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PRODUCTION AND CHARACTERIZATION OF ANTI FIM-C *Salmonella typhi* NATIVE PROTEIN ANTIBODY IN DDY MICE

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Abstract

Typhoid fever is a disease affect many people in developing countries including Indonesia. Prevention can be aided by vaccination to the bacteria; however it still needs further research. This research is aimed to determine the immunogenicity of fim-C native protein *Salmonella typhi in vivo* as a vaccine candidate in ddY mice. Experimental method is used in this research. This research used antigen of pure fim-C native recombinant protein from its previous research. The ddY mice are categorized into five groups, namely experiment group 1 (protein Fim-C+Freund's Complete/Incomplete Adjuvant), experiment group 2 (protein Fim-C *S. typhi*), control group 1 (FCA/FIA adjuvants), control group 2 (PBS1x), and normal group (PBS1x). The results showed that the ddY mice produced antibody response after subcutaneous injection of the Fim-C *S. typhi* native protein +adjuvant or without adjuvant. The antibody responses with Fim-C *S. typhi* native protein as antigen+adjuvant gave higher absorbans than the Fim-C antigen without adjuvant. It is also showed that the antibody titer from the first to fourth injection has gradually increased. These data showed that Fim-C *S. Typhi* native protein had higher immunogenicity. It is concluded that the Fim-C *S. typhi* native protein can be used as a potential recombinant vaccine candidate against typhoid fever.

Key words: *Salmonella typhi*, Typphoi Disease, Fim-C *Salmonella typhi native* Protein Antibody, Recombinant vaccine

INTRODUCTION

Typhoid fever is one of diseases suffered by most people in developing countries, including Indonesia. Typhoid fever is suffered ranging from children to adults. Typhoid fever is easily transmitted to human through food and drink contaminated by *Salmonella typhi* bacteria in the poor standards of environment hygiene. Typhoid fever happens in Indonesia with an average of 900,000 cases per year. The mortality rate is more than 20,000 cases or 91% of all cases ranging in the age of 3-19 years old. The mortality rate increases in every year [Crump and Mintz, 2010; Verry, 2011]. Thus serious action to prevent and overcome typhoid fever in Indonesia is highly needed.

Salmonella typhi bacteria has Fim-C protein, called *fimbriae*. Fim-C protein, a kind of protein on the surface of bacteria cell, acts as important mediators used for interaction or adharence against host cell [Burrows, 2005; Muktiningsih *et al,* 2009]. Proteins on the surface of bacteria cell as Fim-C can be used as an antigen; it also induces immune response well [Verma *et al*.,

2009; Toobak *et al.,* 2013; Yang *et al.*, 2013; Moreno *et al*., 2013]. It is presumed that Fim-C proteins on *Salmonella typhi* can be potentially used as recombinant vaccine for typhoid fever prevention. Due to its higher level of safety, recombinant vaccine is chosen to be further developed rather than conventional non recombinant vaccine that uses attenuated virus.

In the prior research, researchers team from UNJ has succeeded in Fim-C *Salmonella typhi* gene cloning and sub-cloning on the cloning and expression vectors and its protein purification in both native form and inclusion bodies [Anggraeni R *et al*, 2012; Pratiwi E *et al*. 2013; Muktiningsih *et al*, 2013]. This research is aimed to determine the activity of Fim-C *S. typhi* recombinant protein in its native form as an antigen in mice ddY through immune response analysis. This information is very important in the development of the potential recombinant Fim-C *S. typhi* protein as candidate for recombinant vaccine to prevent typhoid fever in human.

RESEARCH METHOD

A. Production of anti Fim-C *S. typhi* antibodies

Production of anti Fim-C *Salmonella typhi* native antibodies consisted of preparation stage for mice ddY as animal test, preparation stage for Fim-C *S. typhi Native* recombinant protein as antigene, immunization and antibodies production, and mice serum/anti-FimC *S. typhi* antibodies isolation [Harlow and Lane, 1988; Deutsher, 1990; Noer, *et al.*, 1992; Jennings, 1995].

