IL-22 diverge considerably in terms of receptor distribution and intracellular signaling. In humans, the receptor complex of IL-17, which includes IL-17RA and IL-1s7RC, is widely expressed on epithelial, mesenchymal, and hematopoietic cells and in contrast, IL-22 binds to a heterodimer formed by the IL-10 receptor b (IL-10Rb) and the IL-22 receptor (IL-22R) where in humans, IL-10Rb is widely expressed, while IL-22R expression is mostly limited to epithelial cells of the skin, lung, and gut including hepatocytes, and kidney. Thus, from receptor distribution, it can be inferred that while IL-17 modulates many cells, including cells of adaptive and innate immunity, IL-22 acts specifically on epithelial cells.

IL-17 functions to provide a protective inflammatory response towards pathogens at boundary tissues at the gut, and lung. Defective IL-17 secretion has been observed in chronic mucocutaneous candidiasis and IL-17 appears crucial for effective immune responses towards Mycobacterium tuberculosis, gastrointestinal infection due to Escherichia coli among others (Isailovic, N. et al., 2015). This defense function

of IL-17 is performed through inducing epithelial cells to release CXCL8 and CXCL1, potent neutrophil chemoattractants, granulocyte colony-stimulating factor (G-CSF), a survival factor for neutrophils, CCL20, which promotes the recruitment of Th17 cells, granulocyte macrophage colony-stimulating factor (GM-CSF), and antimicrobial peptides such as b-defensin-2, mucins (MUC5AC and MUC5B), and S100 proteins such as S100A7, S100A8, and S100A9 (Chiricozzi, A. et al., 2014).

IL-22 exerts its effects on epithelial cells of the skin, pancreas, intestine, liver, and lung. The IL-22 pathway has been shown to modulate the expression of many genes encoding proteins involved in tissue protection, survival, differentiation, and remodeling, and to a lesser extent pro-inflammatory proteins (Eyerich, S, et al., 2009). Similarly to IL-17, IL-22 promotes the release of β-defensin-2 and β-defensin-3, and peptides of the S100 family, including S100A7, S100A8, S100A9 by human keratinocytes (Pennino, D. et al., 2010). Furthermore, both IL-17 and IL-22 support the release of metalloproteinases (MMPs), which facilitate the migration of immune cells to the site of inflammation by inducing the proteolytic degeneration of collagens and proteoglycans (Wolk K et al., 2006). IL-22 appears crucial for effective immune responses in mice against Klebsiella pneumoniae and Citrobacter rodentium in the lung and intestine, and it is involved, together with TNF-α, in the control of Candida albicans infections (Aujla S et al., 2009).

2.4 Oral Vaccination

Mucosal route vaccination is shown to offer advantages for enhanced mucosal immune responses that result in better local protection. Oral delivery of vaccines represents the most attractive mode of administration over other routes of delivery as

the oral vaccination is noninvasive, safe and simple to execute, showing good patient compliance and clinical practicality. For example, the oral polio vaccine, which consists of live attenuated polioviruses, is a clear demonstration of the fact that oral vaccination against a highly contagious human enterovirus has succeeded in eradicating this virus in almost all countries. The polio vaccine is known to mimic the humoral immune response induced by wild strains of poliovirus orally transmitted. The significant property of the vaccine is actually the ability to inhibit invading viruses from propagating in the mucosal tissue of the small intestine and, hence, to effectively control the virus from spreading from mucosal linings to other tissues or being shed (Aylward B and Tangermann R, 2011). Another oral vaccine that is effective primarily against the small intestine infection is the rotavirus oral vaccine. Rotarix and RotaTeq are the two currently used vaccines that confer protection against rotavirus gastroenteritis as effectively as 70%, and the protection can reach 85% to 100% to prevent severe rotavirus gastroenteritis (Ruiz-Palacios GM et al., 2006).

Vaccine efficacy depends on degree if protection conferred to individuals and total coverage, accessibility and cost associated to administering the vaccines. Therefore, oral vaccine have an advantage in improving distribution compared to traditional vaccines as it can be self-administered and does not require a trained healthcare personal. With lesser injection requirements needed , this reduces the overall cost of vaccination programs. Oral immunization has the potential to improve vaccine efficacy simply by increasing accessibility and coverage, however the oral route also provides the additional advantage of stimulating mucosal immunity. The

mucosal epithelium covers the largest surface area in the body and constitutes the first line of defense against external pathogens (Ramirez JE et al., 2017).

However, the oral delivery of antigens needs to overcome multiple physicochemical and biological barriers in the GI tract. The biological barrier of the intestinal epithelium and its mucus secreting layers which serve to digest consumed material for nutrient absorption and to protect the body from the invasion of pathogenic threats, hence to accomplish these tasks, the GI tract includes a highly acidic environment in the stomach, a significant pH range along the length of the GI tract, and the presence of proteolytic enzymes responsible for protein degradation. These characteristics can interfere with the delivery of fragile biomolecules, such as antigenic proteins or peptides, which are highly susceptible to degradation and denaturation. Furthermore, there is a temporal limitation for the absorption of these formulations due to the residence time in the small intestine of 3 to 4 hours, where the majority of absorption processes occur (Renukuntla J et al., 2013).

Another major hurdle in the development of oral vaccines is that a higher dose of antigen is needed to induce an immune response when compared to traditional parenteral immunizations, limiting the possible formulations used as carriers as they must be able to successfully carry the required antigen dosage. Larger doses also increase the risk of inducing tolerance instead of stimulating a protective response, as the GI tract is constantly exposed to a variety of pathogens. If a vaccine does not induce the appropriate danger signals, the body can recognize it as non-pathogenic and avoid triggering an immune response, resulting in immune tolerance instead of

protection. Thus, it is critical in the design of oral vaccine carriers to include potent adjuvants in order to sufficiently stimulate the immune system.

Recombinant techniques have been used to generate protein-based vaccines has resulted in the production of several vaccines, including hepatitis B virus surface antigen vaccine, using different vector systems, including *bacculovirus* and *Saccharomyces cerevisiae*. Recombinant vaccines containing tetanus toxin, diphtheria toxin, and acellular pertussis toxoid are other examples of the use of recombinant technology in generating purified antigen in large quantities for vaccines. Significant progress has also been made in the use of viral agents for antigen delivery as live vectors; vaccinia virus has been particularly favored and used successfully for several antigens. Poliovirus and adenovirus are other attractive vectors for delivery of mucosal vaccines (Piedra PA et al., 1998). It appears that poliovirus can be used as an antigen delivery vehicle to induce CD4 helper T-cell activity, which in turn regulates IgA B-cell response, in addition to specific cytotoxic T lymphocytes, CTL (Ertl HC and Xiang Z, 1996). Upon oral sublingual vaccination, antigen-specific immune responses are induced in the gastrointestinal and the upper and lower respiratory tracts along the gut-lung axis (Lycke N, 2012; Maslowski KM et al., 2009).

2.5 SARS-CoV 2 Vaccination

Vaccines protect from viral pathogens before exposure by generating protective immune memories with harmless agents. The development of neutralizing antibodies from vaccines remains one of the hallmarks of effective vaccines although vaccines that induce cell-mediated immunity also show potential and are under development for viral pathogens such as the influenza virus. Several vaccine platforms

exist with the ability to induce a protective response such as kills all viral vaccines, split-virion vaccine, subunit vaccine, direct attenuated viral vaccine, virus-like particle vaccines, nanoparticle vaccine, and nucleic acid vaccines (DNA and RNA). About selecting vaccine targets and platforms, vaccine candidates must be immunogenic and immune targeting should lead to viral neutralization or strong cytotoxic response. To date, there is no licensed vaccine for SARS-CoV or MERS-CoV although clinical trials have been initiated for the MERS-CoV vaccine. Most of the focus of the development of the SARS-CoV or MERS-CoV vaccine is on spike (S) protein because it is immunogenic and antibodies that target it can neutralize the virus (Agnihothram S et al., 2014; Yong CY et al., 2019). The analysis of S protein suggests that it has potential for vaccine development which can be attributed to work previously done for SARS-CoV and MERS-CoV. The envelope protein (E) is also an attractive vaccine target that has been proposed for use in the development of direct attenuated vaccines. Mutant MERS-CoV with E protein is replication-competent (Almazan F et al., 2013; Yong CY et al., 2019). Similar results were shown for SARS-CoV when E protein was removed (DeDiego ML et al., 2007). Together these suggest that the elimination of E protein from the coronavirus can provide a live-single viral replication that is safe for use in inducing a mucosal immune response. In investigations into the similarity of the E 2019-nCoV protein with a phylogenetic analysis of known coronavirus E protein sequences, it is found that there is a grouping, although somewhat distant, with human SARS-CoV. Given that vaccines have been produced for MERS-Cov and SARS-CoVs by mutating protein E, E-based vaccines could represent replacement candidates for the 2019-nCoV vaccine. When a vaccine

candidate is identified, the requirements of the animal model for vaccine development and evaluation are focused on with high importance.

