The immune responds of balb/C Mice on antigen recombinant fim-C inclusion bodies salmonella typhi protein emulsified with alumina adjuvant

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Abstract. Typhoid fever is still a world health problem, especially in developing countries, including Indonesia. One way to prevent typhoid fever is by vaccination. Different variations have been developed to improve the efficacy of the process. An important part of the current focus is the appropriate adjuvant selection in enhancing the immune response. The purpose of this study is to evaluate the ability of Fim-C Salmonella typhi protein emulsified with adjuvant alumina as candidate vaccine typhus against the immune response generated of Balb-C mice. This study was conducted on 25 male Balb-C mice grouped into 5 groups. That was a normal group (not treated); Control group 1 (given PBS 1x); control group 2 (given alumina); sample group-1 (given recombinant protein Fim-C inclusion bodies S. Typhi and PBS 1x); sample group-2 (given recombinant protein Fim-C inclusion bodies S. Typhi and alumina). Anti-Fim-C antibodies produced by Balb-C mice group 1 (KS-1) and group 2 (KS-2) samples began to increase after the test animals were given Fim-C antigen by 20 μg and experienced a significant increase after given an antigen of 40 μg. The results of immunogenicity testing by ELISA and western immunoblotting methods showed that the anti-Fim-C antibody produced by Balb-C mice was precise to recombinant Fim-C inclusion bodies S. Typhi protein as antigen. Based on the data, it was concluded that the Fim-C Inclusion bodies S. Typhi protein emulsified with Alumina adjuvant can produce a good and specific immune response, potentially as a candidate of typhoid vaccine.

1. Introduction

Typhoid fever is a systemic disease caused by pathogenic bacteria Salmonella enterica serovar Typhi (S. Typhi). Salmonella typhi enters the digestive tract through contaminated food and drinks. One way to prevent typhoid fever is to use a vaccine. At present, there are two types of vaccines used for typhoid fever, Ty21a (oral) and Vi (injection) polysaccharides [1]. Both vaccines are derived from the weakened S. Typhi bacteria. These vaccines turned out to have side effects on users, such as: nausea, vomiting, abdominal discomfort, fever, and headaches. Therefore, a safer and more efficient vaccine is.
Recombinant vaccine is one of the alternatives offered to prevent typhoid fever. That are vaccines produced from a particular gene or protein that is potential in the mechanism of pathogenesis and produced with genetic engineering technology. One of the proteins in S. Typhi that was successfully identified as having a role in the mechanism of pathogenesis is Fim-C protein [2, 3]. The Fim-C or fimbriae protein is a surface protein of S. Typhi, which is used to attach itself to host cells and several other objects. Based on the literature surface proteins can induce a good immune response [3].

As it is known that one of the substances commonly added to help increase the immune response in recombinant protein products is adjuvants. Research on the selection of appropriate adjuvants for a recombinant protein is still widely developed [2]. In a previous study, research on the addition of Freund's Complete/Incomplete Adjuvant (FCA/FIA) was conducted on the production and characterization of Salmonella typhi anti-Fim-C antibodies in ddY mice. The results of this study provide information that the addition of FCA/FIA to the Fim-C-S. Typhi recombinant protein produces an excellent immune response [3, 4]. However, Freund's Complete Adjuvant is known to contain the bacterium Mycobacterium tuberculosis which causes tuberculosis, so that currently the WHO regulation does not recommend the use of adjuvants for humans.

Research on determining the proper adjuvants in vaccine development is still being carried out, because adjuvants used as emulsifiers in the immunization process must be safe, not toxic, and can enhance the immune response for humans. Alumina is one example of adjuvants that have been used for medicine since 1930 [5, 6]. Shaw and Feinberg [7] showed that Alumina Adjuvant or Alum serves two main purposes as an adjuvant. First, it acts as an antigen depot. Vaccine antigens adsorb to alum and elute from it following injection into the host. Second, alum acts a mild irritant, causing the recruitment of leukocytes necessary for generation of an immune response to the site of injection. Adsorption of antigens on to alum routinely improves immunogenicity, particularly the antibody response [7]. Other studies also show that alum treatment leads to the release of intracellular molecules at sites of injection, some of which could potentially be recognized as DAMPs (Damage-associated molecular patterns) [8]. This adjuvant was found to increase the immunogenicity of a mycobacterial vaccine antigen, and offered a comparable protective efficacy to the standard BCG vaccine in mice [9].

