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# Immunogenicity evaluation of recombinant fim-c salmonella typhi protein as typhoid vaccine candidate on wistar rat

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**Abstract.** Typhoid fever is a world health problem and often occurs in developing countries, including Indonesia. In a previous study, the UNJ Salmonella team successfully isolated, cloned, expressed, and purified *inclusion bodies* Fim-C *S. typhi* recombinant protein sized 31 Kilo Dalton (KDa). Furthermore, these proteins have been used as antigen in immunogenicity evaluation with ddY mice as test animals and give excellent results. This study aims to determine the immune response other of rodent test animals against Fim-C *S. typhi* recombinant protein as antigen. Immunogenicity evaluation was performed using male *Wistar* rats. That were divided into five test groups: Normal group, Control Group 1, Control Group 2, and Samples Group 1, Samples Group 2. The results of the ELISA analysis showed an increase in antibody titers produced by *Wistar* rats after subcutaneous immunized with Fim-C protein emulsified adjuvant or without adjuvant. The result of Western Blot showed the specific interaction between *inclusion bodies* Fim-C *S. typhi* recombinant protein as antigen with anti-Fim-C *S. typhi* antibodies. It can be concluded that Fim-C *S. typhi* protein can be used as a potential vaccine candidate for typhoid disease. These results are expected to be an alternative in the discovery of new vaccines.

## 1. Introduction

Typhoid fever is one of the endemic diseases and affects many people in developing countries, including Indonesia [1]. The World Health Organization (WHO) estimates that there are currently 21 million cases of typhoid fever occurring throughout the world with mortality rates ranging from 1 to 4% [2,3]. Along with the increasing cases of typhoid fever, it is necessary efforts to prevent the disease. One of them through vaccination [2]. Currently, two typhoid vaccines of demonstrated safety and efficacy are available on the international market: (1). The oral vaccine based on the live, attenuated mutant strain of *S. typhi* Ty21a (Ty21a vaccine), and (2). The injectable Vi capsular polysaccharide vaccine (ViCPS vaccine) is given Intramuscularly in a single dose [2]. Due to the widespread distribution and diversity of pathogenic *Salmonella* serotype, so that several efforts still need to develop [4, 5].

Literature analysis shows, the biomolecule that is widely developed as a typhoid vaccine is outer membrane protein (Omp) because outer membrane are highly immunogenic and are capable of eliciting protective immune responses [5,6]. Previous studies have reported successful isolation and expression



of recombinant Fim-C protein *Salmonella typhi* measured 31 Kilo Dalton (kDa) in the form of Native and inclusion bodies [7, 8]. Recombinant protein Fim-C *S.typhi* with concentration of 40-60  $\mu\text{g}$  has also been proven able to increase production of anti-Fim-C *S.typhi* antibody and have the ability to protect ddY mice against bacterial infection of *S.typhi*. [8].

This study aims to obtain information regarding the ability of recombinant protein Fim-C *S.typhi* in inducing the formation of antibodies or immunogenicity at the host with higher levels of rodent. The immunogenicity test of recombinant Fim-C *S.typhi* protein was performed on a male Wistar rat model, then observed the changes of body weight, the increase of antibody titre, and the specificity of antibody produced to Fim-C *S.typhi* protein as its antigen [9,10]. The results from this study are expected to provide an alternative product of typhoid fever vaccine that is safe and effective for use by the community, especially in improving the quality of Indonesian society.