Various reports related to SARS-CoV suggest a protective role of both humoral and cell-mediated immune responses. Previously, the resulting antibody response to, the protein most exposed to SARS-CoV, protein S has been shown to protect against infection in mouse models (Yang Z et al., 2004; Deming D et al., 2006; Graham R.L. et al., 2012). Besides, several studies have shown that the antibodies generated against the nucleocapsid (N) protein (a highly immunogenic protein and widely expressed during infection) from SARS-CoV, are particularly prevalent in SARS-CoV infected patients (Lin Y et al., 2003; Wang M et al., 2003; Liu X et al., 2004). While being effective, the antibody response was however found to be short-lived in recovering SARS-CoV patients (Tang F et al., 2011). In contrast, T cell responses have been shown to provide long-term protection, even up to 11 years after infection, and hence have also attracted interest for prospective vaccines against SARS-CoV (Peng H et al., 2006; Fan Y.Y. et al., 2009; Ng O.W. et al., 2016; Liu W.J. et al., 2017). Among all SARS-CoV proteins, T cell responses to structural proteins were found to be the most immunogenic in peripheral blood mononuclear cells of recovered SARS-CoV patients compared to non-structural proteins (Li X. et al., 2008). Furthermore, T cell responses to S and N proteins have been reported to be the most dominant and durable from structural proteins (Channappanavar, R. et al., 2014).

CHAPTER 3

CONCEPT FRAME AND RESEARCH HYPOTHESIS

3.1 Concept Frame

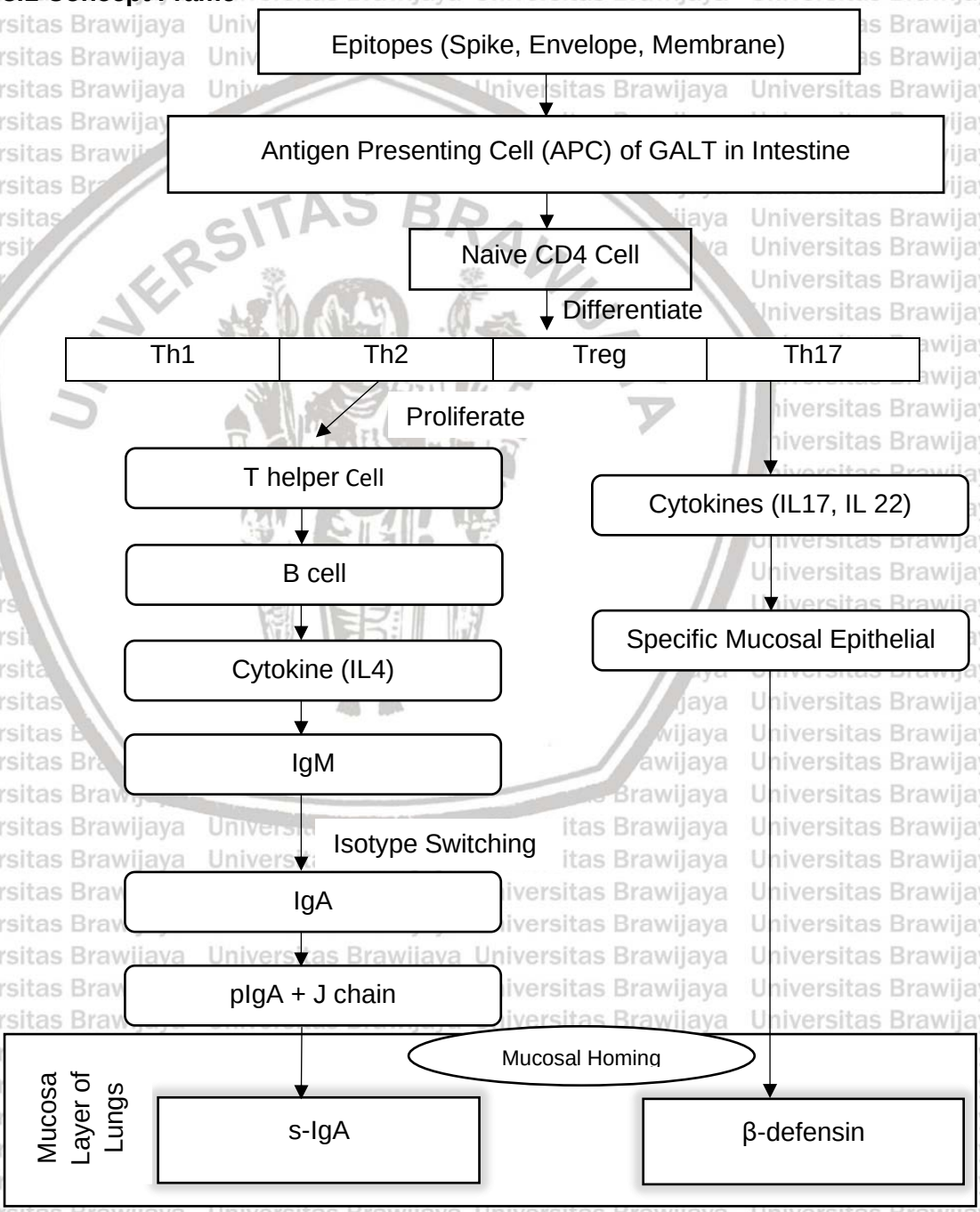

Figure 3.1 Research Framework Concept

3.2 Concept Frame Description

Peptide epitopes (Spike, Envelope, Membrane) are able to trigger humoral and cellular mediated immunity, where the presence of antigenic epitopes are processed by antigen presenting cells (APCs) from isolated lymph follicles (gut-associated lymphoid tissues (GALT)) in intestine and recognized naïve CD4 cells. CD4 cells then differentiate forming Th1, Th2, T-regulatory (Treg) and Th17 subtypes.

Th2 cells later proliferates T helper cells, committing B cell follicle at germinal centers (GCs) secrete IL4 cytokines that promotes IgM. IgM with growth factors in plasma cells undergo isotype switching forming IgA. s-IgA is made and released into luman when two monomers of IgA linked by a junction chain (J chain) and binds to pIgR at basilateral membrane in epithelial cells (Boyaka PN, 2017; Kalenik BM et al., 2018).

Th17 on the other hand activates cytokines IL17 and IL22, that promotes the production β-defensin in induced specific mucosal epithelial of intestine. IgA and β-defensin binds to gut-homing receptors and migrate into the lumen mucosa layer of gut and lungs along the gut-lung axis (Campbell DJ et al., 2002; Maslowski KM et al., 2009).

3.3 Research Hypothesis

Oral immunization of SAR-CoV 2 antigenic epitopes (Epitope SPIKE SARS-CoV 2 - Epitope A, Epitope Envelope protein SARS-CoV 2 - Epitope B and Epitope

Membrane protein SARS-CoV 2- Epitope C) induces production of humoral immunity in lung mucosa of BALB/c mice.

3.4 Research Sub-hypothesis

1. Oral immunization of SAR-CoV 2 antigenic protein epitopes A, B and C, induces production of secretory IgA (s-IgA) levels in lungs of BALB/c mice.

2. Oral immunization of SAR-CoV 2 antigenic protein epitopes A, B and C, induces production of β-defensin levels in lungs of BALB/c mice

3. Oral immunization of SAR-CoV 2 antigenic protein epitopes A, B and C, induces mucosal immunity homing capabilities in lungs of BALB/c mice.

CHAPTER 4
RESEARCH METHODS

4.1 Research Design

This research design is based on a laboratory experimental research with a post test only control group design approach. There are two phases in this research, the first phase is an exploratory study conducted via *in silico* to identify potential epitopes of several SARS-CoV proteins. The second phase is a laboratory experiment done via *in vivo* to identify the immunogenicity of several SARS-CoV protein epitopes by observing the production of secretory IgA (s-IgA) antibodies and β defensin.

4.2 Research Sample

In the first phase of the research where protein epitopes are identified, using amino acid sequences derived from Spike glycoprotein SARS-CoV 2, Envelope SARS-CoV 2 and Membrane SARS-CoV 2.