A.1. Preparation stage for mice ddY as animal test

Production of anti Fim-C antibodies was conducted in LABTIAP (Agricultural Industry Technology Development and Biomedical Laboratory) BPPT-Serpong. The production of anti-Fim C *S. typhi Native* antibodies performed on 40 male mice ddY strains, age of 5-6 weeks and weigh of 17-24 grams. Mice were obtained from PT Biofarma Bandung. Mice were maintained in cages placed in the treatment room with temperature condition of 20-24^{0}C and 20-70% humidity. Treatment room was made sound-proof and impermeable to keep the air pressure lower than the surrounding so the odor will not come out. Every testing was conducted in different room. 4-8 mice are placed in individual cages of polycarbonate (with *stainless steel cover*) with size of 41,5x27x15 cm^3 each. The individual cages were arranged in 3 level stainless steel racks. During the conditioning process, mice were weighed on day 0, day 3 and day 5. Cage, food, health, and activity checks were also done regularly. The conditioning process is as long as a week.

Mice were grouped into three major groups, namely the experiment group (KP), the sick control group (KS) and the normal control group (KN). Experimental group had two sub groups; group immunized by mixture of Fim-C native protein and Frued complete/Incomplete adjuvant (FCA/FIA) which consists of 8 mice (KP-1) and group immunized by Fim-C native protein which consists of 8 mice (KP2). The sick control had two sub groups; group immunized by FCA/FIA which consists of 8 mice (KS-1) and group immunized by Phosphate Buffer Saline (PBS 1x) which consists of 8 mice (KS-2). The normal control group (KN) immunized by Phosphate Buffer Saline (PBS 1x) consists of 4 mice. In the challenge test, only experiment group (KP) and sick control group (KS) were infected by *S. typhi* bacteria. The normal control group (KN) was not be infected by *S. typhi* bacteria. After the conditioning process, the researcher took blood from mice's eyes as much as 1-2 mL as pre immune serum. A day prior to collect pre-immune serum, mice were conditioned to food-fasting.

A.2. Preparation stage for Fim-C *S. typhi Native* recombinant protein as antigene

As many as 25-100 µg Fim-C *S. typhi native* protein from previous results was disolved witth PBS 1x bufer in *Eppendorf* 1,5 mL tube to have a total volume of 100-250 µL. *Freund's complete adjuvant* (FCA) or *Freund's incomplete adjuvant* (FIA) were added with ratio 1:1. Then performed homogenized mix using vortexs until the mix turned white [Harlow and Lane, 1988; Jenings, 1995].

A.3. Immunization and Antibodies Production

Immunization was carried out on the backs of mice in the front section near head subcutaneously as much as 2-3 points for one-time injection. On the first immunization 20 µg Fim C *S. typhi* native protein that has been prepared as an antigen was mixed with *Freund's complete Adjuvant*. One week after the first injection researchers took blood sample from mice's eyes to prepare the serum. One week after the first injection researchers conducted boosting with 40 g Fim-C mixed with *Freund's incomplete Adjuvant*. The second and third boosting were conducted with the 80 g Fim-C after one week of the second and the third injections to obtain the optimal antibody amount [Harlow and Lane, 1988; Noer, 1992].

A.4. Mice serum/anti-FimC *S. typhi* antibodies isolation

Blood sample from mice's eyes was collected on tubes of centrifuges sterile. Blood sample was incubated at temperature of 37^0C for 30-60 minutes to a visible separation between serum and platelets. Centrifugation was carried out for 10 minutes with the speed of 5000 g at a 4 ^{o}C. Clear liquid/serum was taken out and kept in *Eppendorf*. Then the serum was stored in -20^0C [Harlow and Lane, 1988].

