Histopathological Changes in the Lungs of Mice (*Mus musculus*) after Immunization with Inactivated FMD Vaccine Candidates

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Abstract
Foot and Mouth Disease (FMD) is an acute disease that attacks cloven-hoofed animals. The lungs are one of the organs where the FMD virus replicates. Vaccination with inactivated vaccines is one of the efforts to control the disease. The purpose of this study was to determine the level of lung tissue changes after immunization with inactivated FMD vaccine candidates. Histopathological changes were observed using the HE staining method. Inactivated FMD vaccine candidates - this study caused changes in the lung tissue of mice as one of the immune responses.

Keywords: *Foot and Mouth Disease , Histopathological Changes , Lung Tissue.*

1. Introduction

Foot and Mouth Disease is a highly contagious acute disease of the cloven-hoofed species, caused by the FMD virus. Animals infected with the FMD virus will experience lesions on the tongue, muzzle, oral cavity, coronary bands and teats [1]. WOAH (World Organization for Animal Health) states that FMD morbidity reaches 100% with a fairly low mortality of 1-5% in adults and reaches 20% in young livestock. The large number of viruses circulating and then infecting animals on a global scale causes losses in many aspects. The reported global economic losses due to this disease are quite high [2].

Re-emerging FMD in Indonesia in 2022 spread rapidly so that efficient control efforts are needed. The Ministry of Agriculture of the Republic of Indonesia stated that the effort to control the FMD pandemic is through vaccination. The vaccine used is an imported inactivated vaccine that is compatible with the FMD virus serotype currently circulating in Indonesia.

Several vaccines imported by the Indonesian government, none of them have the same serotype and subtype as the FMD virus circulating in Indonesia. For this reason, the development of an inactivated FMD vaccine with local isolates is important to increase protection against the FMD virus circulating in Indonesia [3].

The FMD vaccine development process with the ultimate goal of being a commercial product must ensure that the product does not have adverse and excessive side effects [4]. Based on these reasons, safety testing or safety testing on vaccine candidates is a critical step that must be taken in developing vaccines into commercial products. Safety testing of vaccine candidates can be carried out using the principle of toxicity, namely by observing cell death in the organ tissue of experimental animals. The lungs are the organs used by the virus to replicate so it is important to observe the damage by looking at histopathological changes in the tissue. FMD can cause fatal systemic infections with viruses replicating in all major organs, including the heart, lungs, brain, kidneys, liver, spleen and thymus.

FMD virus inactivation materials that are widely used for vaccine development are binary ethylenimine (BEI) and formaldehyde/formalin. BEI as an inactive material is thought to have side effects related to the immune response [5]. The addition of adjuvants to inactivated vaccines can have an effect on the injection site. At the same time, some adjuvants can also cause serious side effects such as hemolysis, redness, pain, swelling and necrosis [6]. The purpose of this study was to determine the effect of immunization of FMD inactivated vaccine candidates formulated with adjuvants on histopathological changes in the lungs of mice (*Mus musculus*).

2. Materials and Methods

2.1 Place and Materials

This research was conducted at the Pathology Laboratory, Faculty of Veterinary Medicine, Airlangga University. The experimental animals used were BALB/C strains male and female mice (*Mus musculus*), age limit 10-12 weeks. The mices were kept in experimental animal cages that had met the standards and were given ad libitum food and drink. Total 80 mices used in this study, consisted of 40 males and 40 females. The materials used in this study were inactive FMD virus, adjuvant, Unggul II $^{\text{® feed}}$, drinking water, rice husks, 96% alcohol, 70% alcohol, cotton, distilled water, paper towels, 10% formalin buffer , xylol, paraffin, glycerin, Hematoxylin dye, Eosin dye, IFN-γ mouse ELISA Kit, Atropine sulfate, Ketamine and Xylazine.

2.2 FMD Virus Preparation

FMD inactivated virus was formulated and obtained from the Research Center for Vaccine Technology and Development (RCVTD) Laboratory, Institute of Tropical Disease, Airlangga University.

2.3 Provision of Treatment

Male and female mice were each divided into 4 different groups (K1, K2, K3, K4). K1 treatment was an adjuvant (Montanide ISA), K2 was an FMD antigen titer of 10^8 , K3 was adjuvant + titered 10^7 antigen formulation, K4 was adjuvant + 10^6 antigen formulation. The treatment was given 2 times, on D0 (day0) or the first day the study began and D14 (day 14) after the first treatment. Treatment in all groups was given by intramuscular injection into the thigh muscle with an injection volume of 0.1 ml.