At the second phase, experimental animal immunogenicity test are conducted on male Balb/C mice (*Mus musculus*) from Animal House Laboratory, Faculty of Medicine, Universitas Brawijaya. The mice were divided into 5 groups :

1. Phosphate-buffered Saline (PBS) Group
2. Immune Stimulating Complex (ISKOM) Group
3. Spike Epitope (A) Group
4. Envelope Epitope (B) + Membrane Epitope (C) Group
5. Spike Epitope (A) + Envelope Epitope (B) + Membrane Epitope (C) Group

Total sample size is determined using Federer formula (Federer W, 1991):

$$(n-1)(t-1) \geq 15$$

Note: n = the number of samples

t = number of groups

Where 5 groups are used in this experiment, therefore, t = 5.

$$(n-1)(5-1) \geq 15$$

$$(n-1)(4) \geq 15$$

$$4n - 4 \geq 15$$

$$4n \geq 19$$

$$n \geq 4.75 \approx 5$$

Therefore, the number of samples is 5.

A total of 25 samples of experimental animals are used for 5 groups, where each group has 5 mice.

4.3 Place and Time of Research

This research is carried out in the month of October to December 2020, at the Laboratory of Microbiology, Faculty of Medicine, Universitas Brawijaya. The isolation and identification of protein and immunogenicity test are carried out at Biomedical Laboratory of the Faculty of Medicine, Universitas Brawijaya, while analysis to identify

protein epitopes are carried out at Bioinformatics Laboratory (InBio), Universitas Brawijaya.

4.4 Research Variable

4.4.1 Independent Variable

The independent variable in this research are Epitope SPIKE SARS-CoV 2= FLVLLPLVSSQCVNL (Epitope A). 2, Epitope Envelope protein SARS-CoV 2= VNSVLLFLAFVVFLLVTLASS (Epitope B) and Epitope Membrane protein SARS-CoV 2= LYIIKLIFLWLLWPVTLACFVLAAVY (Epitope C)

4.4.2 Dependent Variable

The dependent variable in this research is the level of secretory IgA (s-IgA) dan β defensin.

4.5 Operational Definition

4.5.1 Protein Epitome Identification (Spike Glycoprotein SARS-CoV 2, Envelope SARS-CoV 2, dan Membrane SARS-CoV 2)

Antigenicity analysis and epitope mapping via *in silico* are two methods used to identify protein epitopes (Spike Glycoprotein SARS-CoV 2, Envelope SARS-CoV 2, dan Membrane SARS-CoV 2), where antigenicity analysis is carried out with a threshold value of 1.0 using *in silico* bioinformatics software from the Immune Epitope Database (IEDB) and Analysis Resource, known as the Kolaskar and Tongaonkar antigenicity scale, while with a threshold value of 0.35, Bipipred linear epitope prediction analysis is used from IEDB for epitope mapping (Oany AR et al., 2014;

Sanchez-Trinchado JL et al., 2017). Pyre and Pymol software is used to visualize epitope regions in protein structures (Khasrisma VD et al., 2020).

4.5.2 SARS-CoV 2 Protein Epitope Immunogenicity Test

4.5.2.1 SARS-CoV 2 Protein Epitome and ISCOM Fusion Procedure

The vaccine preparation method is adapted and modified from Mowat AM et al., 2001, where 10 mg of SARS-CoV 2 protein epitope (peptide) in 5 ml of 0.2 M, pH 7.4 Phosphate-buffered Saline (PBS) and homogenized with vortex. Then 10 mg of Quill saponin A (Solution A) is added and homogenized using a vortex. 200 uL of Solution B (1% fosfatidikolon dissolved in 20% lecithin egg yolk and 1% cholesterol) is added to the homogenized peptide and Solution A mixture and again homogenized using a vortex. At room temperature, this mixture is dialyzed in 0.2M PBS of pH 7.4 for 3 hours. Next, it is dialysis solution is changed and the mixture is dialyzed again overnight at 4^{o}C.

Thereafter, at 10,000 g speed, the dialysate is centrifuges at 4^{o}C for 5 minutes, then the centrifuged pellets are resuspended in 25% sucrose diluted at a ratio of 1:1 in 0.2 M PBS of pH 7.4. For two hours, the suspension is then centrifuged in an ultracentrifuge with the speed of 257,000 x g at 4^{o}C. The supernatant is transferred to a different tube while the 2.5 ml PBS is added to the pallets. Nanodrop is used to check the resuspension of pallets, and when the result is positive, Transmission Electron Microscopy (TEM) microscope is used to confirm. Likewise, the supernatant is checked using nanodrop and when positive, TEM microscope is used to reconfirm.

The best results from the supernatant and pallet are used for immunization (Mowat AM et al., 2001).

4.5.2.2 Immunization

BALB/c mice (n=25) aged between 6 to 8 weeks are divided into two groups consisting of 5 mice each. The first group (control group) mice were given 100 µL Phosphate-buffered Saline (PBS) orally through a feeding tube weekly once, for 28 days (4 weeks). The second group of mice were immunized with 100 µL ISCOM orally once a week, for 28 days (4 weeks). The mice of third group were immunized with peptide epitope A conjugated ISCOM at a dose of 30 µg/ 100 µL PBS orally once a week, for 28 days (4 weeks). The mice of fourth group were immunized with peptide epitope B and C conjugated ISCOM at a dose of 30 µg/ 100 µL PBS orally once a week, for 28 days (4 weeks). The final fifth group of mice were immunized with epitope (peptide) A, B and C conjugated ISCOM at a dose of 30 µg/ 100 µL PBS orally once a week, for 28 days (4 weeks). Seven days (1 week) after the last immunization, the mice of both groups were killed by cervical dislocation method (Setyorini D et al., 2013).

4.5.2.3 Measurement of s-IgA and β-defensin Levels

For lungs mucus preparation, lungs were cut into pieces and washed with cold PBS containing 25 µg/ml protease inhibitor cocktail and 1.0 mM EDTA. The lungs is homogenized and collected in tubes containing sterile PBS and protease inhibitors. The suspension is shaken and centrifuged at 12,000 rpm at 4^{o}C for 10 minutes. The

supernatant is purified, where it is resuspended and dialyzed with PBS and is used as samples to measure s-IgA and β-defensin by ELISA (Homenta H et al., 2014).

Using enzyme-linked immunosorbent assay (ELISA) method, levels of s-IgA and β-defensin in bronchus and intestine mucus samples are measured with NovaTEinBio ELISA kit. Before using, the kit is removed from storage temperature of 2-8^{o}C and left at room temperature for 30 minutes. Wash solution is prepared using 1:20 dilution of distilled water and wells are arranged into plates/ strips. 50 µL of diluent sample is put into the first well as a blank, followed by 50 µL of diluent sample in all standard and sample wells. Standard sample of 50 µL is put into second to seventh well (six wells), and 100 µL HRP-conjugated antibody is added into each well then covered with aluminum foil and incubated using agitation at 37^{O}C for 60 minutes (1 hour). Subsequently, 50 µL sample is put into sample well and is washed with washing solution for 5 times. After that, 50 µL chromogenic A substrate and 50 µL chromogenic B substrate is added into each well, closed with aluminum foil to prevent destruction caused by light (protection from light) and put in a shaker incubator and incubated at 37^{O}C for 15 minutes. Later, 50 µL stop solution is added to each well where the reaction is stopped causing blue solution to turn yellow and is immediately observed within 15 minutes at optical density, OD of 450 nm using ELISA reader. The measured concentration is calculated using linear regression from the absorbance results (Setyorini D et al., 2013).