In a different study, IC31 was shown to increase the HI titters and induction of IFN producing CD4+T cells after single vaccination with influenza vaccine in both young and aged mice. Alum has been a component of many vaccines for decades and has an excellent safety record, although its mechanism of action is only now being defined in detail [10]. Based on WHO regulation and some finding from several previous researchers, this study was conducted to determine the effect of the addition of alumina adjuvants on the Fim-C-S. Typhi recombinant protein on the formation of immune responses produced by test animals. The outcome of the research is expected to provide information about alternative uses of Fim-C-S. Typhi as a candidate for typhoid vaccine in humans especially from local product.

2. Methods

2.1. Production of Inclusion Bodies Recombinant Fim-C-S. Typhi Protein
Recombinant protein production in this study refers to previous studies. The stages of production of Inclusion Bodies Fim-C-S. Typhi proteins are (1) Making Inoculum; (2) Overexpression of Fim-C-S. Typhi; (3) Isolation of Fim-C-S. Typhi; (4) Purification of Fim-C-S. Typhi Protein; Measurement of Protein Concentration and (5) Characterization of Recombinant Protein S. Typhi Fim-C with SDS-PAGE [3, 4, 11].

2.2. Testing of Fim-C Inclusion of Salmonella typhi Protein and Adjuvant Alumina in Balb/C Mice
Tests were carried out on Balb / C mice, aged 4-6 weeks, weighing 20-30 grams, number of 25. Experiments began with the acclimatization process following standard procedures for seven days [4, 11, 12]. Mice are grouped into three groups, namely the normal group (KN), the control group (KK), and the sample group (KS). The normal group consisted of five mice, a group of mice who did not inject anything. The control group has two sub groups, namely the group injected PBS 1x (KK-1), and the
group injected with Alumina Adjuvant (KK-2). Both groups consisted of five mice. The sample group (KS) consisted of two sub groups, namely the group injected with the Fim-C-S. Typhi recombinant protein (KS-1), and the group injected with the Fim-C-S. Typhi recombinant protein mixed with adjuvant alumina (KS -2). Each sample group consisted of five mice. The amount of Fim-C.S. Typhi recombinant protein injected in each mouse from sample group 1 (KS-1) and sample group 2 (KS-2) was 20 μg/20 g weight mice, 40 μg/20 g weight mice, and 60 μg/20 g weight mice. Proteins with a predetermined amount are added with PBS 1x with a volume ratio of 1:1 in KS1 and added alumina adjuvant with a volume ratio of 1:1 in KS-2. Every week after the injection blood is taken from the orbital sinus of the eye. The collected blood was inserted into a 1.5 mL Eppendorf tube containing EDTA to prevent clotting. Anti-Fim-C-S. Typhi antibody is taken from blood plasma, by centrifuging blood at 12000 rpm for 10 minutes. Plasma is separated in a sterile centrifuge tube and stored at -20°C. Testing of Fim-C Inclusion bodies recombinant proteins S. Typhi with Alumina Adjuvants in Balb/C Mice were carried out according to the schedule in Table 1 [3, 4, 12].

Table 1. Schedule for Testing Recombinant Protein Fim-C-S. Typhi Inclusion Bodies.

| Group                          | day-1 | day-2 | day-3 | day-4 |
|--------------------------------|-------|-------|-------|-------|
| Normal                         |       |       |       |       |
| Control 1 (PBS 1x)             |       |       |       |       |
| Control 2 (Alumina)            |       |       |       |       |
| Sample 1 (Fim-C + PBS 1x)      |       |       |       |       |
| Sample 1 (Fim-C + PBS 1x + Alumina) |     |       |       |       |
| Acclimatization                | bleed-0 | 500μl | 500μl | 500μl |
|                                | bleed-1 | 20μg /20g weight | 40μg/40g weight | 60μg/60g weight |
|                                | bleed-2 | bleed-3 | bleed-4 | |

2.3. Analysis of amount of anti-Fim-C-S. Typhi antibody with ELISA technique
Analysis of the number of anti-Fim-C-S. Typhi antibody was carried out on bleed-0 (day 8), bleed-1 (day 16), bleed-2 (day 24), bleed-3 (day 32), and bleed-4 (day 36) of each group [12]. ELISA testing follows previous research procedures [2, 4, 13]. The essential substances at this stage are: (1) Fim-C S. Typhi recombinant protein antigen (100ng/50 μL); (2) Primary antibody: Anti-Fim-C S. Typhi antibody obtained from serum at each bleeds [12]; (3) Secondary antibody: Horse Reddish Peroxidase (4) Substrate: TMB (3, 3', 5, 5'-tetramethylbenzidine); (4) Stopper: Sulphuric acid or H2SO4. Next, the absorbance of the reaction results is read out with an ELISA-reader at a wavelength of 450 nm.