Observations of the research indicators were carried out five times: D0, D14, D21, D28 and D56. Sacrifice on each observation day was carried out on 2 mice in each treatment group. Mice necropsies were performed to remove lung organs.

2.4 Sampling

The organ removal was done by surgery according to the necropsy procedure. BALB/C mice were euthanized by giving Atropine Sulfate as a pre-anesthetic dose of 0.02 mg/kg/im then a combination of ketamine 60 mg and xylazine 16 mg was given intramuscularly. After the mice were anesthetized, all four legs were fixed on the preparation board using a needle in a supine position. Then the mice were skinned by injuring the skin of the abdominal area using tweezers and scissors then cut in a medial direction. Then the skin and subcutaneous were separated using the base of the scapel, then the detached skin was stretched and fixed using a needle. The abdominal muscles were incised until the viscera organs were visible, the incision was made slowly. The lungs will be visible on the thorax of the mouse's body and isolated using sharp blunt scissors and anatomical tweezers.

2.5 Preparation of Hematoxylin Eosin (HE) preparations

The lung organs were fixed with 10% formalin and then washed with running water for 30 minutes. The next process is dehydration and clearing by inserting reagents in the order of 70% alcohol, 80%, 96%, absolute alcohol I, II, and II, xylol I and II for 30 minutes each. Paraffin blocks were formed with liquid paraffin at a temperature of 60 ºC for 2 hours. Hematoxylin eosin staining was done using the Harris method. The tissue that had been stained on the object glass was covered with a cover glass that had been dripped with Canada Balsam as a transparent adhesive [7].

2.5 Examination of Histopathological Changes

Histopathological examination of the liver, heart and lungs using a microscope with a magnification of 400x with hematoxylin eosin staining. The level of change will be calculated as a percentage in each of the five fields of view randomly using the scoring method. 0 (normal) no change, 1 (mild) there is <30% change, 2 (moderate) 30-50% change and 3 (severe) >50% change [8].

3. Results

Examination of histopathological changes in the lungs of mice (*Mus musculus*) was carried out using the scoring method on organ preparations with HE staining. The results of identification and scoring of the lungs of mice (*Mus musculus*) in treatment groups K1, K2, K3 and K4 on the 0th observation day showed a normal organ picture with minimal changes. Histopathological changes in the lungs of mice began on the 14th, 21st, 28th and 56th day of observation. There was a slight increase on the 14th to 56th day. Observations with the highest histopathological changes were found on the 28th day after immunization of the inactive PMK vaccine candidate and there was a decrease on the 56th day of observation.

The average results of lung organ scoring of mice (*Mus musculus*) after immunization with the inactivated FMD vaccine candidate are presented in Table 1.

Table 1. Average results of scoring histopathological changes in the heart of mice (*Mus musculus*) after immunization with inactivated vaccine candidate for Foot and Mouth Disease (FMD).

K	D0	D14	D21	D28	D56
	Median	Median	Median	Median	Median
K1B	0	0.1	0.5	0.7	0.9
K2B	0	0.1	0.8	1.3	1.0
K3B	0	0.4	0.8	0.9	0.9
K4B	0	0.4	0.7	1.1	1.1
K1J	0	0.0	0.8	1.2	1.0
K2J	0	0.4	0.5	1.1	0.8
K3J	0	0.5	1.1	0.9	1.1
K4J	0	0.1	1.2	0.9	1.2

Description: K : Group; D : Day; B : Female; J: Male.

Results of data analysis using the ANOVA General Linear Model test on the lungs of mice (*Mus musculus*) after immunization with the inactivated Foot and Mouth Disease (FMD) vaccine candidate is presented in Figure 1.