4.6 Working Diagram

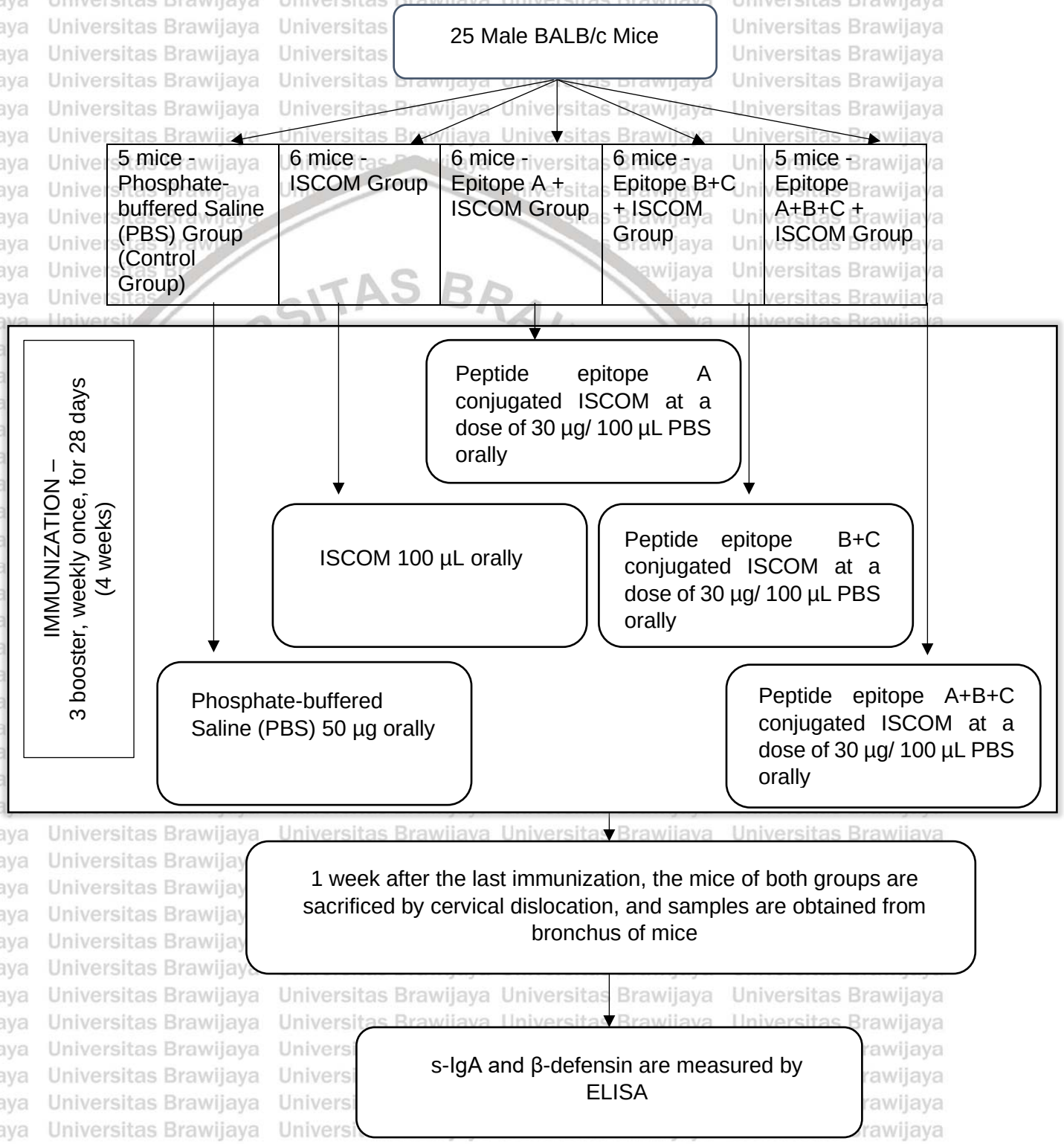

Figure 4.1 Working Diagram

4.7 Data Processing

4.7.1 Data Analysis

SPSS 16.0 for Windows is used to analyze levels of s-IgA and β-defensin. First statistical test used is *Shapiro Wilk* normality test. When a normal result is obtained, homogeneity test is next carried out using *Levene* Test. Results that are normally distributed and homogenous (meets the parametric test requirements) are analyzed using *One Way Anova*. On condition that a significant difference in parametric data is found, *Duncan* test is further carried out. If the data is normal but not homogenous, *Welch* test is carried out. When a significant data is obtained, the test is with *Gomes-Howell* test (Singh A, 2015).

At which the data that does not fulfil parametric test conditions, the data is further transformed. Transformed data that are not normally distributed are tested using nonparametric test and analyzed by *Shapiro Wilk*. When a significant (sig > 0.05) result is obtained, then *Mann-Whitney* test is carried out, but the analysis is stopped when non-significant (sig > 0.05) results are derived.

CHAPTER 5
RESULTS AND DATA ANALYSIS

5.1 The Effect of Immunization of SAR-CoV 2 Protein Antigenic Epitope; Epitope A, Epitope B, Epitope C, on the Levels of Secretory IgA (s-IgA) and β-Defensin in Respiratory Tract Mucosa Layer of BALB/c Mice

5.2 Levels of Secretory IgA (s-IgA) in the Respiratory Tract Mucous Layer

The average levels of secretory IgA (s-IgA) in the respiratory tract mucosa lining of Balb/C mice in each group is explained the the following figure below:

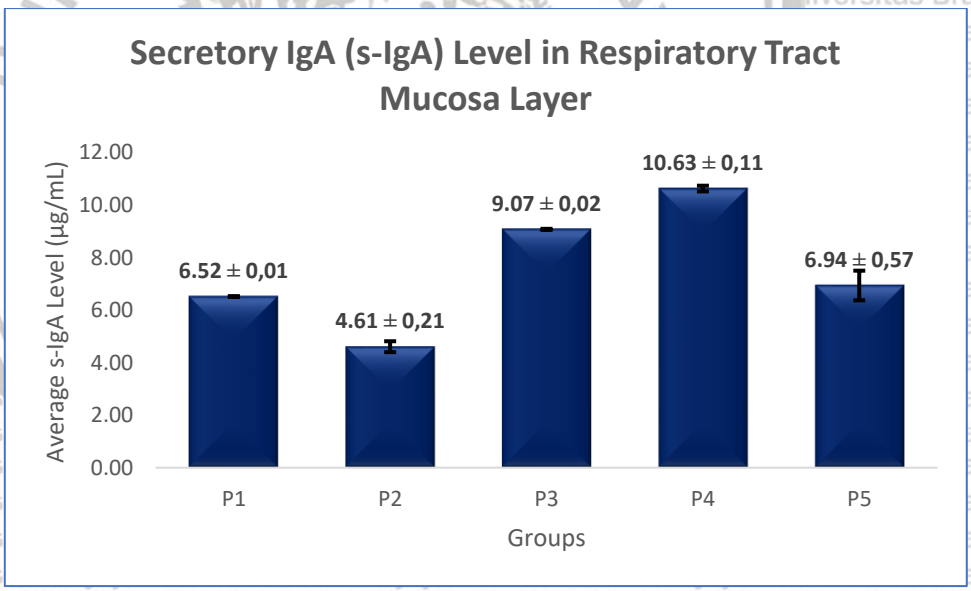

Figure 5.1. Average Levels of Secretory IgA (s-IgA) in Respiratory Tract Mucosa Layer of BALB/c Mice Immunized With 3 Different Epitopes

Description :
P1 - Phosphate-buffered Saline (PBS) Group
P2 - Immune Stimulating Complex (ISCOM) Group
P3 - Spike Epitope (A) Group
P4 - Envelope Epitope (B) + Membrane Epitope (C) Group
P5 - Spike Epitope (A) + Envelope Epitope (B) + Membrane Epitope (C) Group

The figure shows that the average secretory IgA (s-IgA) produced by mice in group P1 (Phosphate-buffered Saline (PBS) Group) is 6.52 ± 0.01 µg/mL, mice in group P2 (Immune Stimulating Complex (ISCOM) Group) produces average s-IgA of 4.61 ± 0.21 µg/mL, while mice immunized by Spike Epitope (A) in group P3 produces 9.07 ± 0.02 µg/mL average s-IgA. Meanwhile, mice from group P4 (Envelope Epitope (B) + Membrane Epitope (C) Group) and P5 (Spike Epitope (A) + Envelope Epitope (B) + Membrane Epitope (C) Group) produces an average s-IgA level of 10.63 ± 0.11 µg/mL and 6.94 ± 0.57 µg/mL, respectively.

Based on the descriptive analysis of the five groups, it is found that the group P4 has the highest average s-IgA level, while group P2 has the lowest average s-IgA levels.

5.3 Levels of β-Defensin in Respiratory Tract Mucous Layer

The average levels of β-defensin in the respiratory tract mucosa lining of Balb/C mice in each group is explained the the following figure below:

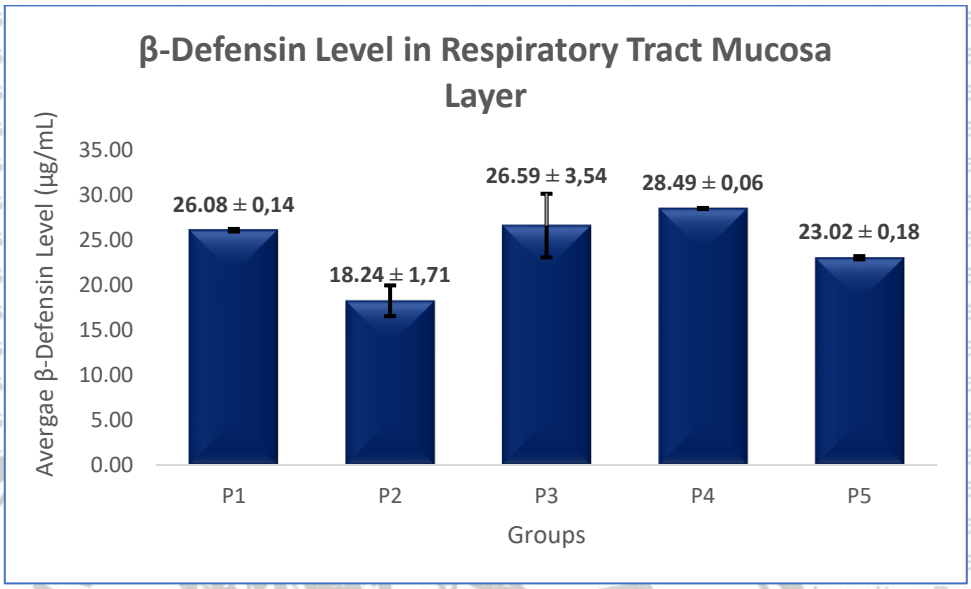

Figure 5.2. Average Levels of β-defensin in Respiratory Tract Mucosa Layer of BALB/c Mice in Immunization With 3 Different Epitopes