B. Characterization of anti Fim-C *S. typhi Native antibodies by* ELISA

Antibodies used in the formation analysis of anti-FimC *S. typhi* antibodies was mice serum from *Bleed* I-*Bleed* IV. Formation analysis of anti-Fim-C protein *S. typhi* antibodies from day 0 (*pre-immune* serum) until the 6 weeks was obtained by *Enzyme Link Immunosorbant Assay* (ELISA).

Antigen (30–300 ng Fim-C *S. typhi native* protein in50 µL fosfat bufer, 1x PBS) was incubated in *microtiter plate wells* (IWAKI) in room temperature overnight. Every well was washed 3 times each with 1x PBS containing 1 mM $MgCl_2$ and 0,05% (v/v) Tween-20 (wash buffer). After they were washed, researchers added into 150 µL 5% *blotto* (5 g skim milk in 100 mL 1x PBS) in every well. And then the microtiter plate was incubated at 37^0C for 1 hour. After the incubation wells were washed as much as 3 times with wash buffer. As many as 50 µL mice serum (from *bleed* I/*pre-immune* serum) - *bleed* V (fifth weeks), by 100x and 300x dilution were added into each well with prepared ELISA design,and then put them into incubation at 37^0C for one hour. The *microtiter plate* wells were washed with wash buffer 3 times. After washing process, 50 µL secondary antibodies was added into each well (*anti IgG-mice-HRP* dilution 1: 5000 with 1x PBS) [Thermo Scientific Biogen, 2013], and incubated in 37^0C for 1 hour. After the incubation, the *microtiter plate* wells were washed with wash buffer 3 times. As much as100 µL substrate TMB (3,3',5,5'-Tetramethylbenzidine) solution was added into each well and incubated at 37^0C for 30 minutes (produced blue color). After the incubation, the reaction was stopped by adding 50 µL H_2SO_4 2M (produced yellow color). Next, the absorbance reading was conducted using *ELISA-Reader* (*Microplate Reader*) at wavelength 450 nm [Noer, 1992; Deutsher, 1990; Muktiningsih, 2005; Thermo Scientific**,** Biogen, 2013]. After the formation of antibodies were known to the maximum researchers collected serum in large

amount or bleeding terminal as much as 5-10 mL of blood.

RESULT AND DISCUSSION

A.Production of anti-Fim-C *S. typhi* native Antibodies

The results of animal conditioning process and pre-immune serum collection process

Health, weight, diet, and physical condition checks and observation during the conditioning process showed good result. This was demonstrated by the increase in weight on average 3-5 gram/mice. The observed physical condition of feathers and motion also showed patterns of activity that corresponds to standard conditions. The conditioning place and picture of test animals are presented in figure 1.

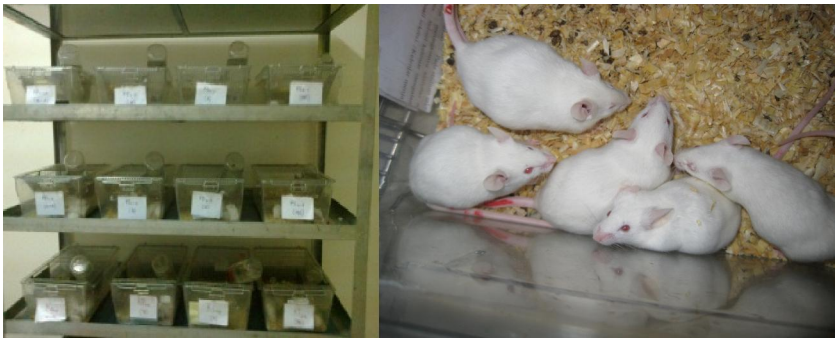

Figure 1. The conditioning place and picture of test animals**.** Mice are grouped into 5 cages. Every cages containing 8 mice for the experimental groups (KP) and the sick control group (KS) and containing 4 mice for normal group (KN).

The retrieval results of pre-immune serum from sinus orbitalis eyes produced 0,5-1 mL blood/mice and it was 0,2-0,5 mL serum of the total blood samples. Serums were stored in frezer with temperture -20^{o}C for further purposes. The pupose of taking *pre-immune* serum is as a negative control to the formation of anti Fim-C antibodies. This is aimed to ensure that there is no interaction occurs between Fim-C protein as antigene with mice antibodies before imunization.