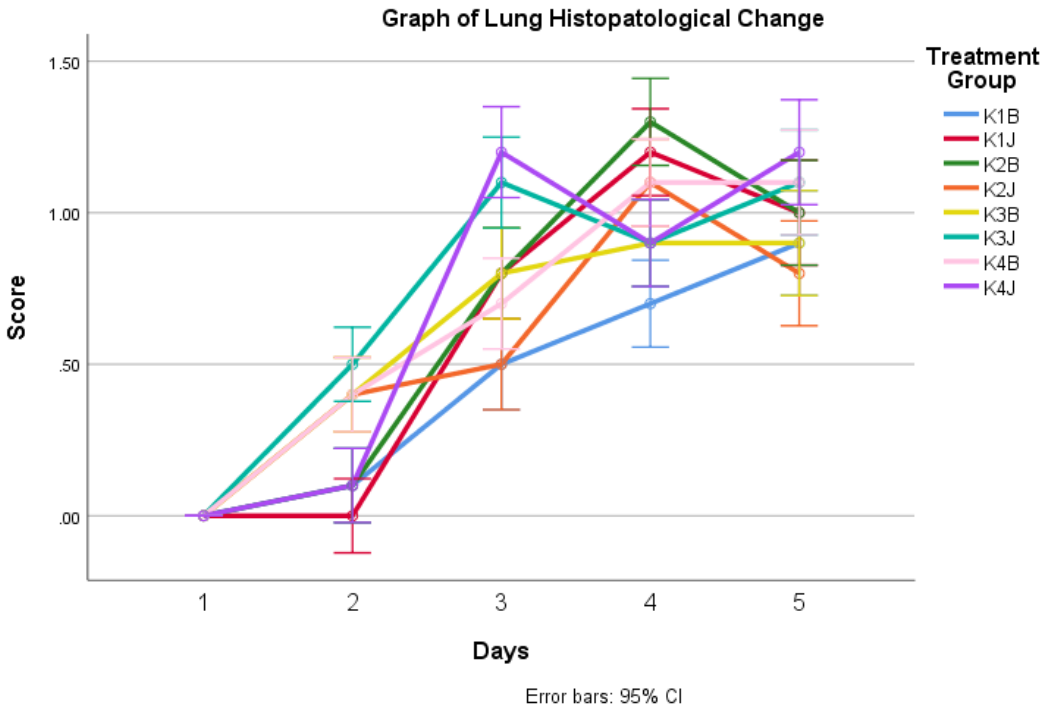

Figure 1 The pattern of increasing and decreasing histopathological changes in the heart of mice (*Mus musculus*) after immunization with inactivated FMD vaccine candidates. Description: B: Female; J: Male.

Treatment groups K1B and K1J began to show an increase in histopathological change scores on the 14th day of observation and continued to increase until the 28th day of observation. The scores shown by K1B and K1J were quite low compared to the other groups. K1B then showed a decrease on the 56th day and K1J still showed an increase until the 56th day of observation.

The K2B and K2J treatment groups showed an increase in histopathological change scores on the 14th observation day with higher scores compared to K1B and K1J. K2B and K2J experienced a significant increase on the 21st day and peaked on the 28th day. The K2B and K2J treatment groups then experienced a decrease in histopathological change scores on the 56th day.

The K3B and K3J treatment groups experienced an increase in histopathological change scores which also started on the 14th day. The K3B and K3J treatment groups had quite high scores starting from the 14th day of observation. K3B showed a constant increase slowly until the 28th day of observation and showed a decrease on the 56th day of observation. The K3J treatment group showed a slight increase in histopathological change scores until the 21st day. The K3J treatment group then experienced a significant increase in histopathological change scores on the 28th day of observation and then decreased on the 56th day of observation.

The K4B and K4J treatment groups began to experience an increase in histopathological change scores on the 14th observation day and continued to increase until the 21st observation day. The K4B treatment group showed an increase until the 28th observation day and a decrease on the 56th observation day. The K4J treatment group experienced a constant score and tended to decrease slightly on the 28th observation day compared to the 21st observation day and continued to show a decrease on the 56th observation day.

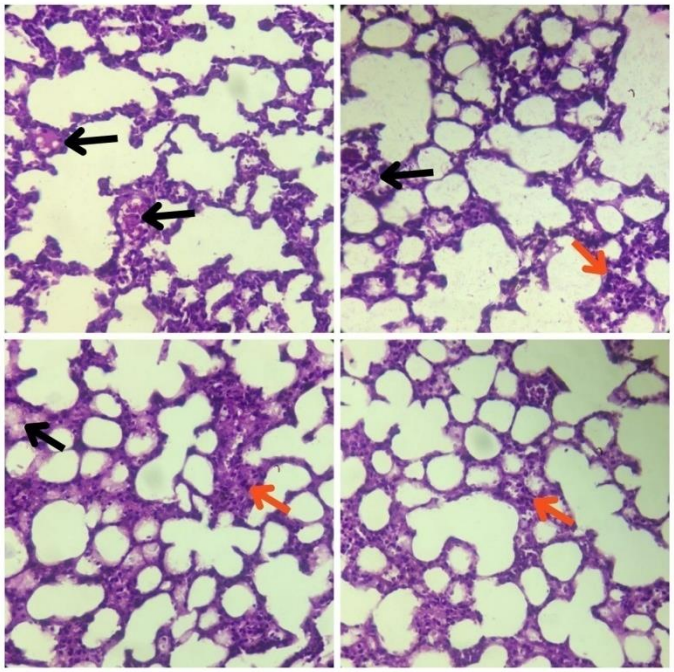

Figure2 Histopathological images of female mouse lung tissue . K1 (top left), K2 (top right), K3 (bottom left) and K4 (bottom right). Black arrows indicate pulmonary edema. Orange arrows indicate inflammatory cell infiltration.