Description :
P1 - Phosphate-buffered Saline (PBS) Group
P2 - Immune Stimulating Complex (ISCOM) Group
P3 - Spike Epitope (A) Group
P4 - Envelope Epitope (B) + Membrane Epitope (C) Group
P5 - Spike Epitope (A) + Envelope Epitope (B) + Membrane Epitope (C) Group

The figure shows the average β-defensin levels produced by mice in group P1 (Phosphate-buffered Saline(PBS) Group is 26.08 ± 0.14 µg/mL, mice in group P2 (Immune Stimulating Complex (ISCOM) Group) produces an average β-defensin level of 18.24 ± 1.71 µg/mL, while mice immunized by Spike Epitope (A) in group P3 produces 26.59 ± 3.54 µg/mL average β-defensin. Meanwhile, mice from group P4 (Envelope Epitope (B) + Membrane Epitope (C) Group) and P5 (Spike Epitope (A) + Envelope Epitope (B) + Membrane Epitope (C) Group) produces an average β-defensin level of 28.49 ± 0.06 µg/mL and 23.02 ± 0.18 µg/mL, respectively.

B Based on the descriptive analysis of the five groups, it is found that the group P4 has the highest average β-defensin level, while group P2 has the lowest average β-defensin levels.

5.4 Normality Test for The Effects of Immunization of SAR-CoV 2 Protein Antigenic Epitope; Epitope A, Epitope B and Epitope C, on The Levels of Secretory IgA (s-IgA) and β-Defensin in Respiratory Tract Mucosa Layer of BALB/c Mice

The test of residual normalitu of the effects of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of secretory Ig-A (s-IgA) and β-defensin in respiratory tract mucosa layer of BALB/c mice is aimed to determine whether the residuals generated from the equation have a normal variety or not. The residual normality test was carried out using Shapiro Wilk, with criteria that if the probability value > level of significance (alpha = 5%), then the residual is declared normal. The results of residual normality test are shown in the following table:

Dependent Variable	*Shapiro Wilk*	Probability
Secretory IgA (s-IgA) Level	0.685	0.000
β-Defensin Level	0.701	0.000

Table 5.1. Normality Test of Data between Immunization of SAR-CoV 2 Protein Antigenic Epitope; Epitope Spike Protein SARS-CoV 2 (Epitope A), Epitope Envelope Protein SARS-CoV 2 (Epitope B) and Epitope Membrane protein SARS-CoV 2 (Epitope C), and The Levels of Secretory IgA (s-IgA) and β-Defensin in Respiratory Tract Mucosa Layer of BALB/c Mice

Based on the above table, it is known that the residual normality testing of the effect of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of secretory IgA (s-IgA) generates Shapiro Wilk statistics of 0.685 with a probability of 0.000. Therefore, it is seen that the residual normality test in a probability < alpha (5%), therefore the residual is concluded to have no normal distribution.

Normality testing of the effect of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of β-defensin, generates Shapiro Wilk statistics of 0.701 with a probability of 0.000. Therefore, it is seen that the residual normality test in a probability < alpha (5%), therefore the residual is concluded to have no normal distribution.

5.5 Homogeneity Test for The Effects of Immunization of SAR-CoV 2 Protein Antigenic Epitope; Epitope A, Epitope B and Epitope C, on The Levels of Secretory IgA (s-IgA) and β-Defensin in Respiratory Tract Mucosa Layer of BALB/c Mice

The test of residual homogeneity of the effects of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of secretory Ig-A (s-IgA) and β-defensin in respiratory tract mucosa layer of BALB/c mice is aimed to determine whether the residuals generated from the equation have a homogeneous variety or not. The residual

homogeneity test was carried out using the Levene test, with the criteria if the probability value > level of significance (alpha = 5%), then the residual is declared homogeneous. The results of the residual homogeneity test are shown in the following table:

Dependent Variable	*Levene Statistic*	Probability
Secretory Ig-A (s-IgA) Level	4.912	0.006
β-defensin Level	5.191	0.005

Table 5.2. Homogeneity Test of Data between Immunization of SAR-CoV 2 Protein Antigenic Epitope; Epitope Spike Protein SARS-CoV 2 (Epitope A), Epitope Envelope Protein SARS-CoV 2 (Epitope B) and Epitope Membrane protein SARS-CoV 2 (Epitope C), and The Levels of Secretory IgA (s-IgA) and β-Defensin in Respiratory Tract Mucosa Layer of BALB/c Mice

Based on the above table, it is known that the residual homogeneity testing of the effect of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of secretory IgA (s-IgA) generates Levene statistics of 4.912 with a probability of 0.006. Therefore, it is seen that the residual homogeneity test in a probability < alpha (5%), therefore the residual is concluded to have no homogeneous variety.

Homogeneity testing of the effect of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of β-defensin, generates Levene statistics of 5.191 with a probability of 0.005. Therefore, it is seen that the residual homogeneity test in a

probability < alpha (5%), therefore the residual is concluded to have no homogeneous variety.

5.6 Testing the Differences in the Effect of Immunization of SAR-CoV 2 Protein Antigenic Epitope; Epitope A, Epitope B and Epitope C, against The Levels of Secretory IgA (s-IgA) in Respiratory Tract Mucosa Layer of BALB/c Mice

Testing the difference in the effect of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of secretory IgA (s-IgA) is performed using Kruskall Wallis with the following hypothesis:

H_0 : There is no significant difference in the influence of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of secretory IgA (s-IgA)

H_1 : A minimum of one pair of the effect of the influence of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of secretory IgA (s-IgA) is significantly different

The test criteria state that when the statistical test Chi-square ≥ $\text{Chi-square}_{\text{table}}$ or a probability ≤ level of significance (alpha = 5%), then H_0 is rejected, therefore it can

be stated that a minimum of one pair of the effect of the influence of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of secretory IgA (s-IgA) is significantly different.

The results of testing the difference in the effect of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of secretory IgA (s-IgA) can be seen in the following table :

Chi-Square Statistic	22.503
Probability	0.000

Table 5.3. Chi-Square Test between The Effect of Immunization of SAR-CoV 2 Protein Antigenic Epitope; Epitope Spike Protein SARS-CoV 2 (Epitope A), Epitope Envelope Protein SARS-CoV 2 (Epitope B) and Epitope Membrane protein SARS-CoV 2 (Epitope C), and The Levels of Secretory IgA (s-IgA) in Respiratory Tract Mucosa Layer of BALB/c Mice

The table above shows that the testing difference between the effect of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of secretory IgA (s-IgA) produces Chi-square test statistics of 22.503 with a probability of 0.000. It is known that the probability is < alpha (5%), therefore H_0 is rejected. Hence, it can be stated that a minimum of one pair of the effect of the influence of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope

Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of secretory IgA (s-IgA) is significantly different.

To determine the influence of the effect of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of secretory IgA (s-IgA) with significant difference, Mann Whitney test is carried out with the criteria that one pair results in probability ≤ level of significance (alpha = 5%), then it can be stated that there is a significant difference in the effect of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of secretory IgA (s-IgA). The results of the Mann Whitney test analysis of differences in the effect of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of secretory IgA (s-IgA) are shown in the following table:

Group	Average	Mann Whitney Probability					Notation
		P2	P1	P5	P3	P4	
P2	4.61		0.008	0.008	0.008	0.008	a
P1	6.52	0.008		0.107	0.007	0.007	b
P5	6.94	0.008	0.107		0.007	0.007	b
P3	9.07	0.008	0.007	0.007		0.007	c
P4	10.63	0.008	0.007	0.007	0.007		d

Table 5.4. Mann Whitney Probability Test Between The Effect of Immunization of SAR-CoV 2 Protein Antigenic Epitope; Epitope Spike Protein SARS-CoV 2 (Epitope A), Epitope Envelope Protein SARS-CoV 2 (Epitope B) and Epitope Membrane protein SARS-CoV 2 (Epitope C), and The Levels of Secretory IgA (s-IgA) in Respiratory Tract Mucosa Layer of BALB/c Mice

Description :
P1 - Phosphate-buffered Saline (PBS) Group
P2 - Immune Stimulating Complex (ISCOM) Group
P3 - Spike Epitope (A) Group
P4 - Envelope Epitope (B) + Membrane Epitope (C) Group
P5 - Spike Epitope (A) + Envelope Epitope (B) + Membrane Epitope (C) Group

The result of the above analysis indicates that the group P4 has the highest average s-IgA level and significantly different with all groups, namely group P1, P2, P3 and P5. Meanwhile, group P2 has the lowest average s-IgA level and significantly different with all groups, namely group P1, P3, P4, and P5.