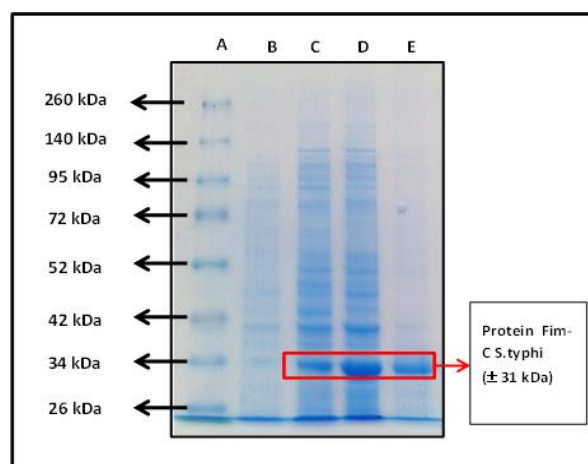
## 2. Methods

This study consists of several stages (1) Productions of Fim-C inclusion bodies *Salmonella typhi* protein according to pET system procedure [11-15]; (2) Characterization of proteins with SDS PAGE according to Laemmli procedure and Amersham Pharmacia [16]; (3) Immunogenicity test in Wistar rats follow The Guide and Harlow and Lane [9,17], consist of (a) The acclimatization process and taking sera pre-immunization, (b) preparation of recombinant protein Fim-C *S. typhi* inclusion bodies as antigen with concentrations of 50-100 micrograms and mixed with Freund's Complete incomplete Adjuvant [18], ((c) Subcutaneous immunization, (d) Isolation of plasma; (4) Analysis of antibody development of anti Fim C *S. typhi* by ELISA method (5) Analysis of anti-Fim-C specificity of *S. typhi* antibody with Western Blot method with DAB staining [19].

## 3. Result and discussion

### 3.1. Production of recombinant fim c salmonella typhi protein

In this study, protein samples of Fim-C Inclusion Bodies *Salmonella typhi* have been successfully produced through protein overexpression with host cell *E. coli BL21 (DE3) pLysS* containing recombinant plasmid pET-30a-Fim-C *S.typhi*. The isolation of overexpressed cells results in a protein extract dissolved in the cytoplasm and forming an aggregate (*Inclusion Bodies*) [12]. The purification of recombinant Fim-C Inclusion Bodies *S.typhi* is done by using Ni-NTA resin columns and yielding pure Protein Fim-C Inclusion Bodies *S.typhi* [14]. The extracted proteins, and the purified results with Ni-NTA system were characterized by SDS-PAGE electrophoresis shown in figure 1 [20].



**Figure 1.** Recombinant Protein Characterization of Fim-C *S.typhi*. Lane A Protein Marker; Lane B extract protein Fim-C *S.typhi* before induction; Lane C recombinant protein Fim-C *S.typhi* in native form; Lane D recombinant protein Fim-C *S.typhi* inclusion bodies; Lane E recombinant protein Fim-C *S.typhi* after purification with Ni-NTA.

The characterization results show the presence of a single band on line E with a molecular weight of  $\pm 31$  kDa. This suggests that the process of overexpression of the Fim-C Inclusion Bodies *S.typhi* protein has been successfully performed [7, 8,12,14].

### 3.2. Production of anti-fim-c antibodies *s. Typhi* in wistar rats

**3.2.1. Preparation of test animals and pre immune blood plasma.** During rat conditioning fed and drank regularly monitored the condition of the room as well as its physical kindness and weighing weight according to the Guide [9]. Weighing results shows each rat in each group experienced a rise indicating a mouse can adapt well to its new environment. The results of pre-immune plasma blood taking from the orbital sinuses result in 0.2-0.5 mL of blood [9, 17], the resulting plasma is 0.1-0.2 mL of the total blood taken. The result stored at  $-20^{\circ}\text{C}$ .

**3.2.2. Injection of antigen.** The injection process was performed on subcutaneous groups of mice, namely on the front of the back near the head. The Normal Group (KN) consists of 5 untreated rats; The Control Group 1 (KK1) consisted of 5 mice injected with PBS 1x; The Control Group 2 (KK2) consisted of 5 mice injected with Freund's Complete/ incomplete adjuvant; Sample Group 1 (KS1) consisted of 5 mice injected with recombinant protein Fim-C Inclusion bodies *S. typhi* dissolved with PBS1x; Sample Group 2 (KS2) consisted of 5 mice injected with recombinant protein Fim-C Inclusion bodies *S. typhi* diluted in PBS 1X plus Freund's Complete/incomplete adjuvant. Injecting recombinant protein dose Fim-C Inclusion bodies *S. typhi* as antigen consecutively as much as 50  $\mu\text{g}/200$  g weight, 75  $\mu\text{g}/200$  g weight, and 100  $\mu\text{g}/200$  g weight with booster every 8 days [9].