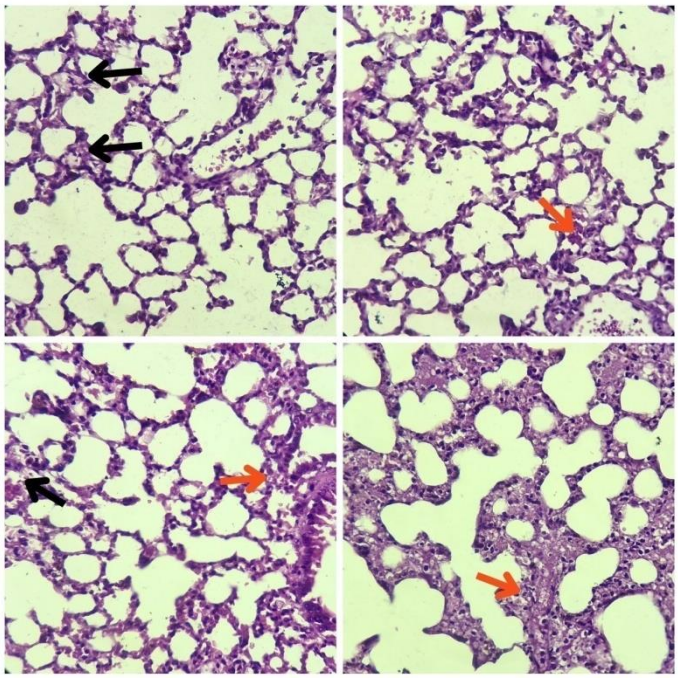

Figure3 Histopathological images of male mouse lung tissue . K1 (top left), K2 (top right), K3 (bottom left) and K4 (bottom right). Black arrows indicate pulmonary edema. Orange arrows indicate inflammatory cell infiltration.

4. Discussion

Histopathological changes in the lungs can occur due to various conditions such as infection, inflammation, injury, autoimmune diseases, and chronic exposure to pollutants or allergens. Each condition can trigger certain different histopathological characteristics. Some of the histopathological changes observed in this study were pulmonary edema, inflammatory cell infiltration, vascular congestion and alveolar hemorrhage. Pulmonary edema is the accumulation of fluid in the alveoli that interferes with gas exchange and causes respiratory distress [9].

Pulmonary edema can be caused by the presence of inflammatory processes and excessive immune reactions. Infiltration of inflammatory cells in the lungs is an immune process where immune cells will move to lung tissue in response to injury, infection or other immune stimuli. This process is part of the body's defense mechanism to fight pathogens, repair damaged tissue, or trigger other immune reactions [10].

The increase in histopathological changes in the lungs of mice starting on day 14 indicates that there is an immune response in the body of mice after immunization with the inactive FMD vaccine candidate. The immune response triggered by vaccination can cause pathological changes in lung tissue. This reaction may occur due to hypersensitivity or inflammation mediated by the immune system triggered by antigens in the vaccine or adjuvant components [11]. Some histopathological conditions that may be found in studies related to vaccine safety are the occurrence of inflammatory cell infiltration in the walls of the alveoli and bronchioles.

The increase that occurred on day 21 and day 28 indicated that the immune response induced by the inactivated PMK vaccine candidate persisted and even increased until day 28. This was due to the presence of adjuvants added to the vaccine formulation so that it could maintain the immune response in the mice's body [12]. In addition, the administration of boosters on day 14 also affected the resistance of the immune response in the mice's body. Adjuvants that can provide antigen exposure for a long time are responsible for the antigen exposure time in the mice's body. As long as the adjuvant can still maintain antigen exposure in the body, the immune response to the vaccine will be formed [13][12]. Histopathological changes that peaked on the 28th day of observation are a sign of maximum immune activity. In this process, the body tries very hard to fight antigens that are considered a threat.