2.4. Analysis of anti-Fim-C-S. Typhi antibody specificity with western immunoblotting
Western immunoblotting procedures use Bio-Rad procedures [14]. Antigens, primary antibodies, and secondary antibodies used are the same as the ELISA process. The dye in the Western Blot technique is DAB.

3. Result and Discussion

3.1. Production and Characterization of Recombinant Fim-C Inclusion Bodies S. Typhi Protein
Recombinant Fim-C-S. Typhi inclusion bodies protein has been successfully produced. Cell culture of E. coli recombinant bacteria BL21 (DE3) pLysS containing pET-30a-fim-C-S. Typhi plasmid was induced for four hours and produced 2.88 grams of cell pellets. Protein isolation yielded four mL of recombinant Fim-C-S. Typhi of inclusion bodies protein extracts. The recombinant Fim-C-S. Typhi protein inclusion bodies were purified by the Immobilized Metal Affinity Chromatography (IMAC) method in columns containing Ni-NTA resin. The purification results produced pure Fim-C Protein S.
Typhi inclusion bodies with the first elution concentration of 270.5 μg/mL. The second elution result was 168.8 μg/mL, and the third elution result were 155.5 μg/mL [15]. The concentration of S. Typhi Fim-C recombinant protein extracted was 2338.3 μg/mL. Based on the data, the obtained recombinant content of S. Typhi Fim-C inclusion bodies was 25.44%. Recombinant Fim-C S. Typhi inclusion bodies was characterized by SDS-PAGE shown in Fig. 1.

Characterization results show that the Fim-C-S. Typhi recombinant protein inclusion bodies has been successfully produced and purified by the presence of protein bands at a molecular weight of ± 31 kDa in lane 5 and single protein bands at a molecular weight of ± 31 kDa in lanes 6. Recombinant protein molecular weight The Fim-C-S. Typhi obtained based on theoretical calculations using the DNAstar program, specifically EditSeq where the Fim-C-S. Typhi fusion protein molecule contains 10 histidine amino acids at the 5’end and 12 amino acids, which are recognized by factor Xa, which ranges in weight 31 kDa molecule [1, 3].

![Results of Characterization of SDS-PAGE of S. Typhi Fim-C Recombinant Protein.](image)

Lane 1: 10 μL marker protein; Lane 2: 20 μL of Fim-C recombinant protein before induction; Lane 3: 20 μL of recombinant Fim-C protein after induction; 4: 20 lane μL extracts of native Fim-C recombinant protein resulting from overexpression with 5 μg protein; Lane 5: 20 μL of Fim-C inclusion body recombinant protein extracts resulting from overexpression with 5 μg protein; 6: 20 μL lane of Fim-C inclusion bodies with 3 μg protein.

3.2. Analysis of Titer Anti-Fim-C Salmonella typhi Antibody

The results of the analysis of the increase in the number of S. Typhi anti-Fim-C antibodies are shown in Fig. 2.
Based on the data presented in Fig. 2, the normal group, control 1, and control 2 have an absorbance value below 0.2. This shows that mice in these groups did not form anti-Fim-C antibodies [12]. The three groups are used as negative controls. In the sample group 1 and sample 2 there was a significant increase in absorbance at bleed-2, after 40 Fim-C inclusion bodies recombinant protein was injected as much as 40 μg/20 g weight mice. These results indicate that the Fim-C-S. Typhi recombinant protein and adjuvant alumina protein produced a good immune response in Balb/C mice and potentially as typhoid vaccine.