The production results of anti-FimC *S. typhi antibodies*

Production of anti-FimC *S. typhi native* antibodies in experimental and control groups was performed in 6 weeks. The process consisted of the first imunization with 20 µg Fim C *S. typhi native* protein, *boosting*-1 with 40 µg Fim-C *S. typhi* native protein. *Boosting-2* and *boosting*-3 were conducted by using 80 µg Fim-C *S. typhi* native protein. Each step gave a total amount serum 0,2-0,5 mL. After ELISA data was obtained with highest absorbance values, in the sixth week researchers collected blood samples of 4 mice from experimental groups by the maximum as much as 5-10 mL. The blood was prepared to serum containing anti-Fim C *S. typhi* antibodies as many as 2-4 mL/mice.

B. Characterization of anti Fim-C *S. typhi* native antibodies by ELISA

The formation analysis of antibodies used as a primer was taken from mice serum of *Bleed* I-*Bleed* IV. ELISA analysis was performed on 4 mice from each group,that are: experimental

group I (immunized by Fim-C *S. typhi native* Protein mixed with Freud complete/incomplete adjuvant-KP-1), experimental group 2 (immunized by Fim-C *S. typhi native* Protein without adjuvant-KP2), control group-1 (immunized by Freud complete/ incomplete adjuvant, KS1), control group-2 (immunized by 1x PBS buffer, KS-2), and normal group (immunized by 1x PBS buffer, KN). The result of development of antibodies formation from 4 mice ddY from each group are presented in table 1. While the analysis results of development formation of anti-Fim C *S. typhi* antibodies is presented in figure 2 and figure 3.

Table 1. The data values of absorbance on development anti Fim-C *S. typhi native* antibodies at experimental group (KP) and the sick control group (KS) at antigene concentration 100 ng and 300 ng

bleed to-	Experimental Group- 1 (KP1)		Experimental Group- 2 (KP2)		The Sick Control Group-1 (KS1)		The Sick Control Group-2 (KS2)	
	100 ng	300 ng	100 ng	300 ng	100 ng	300 ng	100 ng	300 ng
bleed -0	0,0285	0,04	0,01125	0,00975	0,048333	0,009333	0,01	0,01
bleed -1	0,0055	0,01825	0,00275	0,008	0,027333	0,009667	0,0165	0,0165
bleed -2	0,19675	0,23275	0,04125	0,09575	0,029	0,037	0,0365	0,0365
bleed -3	0,2715	0,44175	0,19	0,2225	0,042667	0,037667	0,0335	0,0335
bleed -4	0,27925	0,5175	0,1805	0,20275	0,04	0,043	0,047	0,047

Based on the results of analysis on the development of formation anti-Fim C *S. typhi* antibodies by ELISA shown in Table 1 that Fim-C *S. typhi native* protein with or whithout adjuvants give satisfied immune response. This is shown by an increase absorbance values from *Bleed* 0-*Bleed* 4. The increase of color intensity or absorbance values showed increase in the amount of antibody titer that interacted with Fim-C *S.typhi* antigene. The blue color was from the oxidation TMB substrate (3,3',5,5'-Tetramethylbenzidine) to 3,3',5,5'-tetramethylbenzidine diimine by *Horse Redish Peroksidase* enzyme which bounded to secondary antibodies. Color formation reaction was stopped by adding 50 µL H_2SO_4 and resulted yellow color and measured at wavelenghts 450 nm [Thermo scientific Biogen, 2013]

Data presented in table 1 or figure 2 and figure 3 give information that induction by protein Fim-C *S. typhi Native* +Adjuvant FCA/FIA as antigene produced higher antibodies than by protein Fim-C *S. typhi Native*+ PBS 1x. This is in accordance with the literature review stated that the Frued complete/incomplete adjuvant (FCA/FIA) can enhance the formation of immune response [Harlow and Lane, 1988; Fiorino *et al*, 2012; Moreno *et al*., 2013]. These results also provide information on specific characters of Fim-C *S. typhi* native protein that if recombinant protein mixed with adjuvants the resulting immune response will be higher than without adjuvants. The information is also essential that Fim-C *S. typhi* native protein without adjuvant can also provide a good immune response.