5.7 Testing the Differences in the Effect of Immunization of SAR-CoV 2 Protein Antigenic Epitope; Epitope A, Epitope B and Epitope C, against The Levels of β-Defensin in Respiratory Tract Mucosa Layer of BALB/c Mice

Testing the difference in the effect of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2

(Epitope C), on the levels of β-defensin is performed using Kruskall Wallis with the following hypothesis:

H_0 : There is no significant difference in the influence of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of β-defensin

H_1 : A minimum of one pair of the effect of the influence of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of β-defensin is significantly different

The test criteria state that when the statistical test Chi-square ≥ $\text{Chi-square}_{table}$ or a probability ≤ level of significance (alpha = 5%), then H_0 is rejected, therefore it can be stated that a minimum of one pair of the effect of the influence of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of β-defensin is significantly different.

The results of testing the difference in the effect of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of β-defensin can be seen in the following table :

Chi-Square Statistic	20.476
Probability	0.000

Table 5.5. Chi-Square Test between The Effect of Immunization of SAR-CoV 2 Protein Antigenic Epitope; Epitope Spike Protein SARS-CoV 2 (Epitope A), Epitope Envelope Protein SARS-CoV 2 (Epitope B) and Epitope Membrane protein SARS-CoV 2 (Epitope C), and The Levels of β-Defensin in Respiratory Tract Mucosa Layer of BALB/c Mice

The table above shows that the testing difference between the effect of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of β-defensin produces Chi-square test statistics of 20.476 with a probability of 0.000. It is known that the probability is < alpha (5%), therefore H_0 is rejected. Hence, it can be stated that a minimum of one pair of the effect of the influence of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of β-defensin is significantly different.

To determine the influence of the effect of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of β-defensin with significant difference, Mann Whitney test is carried out with the criteria that one pair results in probability ≤ level of significance (alpha = 5%), then it can be stated that there is a significant difference in the effect of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope

Membrane protein SARS-CoV 2 (Epitope C), on the levels of β-defensin. The results of the Mann Whitney test analysis of differences in the effect of immunization of SAR-CoV 2 protein antigenic epitope; Epitope Spike protein SARS-CoV 2 (Epitope A), Epitope Envelope protein SARS-CoV 2 (Epitope B), and Epitope Membrane protein SARS-CoV 2 (Epitope C), on the levels of β-defensin are shown in the following table:

Group	Average	Mann Whitney Probability					Notation
		P2	P5	P1	P3	P4	
P2	18.24		0.008	0.009	0.016	0.008	a
P5	23.02	0.008		0.008	0.112	0.007	bc
P1	26.08	0.009	0.008		0.115	0.008	c
P3	26.59	0.016	0.112	0.115		0.008	c
P4	28.49	0.008	0.007	0.008	0.008		d

Table 5.6. Mann Whitney Probability Test Between The Effect of Immunization of SAR-CoV 2 Protein Antigenic Epitope; Epitope Spike Protein SARS-CoV 2 (Epitope A), Epitope Envelope Protein SARS-CoV 2 (Epitope B) and Epitope Membrane protein SARS-CoV 2 (Epitope C), and The Levels of β-Defensin in Respiratory Tract Mucosa Layer of BALB/c Mice

Description :
P1 - Phosphate-buffered Saline (PBS) Group
P2 - Immune Stimulating Complex (ISCOM) Group
P3 - Spike Epitope (A) Group
P4 - Envelope Epitope (B) + Membrane Epitope (C) Group
P5 - Spike Epitope (A) + Envelope Epitope (B) + Membrane Epitope (C) Group

The result of the above analysis indicates that the group P4 has the highest average β-defensin level and significantly different with all groups, namely group P1, P2, P3 and P5. Meanwhile, group P2 has the lowest average β-defensin level and significantly different with all groups, namely group P1, P3, P4, and P5.

CHAPTER 6

DISSCUSSION

6.1 Disscussion

Epitope-based vaccines are primarily derived to target specific direction of cellular and humoral immune responses, overcoming the difference is virus strains of SAR-CoV 2 (Testa JS and Philip R, 2012). Therefore, targeted immunogenous surface of SAR-CoV 2 are analyzed and mapped via *in silico* bioinformatics software from the Immune Epitope Database (IEDB), giving rise to 3 antigenic protein epitopes namely, Epitope SPIKE SARS-CoV 2= FLVLLPLVSSQCVNL (Epitope A). 2, Epitope Envelope protein SARS-CoV 2= VNSVLLFLAFVVFLLVTLASS (Epitope B) and Epitope Membrane protein SARS-CoV 2= LYIIKLIFLWLLWPVTLACFVLAAVY (Epitope C). Surface presentation of relevant cells and capacity of prospective epitopes to bind appropriate molecules play major role in influencing immunodominance, therefore the choice of active site protein is a major factor in determining the epitopes derived to reach optimal antigen interaction (Sette A and Fikes J, 2003).

Previous studies on SARS-CoV and MERS-CoV vaccines have shown that antibodies targeting spike (S) protein neutralizes the virus and envelope (E) protein is replication-competent therefore, contributes to inducing mucosal immune response in host (Agnihothram S et al., 2014; Yong CY et al., 2019; DeDiego ML et al., 2007). Introduction of epitopes with similar surface of SAR-CoV 2 induces virus specific humoral T cell and B cell responses in host plasma cells, later switching into IgA and β-defensin. This is in line with a recent finding that increasing number of CD4 cells and

natural killer T cells at mucosal inductive sites stimulates Th2 (IL4 cytokines), inducing s-IgA, as well as mast cell activator compound stimulates migration of CD4 cells into T-cell areas of nasopharyngeal-associated lymphoid tissue (NALT) and the development of TH2 and TH17 cells, therefore targeting mucosal tissue cells and capable of releasing pro-inflammatory mediators via oral immunization induces s-IgA and β-defensin. Gut-homing receptors are induced, therefore IgA is secreted in mucosa layer as secretory IgA (s-IgA) and β-defensin (Maslowski KM et al., 2009; Boyaka PN, 2017).

The objective of this study is to determine the increase in humoral immune response when induced by SARs-CoV 2 antigenic epitopes; Epitope Spike protein (Epitope A), Epitope Envelope protein (Epitope B) and Epitope Membrane protein (Epitope C). Similar *in silico* study of multi-epitope peptide vaccine has shown that potential antigenicity and induction of humoral and cellular immune responses against SARS-CoV 2 (Yazdani Z et al., 2020). Another study on Middle East Respiratory Syndrome (MERS) coronovirus infection in mice shows induced mucosal IgA when human beta defensins are used as adjuvants enhancing the immunogenicity of subunit vaccine candidate against MERS-CoV (Kim J et al., 2020).

The results shows that the levels of secretory IgA (s-IgA) and β-defensin produced in the respiratory tract of BALB/c mice that are immunized by all three epitopes show a positive effect in comparison to mice of control group. Therefore, this is evident that a cocktail of major structural protein epitopes such as spike protein, envelope protein and membrane protein induce humoral immune response and mucosal immunity in respiratory tract of mice as well as has bigger potency in

coronavirus pathogenesis. Combination of these structural proteins have shown promising evidence in development of a ideal novel SAR-Cov 2 vaccine (Mahapatra SR et al., 2020).

Nevertheless, mice of control group showed higher levels of s-IgA and β-defensin in comparison to mice of Immune Stimulating Complex (ISCOM) group. This can be explained that free s-IgA in lungs of control group mice are lesser in mice of ISCOM group, with a possibility of hollow ISCOM used to immunize the mice of this group bound to s-IgA in mucosa of intestine, reducing the total s-IgA switched to lungs mucosa layer. Although there is no evidence to support this reaction, Kaufmann SH explains ISCOM is a potent adjuvant when is covalently linked to the antigen or as a fusion protein together with the antigen enhancing immune stimulation, than when used by itself (Kaufmann SH, 1996).