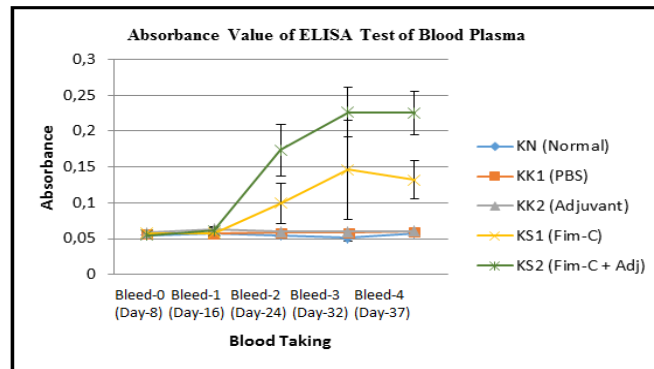
Observation of the injection process on body weight of mice showed that normal group mice, control group and treatment/sample group had increased. This means that injecting PBS buffers, Freund's Complete/incomplete Adjuvant, and recombinant Fim-C *S.typhi* proteins do not affect the diet of mice, and mice remain healthy [7, 9, 17].

**3.2.3. Blood plasma taking after injection of antigen.** Blood sampling is done one week after antigen injection, blood collection is performed for antibody production analysis, and is done through the eye orbital sinus using capillary pipe as much as 1-1.5 ml. Furthermore, the blood is prepared to produce plasma, and obtained blood plasma containing anti-Fim-C *S. typhi* antibody as much as 0.5-0.7 ml. At the bleeding terminal the blood takes 2-3 mL, and the plasma is obtained by 1-1.5 mL for each mouse experiment animal [9, 17].

The results of Bleed-0 plasma isolations were blood plasma prior to injection of antigen (pre-immune), obtained in the day 8. Blood plasma in bleed-1, bleed-2, and bleed-3 are blood plasma after injection of antigen dose 50  $\mu\text{g}/200$  g weight, 75  $\mu\text{g}/200$  g weight, and 100  $\mu\text{g}/200$  g weight, at day 16, 24, and 32. Bleed-4 or terminals bleeding were performed with the aim of obtaining blood plasma as much as possible, obtained at day 37. The antibody of each stage is monitored by ELISA analysis [7, 17].

### 3.3. Analysis of the development of anti-Fim-C *S. typhi* antibody using ELISA method

Blood plasma obtained from each group of mice then analyzed the development of the amount of antibody formation by ELISA (enzyme-linked immunosorbent assay) method. The ELISA process utilizes recombinant FIM-C antigen *S. typhi* 50-300 ng/well, blood plasma containing anti-Fim-C *S. typhi* antibody with 100x dilution, secondary antibody Horse Reddish Peroxidase with 5000x dilution, and dye/substrate TMB as much as 100  $\mu\text{L}/\text{well}$ . The interaction of the antigen is measured its absorbance at a wavelength of 450 nm [21, 22]. The results of ELISA analysis of the development of anti-Fim C *S. typhi* antibody formation in Wistar rats are presented in figure 2.



**Figure 2.** Graph of Absorbance Value of the Rats Blood Plasma by ELISA Test. The ELISA condition was performed on 1/100th plasma dilution and 1/5000th.

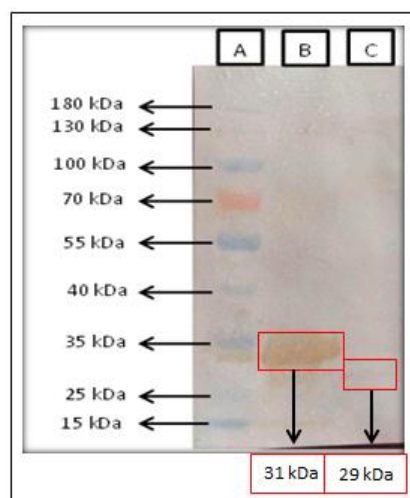
Based on the graph in figure 2 it is known that there is an increase in absorbance in the sample group 1 and the sample group 2 indicating that the antibody produced increases, then from the data obtained by the analysis with one way ANOVA (factorial ANOVA), and obtained the result that the sample group 1 (KS1) has significant differences in bleed-3 and bleed-4 against bleed-0 with  $p$  value 0.007 ( $P < 0.05$ ). Sample Group 1 (KS1) also had significant differences to the normal group (KN) and control group 1 (KK1) with  $p$  values of  $5.7 \times 10^{-6}$  and  $9.8 \times 10^{-6}$  ( $p < 0.05$ ) respectively.