This information is very important as the scientific foundation to conclude that the withdrawal of recombinant protein molecule of Fim-C *S. typhi native* is candidate vaccine recombinant. The results of literature analysis stated that the recombinant vaccine has many advantages. It (1) has higher safety for patients (2) has higher purity (3) generates more spesific immune response (4)

can be produce in large quantities (5) is easy in production and storage, and some of other advantages [Harlow and Lane, 1988; Fiorino *et al*, 2012].

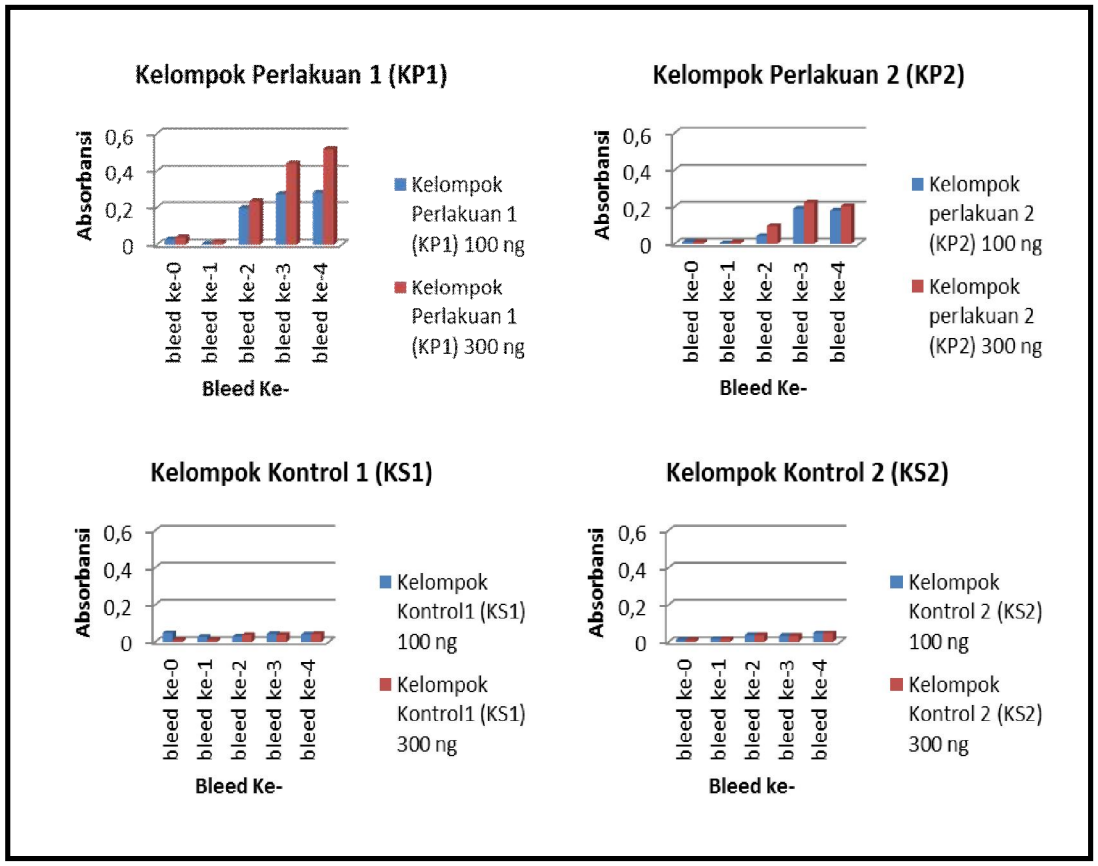

Figure 2. **Graphic analysis of the formation of anti Fim-C *S. typhi native* antibodies in the experimental and the control group.** (A) graphic of formation of antibodies in experimental group-1 (KP-1). (B) graphic of formation of antibodies in experimental group-2 (KP2), (C) graphic of control group-1 (KS1), (D) graphic of control group-2 (KS2). The x-axis showed the development of immunization from each bleed 0-4. The y-axis shows the value of absorbance reading of ELISA reader. Blue and red colored diagram shows the amount of protein FimC *S. typhi* recombinant 100 ng dan 300 ng as antigenes.

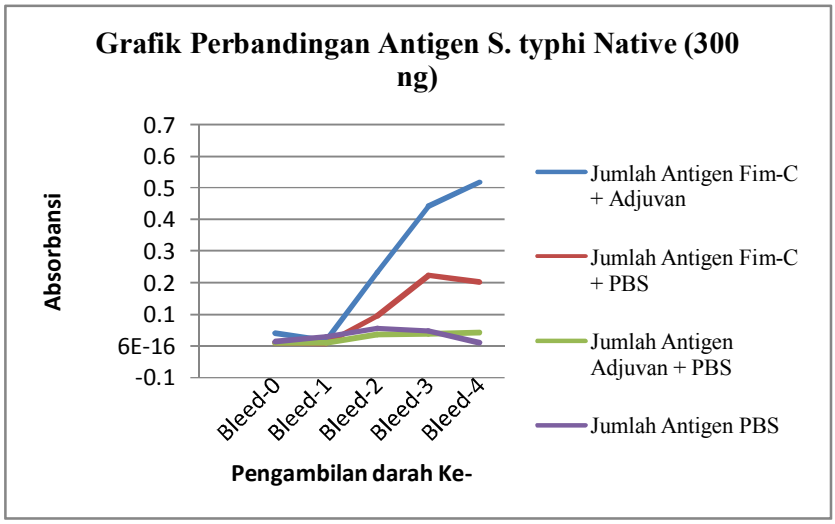

Figure 3. The graphic result of immunogenicity test on antigens Fim-C *S. typhi Native* protein. The blue line shows the value of absorbance for a group of mice immunized by Fim-C *S. typhi* native+Adjuvant FCA/FIA as antigens. The red line shows the value of absorbance for a group of mice immunized by Fim-C *S. typhi* native+1x PBS as antigens. The green line shows the value of absorbance for a group of mice immunized by Adjuvant