The results of spike epitope group in comparison to the combination of envelope protein epitope and membrane protein epitope group which produces the highest levels of average s-IgA and β-defensin levels, and s-IgA level is significantly different from all the other groups, contradicts to a current study that states spike protein has the highest specificity to binding ACE2 protein receptors in SAR-Cov 2 and is dominantly responsible in neutralizing antibodies with immunodominant epitopes as spike protein latches into cells forcing entry through the cell membrane (Mahapatra SR et al., 2020). As seen in a study with respiratory syncytial virus (RSV) envelope antigens inducing high level of IgA responses, high immune responses are induced by ISCOMs combined with envelope antigenic protein epitope as they indicate prominent mucosal delivery and adjuvant properties of ISCOM, due to functional

property of fusion (F) protein of the virus particle (Hu KF et al., 1998). This is evident in a multi-epitope based vaccines against SAR-CoV 2 study showing E glycoprotein having the highest antigenicity score and the most potent candidate to generate immune response. A recent report by Schoeman and Fielding states that coronaviruses lacking E protein make promising candidate vaccine (Schoeman D and Fielding BC, 2019).

Envelope and membrane epitope group has higher levels of average s-IgA and β-defensin levels when compared to group with all three (spike, envelope and membrane) epitopes used. Spike protein induces neutralizing antibody (NAb) and T-helper 1 (Th1) responses, as well as balances Th1/Th2 responses that suppresses Th2-bias modality (Prompetchara E et al., 2021). The study of balance of Th1 and Th17 effector and peripheral regulatory T cells showed that differentiation of early Th17 also gives rise to subsequent Th1 development, inhibiting T-regulatory (Treg), limiting IL17 production in peripheral organ and lymphatic system (Lohr J et al., 2009). Therefore, based on homeostasis between T-helper 1 (Th1) and T-helper 2 (Th2) activity hypothesis, it is inferred that overreaction of either Th1 or Th2 can down-regulate the other, in this case, spike of T1 can cause down-regulation of Th2 and Th17, causing an overall low production of s-IgA and β-defensin (Kidd P., 2003). Besides that, it is found that T regulatory (T-reg) undergoes suppression activity activated through TLR2 in mice and humans, therefore it decreases adaptive immune responses by enhancing Treg suppressive function (Lui H et al., 2006). In addition, ISCOM is used in this experiment as an mucosal delivery system for peptide epitopes used, however ISCOM itself does not generate immune complexes as antigenic

peptides to stimulate specific immune complexes are absent, explaining lowest s-IgA and β-defensin levels compared to groups with peptides epitope present (Hu KF et al., 1998).

6.2 Limitations of the Study

This study only used lungs samples as a single indicator of measurement of mucosal humoral immunity. Mucosal immunity are primarily accumulated and transited between mucosa-associated lymphoid tissues (MALT), therefore increase mucosal immunity closely corelates to measurement in MALT organs and gut-associated lymphoid tissues (GALT) as the immunizations are given orally. Suggested subsequent research to use indicators from MALT and GALT organs to see the difference in secretory IgA (s-IgA) and β-defensin levels more significantly (Holmgren J and Czerkinsky C, 2005).

Besides that, this study is solely an animal model preclinical study, hence the time course of antibodies with clinical status on human samples are not known. Preclinical and clinical trials are vital in ensuring a successful and safe preventive vaccine, hence proper clinical trials are required especially clinical trials with large control groups such as in phase III and phase IV to achieve a conclusive report (Green DR, 2020). In 1966, the respiratory syncytial virus (RSV) failed as a result of insufficient antibody affinity maturation, therefore clinical trials should not be fast tracked to avoid such phenomenon with COVID-19 vaccine (Glezen WP et al.,1986). Therefore, further research has to be carried out with human samples and larger control groups in line with the World Health Organization's research protocol in clinical trails.

CHAPTER 7

CONCLUSION

7.1 Conclusion

1. The effect of SAR-CoV 2 antigenic protein epitopes A, B and C oral immunization, increases secretory IgA (s-IgA) levels in lungs of BALB/c mice.

2. The effect of SAR-CoV 2 antigenic protein epitopes A, B and C oral immunization, increases β-defensin levels in lungs of BALB/c mice

3. The effect of SAR-CoV 2 antigenic protein epitopes A, B and C oral immunization, increases mucosal immunity homing capabilities in lungs of BALB/c mice.

7.2 Suggestion

1. Further study should be carried out using more than one measurement indicator of mucosal humoral immunity to see significant difference in secretory IgA (s-IgA) and β-defensin levels of other mucosa-associated lymphoid tissues (MALT).

2. Preclinical and clinical studies on human samples should be conducted in accordance with World Health Organization's research protocol in clinical trials to ensure a safe and successful epitope-based COVID-19 vaccine candidate.

3. Inoculation of SAR-CoV 2 antigenic protein epitopes A, B and C should be administered with complete components in line with facilities and infrastructure that compact.

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APPENDICES

Appendix 1: Descriptive Analysis

Descriptive Analysis of Secretory IgA on BALB/c Mice

Descriptive Statistics

Dependent Variable: sIgA

Kelompok	Mean	Std. Deviation	N
P1	6.5166	.00593	5
P2	4.6060	.20820	5
P3	9.0741	.02153	5
P4	10.6282	.10933	5
P5	6.9376	.56534	5
Total	7.5525	2.15101	25

Descriptive Analysis of β defensin on BALB/C mice

Descriptive Statistics

Dependent Variable: β defensin

Kelompok	Mean	Std. Deviation	N
P1	26.0789	.14177	5
P2	18.2364	1.70997	5
P3	26.5889	3.53648	5
P4	28.4866	.06097	5
P5	23.0164	.18224	5
Total	24.4814	3.99405	25

Appendix 2: Testing the Effect of Epitope A, B and C on Level of Secretory Ig-A in BALB/c Mice Lungs'

Residual Normality Test

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Residual for Y1	.302	25	.000	.685	25	.000

a. Lilliefors Significance Correction

Residual Homogeneity Test

Levene's Test of Equality of Error Variances[a]

Dependent Variable: sIgA

F	df1	df2	Sig.
4.912	4	20	.006

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + X

Kruskall Wallis Analysis

Ranks

	Kelompok	N	Mean Rank
sIgA	P1	5	9.00
	P2	5	3.00
	P3	5	18.00
	P4	5	23.00
	P5	5	12.00
	Total	25	

Test Statistics[a,b]

	sIgA
Chi-Square	22.503
df	4
Asymp. Sig.	.000

a. Kruskal Wallis Test

b. Grouping Variable: Kelompok

Multiple Comparison (Post Hoc) – Mann Whitney Test

Ranks

	Kelompok	N	Mean Rank	Sum of Ranks
sIgA	P1	5	8.00	40.00
	P2	5	3.00	15.00
	Total	10		

Test Statistics[a]

	sIgA
Mann-Whitney U	.000
Wilcoxon W	15.000
Z	-2.660
Asymp. Sig. (2-tailed)	.008
Exact Sig. [2*(1-tailed Sig.)]	.008[b]

a. Grouping Variable: Kelompok

b. Not corrected for ties.

Ranks

	Kelompok	N	Mean Rank	Sum of Ranks
sIgA	P1	5	3.00	15.00
	P3	5	8.00	40.00
	Total	10		

Test Statistics[a]

	sIgA
Mann-Whitney U	.000
Wilcoxon W	15.000
Z	-2.694
Asymp. Sig. (2-tailed)	.007
Exact Sig. [2*(1-tailed Sig.)]	.008[b]

a. Grouping Variable: Kelompok

b. Not corrected for ties.

Ranks

	Kelompok	N	Mean Rank	Sum of Ranks
sIgA	P1	5	3.00	15.00
	P4	5	8.00	40.00
	Total	10		

Test Statistics[a]

	sIgA
Mann-Whitney U	.000
Wilcoxon W	15.000
Z	-2.685
Asymp. Sig. (2-tailed)	.007
Exact Sig. [2*(1-tailed Sig.)]	.008[b]

a. Grouping Variable: Kelompok

b. Not corrected for ties.

Ranks

	Kelompok	N	Mean Rank	Sum of Ranks
sIgA	P1	5	4.00	20.00
	P5	5	7.00	35.00
	Total	10		

Test Statistics[a]

	sIgA
Mann-Whitney U	5.000
Wilcoxon W	20.000
Z	-1.611
Asymp. Sig. (2-tailed)	.107
Exact Sig. [2*(1-tailed Sig.)]	.151[b]

a. Grouping Variable: Kelompok

b. Not corrected for ties.