The mechanism of alumina induction on the formation of immune responses has been widely stated, one of the models proposed by Ghimire is as follows [6]: Alum immunization leads to recruitment of neutrophil, natural killer cell, macrophage, eosinophil, and immature Dendritic Cell (DC) at the injection site. Immature DCs take up soluble Ag (Antigen) released from alum or particulate Ags mixed in alum in the subcutaneous areas and migrate towards draining lymph node (DLN). Soluble Ag can reach to DLN without help of DCs. In T cell area (paracortex), soluble Ags leaked out of conduits are taken up by resident DCs. DCs present Ag to the naïve T cells or transfer Ag to the resident DCs that present Ag to those T cells. In addition, B cells are capable of binding to this Ag with their surface immunoglobulins. B cells undergo activation, produce effector B cells (eB cells), and rapidly differentiate in plasma cells (PCs). Plasma cells produce low-affinity antibodies (LAb). B cells also migrate to B cell follicle. On the other hand, as a result of CD8 and CD4 T cell activation, effector CD8 (eCD8) T cells and effector CD4 (eCD4) T cells are produced. The eCD4 polarizes into T helper (Th) 1, 2, 17 or T follicular helper (Tfh) cells though alum induces development of mostly Th2 and Tfh cells. Tfh or Th2 may reach to the border of B cell follicle to activate B cells that produce eB cells and then PCs. The PCs consequently produce and secrete high-affinity antibody (HAb). Few alum-fed DCs and eCD4 may travel to efferent lymph vessels to reach into the distant LN in vivo[6, 8, 9]. Figure Model of the mechanism of induction of immune responses caused by recombinant proteins as antigens that are mixed with the alumina adjuvant presented in Fig. 3 [6].
Figure 3. Model of the mechanism of induction of immune responses caused by recombinant proteins as antigens that are mixed with the alumina adjuvant. Detailed descriptions are in the text [6].

The results of the analysis of static antibody formation with one-way ANOVA on the normal group, control 1, and control 2 have p-values (p-value) are 0.968, 0.079, and 0.607 respectively. Because the p-value > 0.05 or (p > 0.05) it can be concluded that the change in the increase in S. Typhi anti-Fim-C antibody production in Balb/C mice in the normal group (KN), control 1 (KK-1), and control 2 (KK-2) does not have a significant difference. In the sample group 1 (KS-1) has a p-value (p-value) of 0.00001, which means that (p < 0.05), it can be concluded that the increase in anti-Fim-C-S. Typhi antibody production in Balb/C mice in the group sample 1 (KS-1) had significant differences on bleed-2 (day-24), bleed-3 (day-32), and bleed-4 (day-36) against bleed-0 (day-8). The sample group 1 (KS-1) also had a significant difference (p < 0.05) to the normal group (KN) with a p-value of 2x10^-10 [12]. Data analysis of the increase of anti-Fim-C antibody production by non-parametric test in sample group 2 (KS-2) was obtained p-value of 0.005. It can be concluded that the change in the increase of anti-Fim-C-S. Typhi antibody production in Balb/C mice in sample group 2 (KS-2) has a significant difference at bleed-2 (day-24), bleed-3 (day-32), and bleed-4 (day 36) against bleed-0 (day 8). If the change in the increase of anti-Fim-C-S. Typhi antibody production in Balb/C mice in sample group 2 (KS-2) was compared to the normal group (KN), then KS-2 has a significant difference to KN with p = 0.005.

3.3. Specificity Analysis of S. Typhi Anti-Fim-C Antibodies with Western Immunoblotting
The results of the specificity analysis of the S. Typhi anti-Fim-C antibody to the Fim-C-S. Typhi antigen (Fim-C-S. Typhi recombinant protein) was shown in Fig. 4.
Figure 4. Results of *S. Typhi* Anti-Fim-C Antibody Characterization with Western Blot.

Lane 1: Thermo scientific™ 10 µL marker protein. Lane 2: 20 µL of pure Fim-C recombinant protein *S. Typhi* inclusion bodies as much as 3 µg of staining was done with DAB, primary antibody dilution was 1: 100x, and secondary antibody dilution were 1: 5000x.

The interaction of Fim-C antigen and anti-Fim-C antibody is characterized by brown precipitate formation on nitrocellulose membranes. The formation of brown color occurs due to the formation of Quinone Iminium compounds resulting from the reaction of the substrate DAB by Horse reddish peroxidase enzymes [11].

4. Conclusions
This study concludes that: (1) Adjuvant alumina and *Salmonella typhi* Fim-C inclusion bodies recombinant protein provided a good immune response in Balb/C mice. Anti-Fim-C antibodies produced by Balb/C mice sample group 1 (KS-1) and sample group 2 (KS-2) began to increase after test animals were given Fim-C antigen as much as 20 µg/20 g weight and experienced a significant increase after being given antigen 40 µg/20 g weight (2). The *S. Typhi* Anti-Fim-C antibody produced specifically for *S. Typhi* Fim-C antigen was proven by the formation of brown bands at a molecular weight of ± 31 kDa. These results can be used as a basis for Adjuvant selection and verification that recombinant protein antigens of Fim-C- *S. Typhi* can be used as candidates for typhoid vaccine.

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