The decrease in the value of histopathological changes in the lungs of mice after immunization with the inactivated FMD vaccine candidate occurred on the 56th day. This decrease indicates that there has been an adjustment by the immune system in the body to the antigen given. In this process, inflammation begins to subside. Immune cells such as regulatory T cells and anti-inflammatory responses have begun to work to reduce inflammation and begin to repair previously damaged tissue. Treg cells play an important role in controlling and regulating the immune response, including reducing inflammation and increasing tolerance. These cells will likely continue to function, contributing to continuing to reduce inflammation and repair tissue [14].

The K1 treatment group given adjuvant had the lowest histopathological change value but lasted longer. This is because adjuvants can induce immune responses but not on a large scale in the absence of antigens. Adjuvants have an important role in extending the duration of the immune response by increasing the activation of innate immune cells, strengthening T and B cell responses, and prolonging the formation of memory cells. In this way, adjuvants allow vaccines to provide longer and more effective protection, especially when the antigen itself is not strong enough to stimulate a long-lasting immune response [15][16].

The K2 treatment group was given a high titer of viral antigen, namely 10^8. Inactive antigens with high titers can affect the histopathological picture of the lungs of mice through immunological mechanisms involving infiltration of inflammatory cells and changes in tissue structure [17]. In general, inactive antigens themselves are usually not pathogenic because they have been killed or deactivated, but the immune response produced by the body to this antigen can cause histological changes in tissue, including the lungs.

FMD virus that replicates in the lungs can affect the histopathology of mouse lungs through several mechanisms, such as inflammatory cell infiltration. The immune system will recognize antigens that have been immunized in the body which can trigger immune reactions involving various immune cells such as macrophages, neutrophils and T and B lymphocytes. Inflammatory cell infiltration can cause changes in lung tissue such as swelling, thickening of the alveolar walls and lung tissue damage [18]. Pulmonary edema is caused by increased capillary permeability due to inflammation triggered by inactive antigens which is characterized by fluid accumulation in the alveoli [10].

In certain conditions, acute inflammation caused by immune reactions to inactive antigens or adjuvants can trigger damage to alveolar capillaries, resulting in bleeding in the alveoli [19].

Groups K3 and K4 have different histopathological change mechanisms. Treatment group K3, which was given a higher antigen titer than K4, which was 10^7, experienced an increase starting from day 14 and continued to increase until day 21. However, group K3 experienced a decrease on day 28 and then increased again on day 56. This indicates that the immune response induced by K3 treatment has a faster response compared to K4 and experiences immunological adaptation that is also faster than K4. The increase in the value of histopathological changes in K3 that occurred on day 56 was likely due to the ongoing inflammatory reaction from the adjuvant. Montanide ISA, which is an adjuvant in oil form, can induce a strong immune response for a longer time [20].

The K4 treatment group formulated with a lower titer, 10^6 , experienced a continuous increase from the 14th, 21st, 28th observation days to the last observation day, namely the 56th day. The K4 treatment group also showed that the changes experienced did not immediately increase like the other groups, but increased slowly but consistently. This is because the antigen dose formulated for K4 is lower than K3. Lower antigen titers are known to induce immune responses that are not stronger than high antigen titers because the number of viruses that must be handled by the immune response is also much lower. Histopathological changes as an effect of the immune response induced by low titer antigens are not higher than those with high titer antigens.

The adjuvant used in the formulation of the inactivated PMK vaccine is Montanide ISA which is a type of water in oil adjuvant. This adjuvant has a working mechanism as a depot effect that can keep the antigen in place and release it slowly and help keep the antigen in its original form longer, thereby increasing its immunogenicity [21]. In addition, this adjuvant can stimulate innate immune cells such as macrophages and dendritic cells, triggering the release of pro-inflammatory cytokines that can strengthen the activation of other immune cells [16].

The viral antigen used in this study is an antigen from the whole virus. Whole virus antigen uses the entire inactivated or “killed” virus. This shows that there is still a possibility of a protein that can be “toxic” from the virus included in the vaccine formulation. Viral proteins that can be toxic can help the virus avoid the immune response, replicate and/or destroy host cells to spread further virus particles. Some proteins from FMD viruses that can potentially be toxic are structural proteins that form capsids, Lpro which breaks intracellular communication of host cells and immune responses and 3Cpro which can break down host proteins [22][23][24].

5. Conclusion

Based on this research, it can be concluded that immunization of inactivated FMD vaccine candidates can induce immune responses. Histopathological changes in the lungs of mice increased after immunization of inactivated FMD vaccine candidates. The peak increase occurred on day 28 with K3 and K4 having the highest level of change.

Conflict of Interest

The authors have no conflict of interest regarding this investigation.

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