Ranks

	Kelompok	N	Mean Rank	Sum of Ranks
sIgA	P2	5	3.00	15.00
	P3	5	8.00	40.00
	Total	10		

Test Statistics[a]

	sIgA
Mann-Whitney U	.000
Wilcoxon W	15.000
Z	-2.660
Asymp. Sig. (2-tailed)	.008
Exact Sig. [2*(1-tailed Sig.)]	.008[b]

a. Grouping Variable: Kelompok

b. Not corrected for ties.

Ranks

	Kelompok	N	Mean Rank	Sum of Ranks
sIgA	P2	5	3.00	15.00
	P4	5	8.00	40.00
	Total	10		

Test Statistics[a]

	sIgA
Mann-Whitney U	.000
Wilcoxon W	15.000
Z	-2.652
Asymp. Sig. (2-tailed)	.008
Exact Sig. [2*(1-tailed Sig.)]	.008[b]

a. Grouping Variable: Kelompok

b. Not corrected for ties.

Ranks

	Kelompok	N	Mean Rank	Sum of Ranks
sIgA	P2	5	3.00	15.00
	P5	5	8.00	40.00
	Total	10		

Test Statistics[a]

	sIgA
Mann-Whitney U	.000
Wilcoxon W	15.000
Z	-2.652
Asymp. Sig. (2-tailed)	.008
Exact Sig. [2*(1-tailed Sig.)]	.008[b]

a. Grouping Variable: Kelompok

b. Not corrected for ties.

Ranks

	Kelompok	N	Mean Rank	Sum of Ranks
sIgA	P3	5	3.00	15.00
	P4	5	8.00	40.00
	Total	10		

Test Statistics[a]

	sIgA
Mann-Whitney U	.000
Wilcoxon W	15.000
Z	-2.685
Asymp. Sig. (2-tailed)	.007
Exact Sig. [2*(1-tailed Sig.)]	.008[b]

a. Grouping Variable: Kelompok

b. Not corrected for ties.

Ranks

	Kelompok	N	Mean Rank	Sum of Ranks
sIgA	P3	5	8.00	40.00
	P5	5	3.00	15.00
	Total	10		

Test Statistics[a]

	sIgA
Mann-Whitney U	.000
Wilcoxon W	15.000
Z	-2.685
Asymp. Sig. (2-tailed)	.007
Exact Sig. [2*(1-tailed Sig.)]	.008[b]

a. Grouping Variable: Kelompok

b. Not corrected for ties.

Ranks

	Kelompok	N	Mean Rank	Sum of Ranks
sIgA	P4	5	8.00	40.00
	P5	5	3.00	15.00
	Total	10		

Test Statistics[a]

	sIgA
Mann-Whitney U	.000
Wilcoxon W	15.000
Z	-2.677
Asymp. Sig. (2-tailed)	.007
Exact Sig. [2*(1-tailed Sig.)]	.008[b]

a. Grouping Variable: Kelompok

b. Not corrected for ties.

Appendix 3: Testing the Effect of Epitope A, B and C on Levels of β-Defensin in BALB/c Mice Lungs'

Residual Normality Test

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Residual for Y2	.257	25	.000	.701	25	.000

a. Lilliefors Significance Correction

Residual Homogeneity Test

Levene's Test of Equality of Error Variances[a]

Dependent Variable: β defensin

F	df1	df2	Sig.
5.191	4	20	.005

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + X

Kruskall Wallis Analysis

Ranks

	Kelompok	N	Mean Rank
β defensin	P1	5	14.00
	P2	5	3.20
	P3	5	15.80
	P4	5	23.00
	P5	5	9.00
	Total	25	

Test Statistics[a,b]

	β defensin
Chi-Square	20.476
df	4
Asymp. Sig.	.000

a. Kruskal Wallis Test

b. Grouping Variable: Kelompok

Multiple Comparison (Post Hoc) – Mann Whitney Test

Ranks

	Kelompok	N	Mean Rank	Sum of Ranks
β defensin	P1	5	8.00	40.00
	P2	5	3.00	15.00
	Total	10		

Test Statistics[a]

	β defensin
Mann-Whitney U	.000
Wilcoxon W	15.000
Z	-2.619
Asymp. Sig. (2-tailed)	.009
Exact Sig. [2*(1-tailed Sig.)]	.008[b]

a. Grouping Variable: Kelompok

b. Not corrected for ties.

Ranks

	Kelompok	N	Mean Rank	Sum of Ranks
β defensin	P1	5	4.00	20.00
	P3	5	7.00	35.00
	Total	10		

Test Statistics[a]

	β defensin
Mann-Whitney U	5.000
Wilcoxon W	20.000
Z	-1.576
Asymp. Sig. (2-tailed)	.115
Exact Sig. [2*(1-tailed Sig.)]	.151[b]

a. Grouping Variable: Kelompok

b. Not corrected for ties.

Ranks

	Kelompok	N	Mean Rank	Sum of Ranks
β defensin	P1	5	3.00	15.00
	P4	5	8.00	40.00
	Total	10		

Test Statistics[a]

	β defensin
Mann-Whitney U	.000
Wilcoxon W	15.000
Z	-2.660
Asymp. Sig. (2-tailed)	.008
Exact Sig. [2*(1-tailed Sig.)]	.008[b]

a. Grouping Variable: Kelompok

b. Not corrected for ties.

Ranks

	Kelompok	N	Mean Rank	Sum of Ranks
β defensin	P1	5	8.00	40.00
	P5	5	3.00	15.00
	Total	10		

Test Statistics[a]

	β defensin
Mann-Whitney U	.000
Wilcoxon W	15.000
Z	-2.652
Asymp. Sig. (2-tailed)	.008
Exact Sig. [2*(1-tailed Sig.)]	.008[b]

a. Grouping Variable: Kelompok

b. Not corrected for ties.

Ranks

	Kelompok	N	Mean Rank	Sum of Ranks
β defensin	P2	5	3.20	16.00
	P3	5	7.80	39.00
	Total	10		

Test Statistics[a]

	β defensin
Mann-Whitney U	1.000
Wilcoxon W	16.000
Z	-2.410
Asymp. Sig. (2-tailed)	.016
Exact Sig. [2*(1-tailed Sig.)]	.016[b]

a. Grouping Variable: Kelompok

b. Not corrected for ties.

Ranks

	Kelompok	N	Mean Rank	Sum of Ranks
β defensin	P2	5	3.00	15.00
	P4	5	8.00	40.00
	Total	10		

Test Statistics[a]

	β defensin
Mann-Whitney U	.000
Wilcoxon W	15.000
Z	-2.652
Asymp. Sig. (2-tailed)	.008
Exact Sig. [2*(1-tailed Sig.)]	.008[b]

a. Grouping Variable: Kelompok

b. Not corrected for ties.

Ranks

	Kelompok	N	Mean Rank	Sum of Ranks
β defensin	P2	5	3.00	15.00
	P5	5	8.00	40.00
	Total	10		

Test Statistics[a]

	β defensin
Mann-Whitney U	.000
Wilcoxon W	15.000
Z	-2.643
Asymp. Sig. (2-tailed)	.008
Exact Sig. [2*(1-tailed Sig.)]	.008[b]

a. Grouping Variable: Kelompok

b. Not corrected for ties.

Ranks

	Kelompok	N	Mean Rank	Sum of Ranks
β defensin	P3	5	3.00	15.00
	P4	5	8.00	40.00
	Total	10		

Test Statistics[a]

	β defensin
Mann-Whitney U	.000
Wilcoxon W	15.000
Z	-2.660
Asymp. Sig. (2-tailed)	.008
Exact Sig. [2*(1-tailed Sig.)]	.008[b]

a. Grouping Variable: Kelompok

b. Not corrected for ties.

Ranks

	Kelompok	N	Mean Rank	Sum of Ranks
β defensin	P3	5	7.00	35.00
	P5	5	4.00	20.00
	Total	10		

Test Statistics[a]

	β defensin
Mann-Whitney U	5.000
Wilcoxon W	20.000
Z	-1.591
Asymp. Sig. (2-tailed)	.112
Exact Sig. [2*(1-tailed Sig.)]	.151[b]

a. Grouping Variable: Kelompok

b. Not corrected for ties.

Ranks

	Kelompok	N	Mean Rank	Sum of Ranks
β defensin	P4	5	8.00	40.00
	P5	5	3.00	15.00
	Total	10		

Test Statistics[a]

	β defensin
Mann-Whitney U	.000
Wilcoxon W	15.000
Z	-2.685
Asymp. Sig. (2-tailed)	.007
Exact Sig. [2*(1-tailed Sig.)]	.008[b]

a. Grouping Variable: Kelompok

b. Not corrected for ties.