FCA/FIA+1x PBS as antigens. The green line shows the value of absorbance for a group of mice immunized by Adjuvant FCA/FIA+1x PBS as antigens. The violet line shows the value of absorbance for a group of mice immunized by 1x PBS as antigens. Condition of ELISA performed on serum dilutiom 100x and secondary antibodies dilution 5000x.

CONCLUSION AND SUGGESTION

Fim-C *S, typhi* Protein in *native* form has ben succesfully used as an antigen specific in production of anti Fim-C *S. typhi* antibodies *in vivo* in mice ddY. The generated formation of immune response is indicated by the color change of substrate after interaction between Fim-C *S. typhi* antigen with anti Fim-C *S. typhi* antibodies. From Bleed 1-Bleed IV, the establishment of spesific anti Fim-C *S. typhi* antibodies with Fim-C + adjuvant FCA/FIA as antigene gives higher immune response than without adjuvants. The result also provides information that Fim-C *S. typhi* Typhi protein has good nature of imunogenicity because it is able to make higher response immune without the addition of adjuvant and adjuvant FCA/FIA. So that it can be inferred that Fim-C *S. typhi* native Protein can be used as vaccine candidates.

Significant supporting data about vaccine's standardized test and clinical trials for human are still needed to make recombinant protein molecules of Fim-C *S. typhi* native serve as a safe and inexpensive molecular vaccine. The information generated in this study could be made of the scientific basis for the further development of recombinant vaccines for typhoid fever in humans, especially in Indonesia.

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