Sample Group 2 (KS2) had significant differences in bleed-2, bleed-3, and bleed-4 against bleed-0 with  $p$  values of  $4.7 \times 10^{-8}$  ( $P < 0.05$ ). The Sample Group 2 (KS2) also had significant differences to the normal group (KN) and control group 2 (KK2) with  $p$  values of  $2.9 \times 10^{-17}$  and  $5.1 \times 10^{-17}$  ( $p < 0.05$ ) respectively.

The results show that recombinant protein Fim-C *S. typhi* Inclusion bodies can improve the immune response of Wistar rat test. Literature analysis also showed that subcutaneous injection caused antigen release gradually, and induced the formation of antibodies well.

#### 3.4. Analysis specificity of anti-protein fim-c *s.typhi* antibody by western immunoblotting

Antibodi used for the analysis of specificity with Western Blot technique ie antibodies contained in blood plasma from the group of rats (KS2) immunized with antigen antigen (Fim-C + protein adjuvant). The Western blot result is shown in figure 3.



**Figure 3.** Results of Western Blot Sample Group 2. Lane A Protein marker 5  $\mu$ L (Thermo). Lane B The purification result of Fim-C Inclusion Bodies protein 3  $\mu$ g/20  $\mu$ L sample; Lane C Extract protein bacteria *Salmonella typhi* 20  $\mu$ L.S.



The results obtained from the specificity test using Western Blot indicate that in Lane B there is brown ribbon with high intensity at molecular mass of  $\pm 31$  kDa protein. This result proves that Fim-C *S. typhi* antigen has successfully identified its specific antibodies Anti-Fim-C *S. typhi* Antibodies characterized by the formation of brown ribbon on the nitrocellulose membrane. It also shows that in rats blood plasma antibodies have been produced anti-Fim-C as an immune response to Fim-C protein induced into Rats.

Anti-Fim C *S. typhi* antibody interactions produced in Wistar rats were also observed against *Salmonella typhi* bacteria extract protein from pure culture. The results showed that there was an interaction between antibody anti-Fim C *S. typhi* and protein measuring 29 kDa. The protein is predicted to be a Fim C. *S. typhi* protein prior to recombination through a genetic engineering process. The results show that recombinant protein tagging Fim-C *S. typhi* is six amino acids Histidine and 10 amino acid constituent Xa factor having molecular mass of two Kilo Dalton. To ensure that proteins are recognized antibodies can be done further process is a protein sequencing. Currently, UNJ salmonella team utilizes the invention for the development of anti-Fim-C antigen *S. typhi* s from Wistar rat as a detection tool, so this research is very useful not only for the development of typhoid fever vaccine but also for developing detection tool for typhoid disease.

#### 4. Conclusion

The results of the immunogenicity assay showed that Fim-C Inclusion Bodies *S. typhi* protein can induce *Wistar* rat's immune response. This is evidenced by the increase of absorbance value in the sample ELISA test group 1 (KS 1) immunized with Fim-C Inclusion Bodies *S. typhi* and group 2 samples (KS 2) immunized with Fim-C Inclusion Bodies *S. typhi* plus Adjuvant FCA / FIA. The results of this study were reinforced by statistical data processing using one way ANOVA which showed that Fim-C Inclusion bodies *S. typhi* have significant effect ( $P < 0,05$ ) to the increase of ELISA test absorbance that plays an important role in immunogens. Specificity test results using the Western Blot method showed that the antibodies produced were anti-Fim-C *S. typhi* antibodies characterized by the formation of brown ribbon on the nitrocellulose membrane. It can be concluded that Fim-C *S. typhi* protein can be used as a potential vaccine candidate for typhoid disease. These results are expected to be an alternative in the discovery of new vaccines.

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