PAPER • **OPEN ACCESS**

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

To cite this article: Muktiningsih Nurjayadi *et al* 2019 *IOP Conf. Ser.: Mater. Sci. Eng.* **509** 012089

IOP Conf. Series: Materials Science and Engineering **509** (2019) 012089 doi:10.1088/1757-899X/509/1/012089

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

Muktiningsih Nurjayadi[1], Dwi Ariastuti[1], Kurnia Agustini[2], Asri Sulfianti[2], Wibowo Mangunwardoyo[3]

[1] Department of Chemistry, Faculty of Mathematics and Natural Science, Universitas Negeri Jakarta, Gedung KH. Hasjim Asj'ari, 6th Floor, Jl. Rawamangun Muka Jakarta Timur 13220, Indonesia
[2] Laboratory for the Development of Industrial Technology for Agriculture and Biomedical (LABTIAP), BPPT- Indonesia.
[3] Department of Biology. Faculty of mathematic and Sciences, Universitas Indonesia, Depok Indonesia 16424.
* Coresponding Author : muktiningsih@unj.ac.id

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-								
	0-7	8	9	16	17	24	25	32	36
Normal	Acclimatization	bleed-0	-	bleed-1	500µl	bleed-2	500µl	bleed-3	bleed-4
Control 1 (PBS 1x)			500µl						
Control 2 (Alumina)									
Sample 1 (Fim-C + PBS 1x)			20µg / 20g weight		40µg/40g weight		60µg/60g weight		
Sample 1 (Fim-C + PBS1x + Alumina)									

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 H_2SO_4. 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].

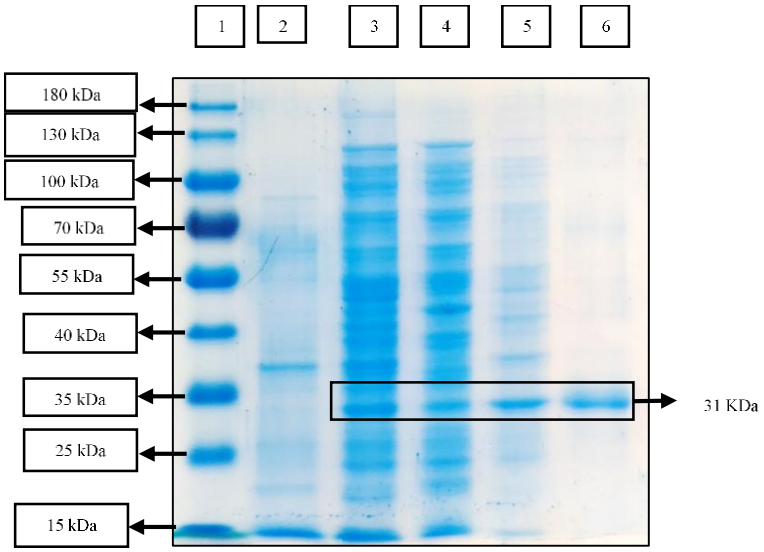

Figure 1. Results of Characterization of SDS-PAGE of *S. Typhi* Fim-C Recombinant Protein.

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.**

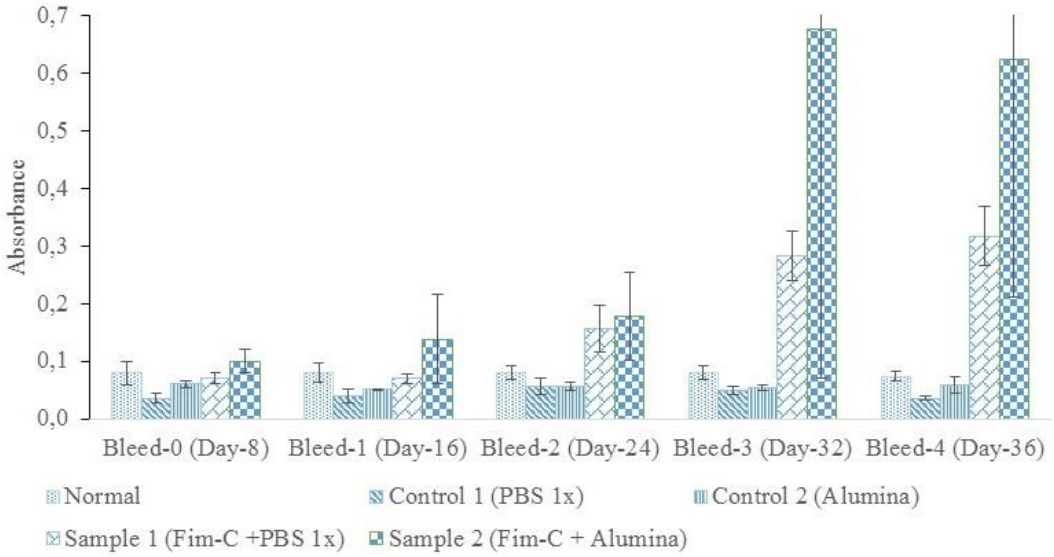

Figure 2. Graph of Formation of *S. Typhi* Anti-Fim-C Antibodies. Detailed descriptions are in the text.

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].

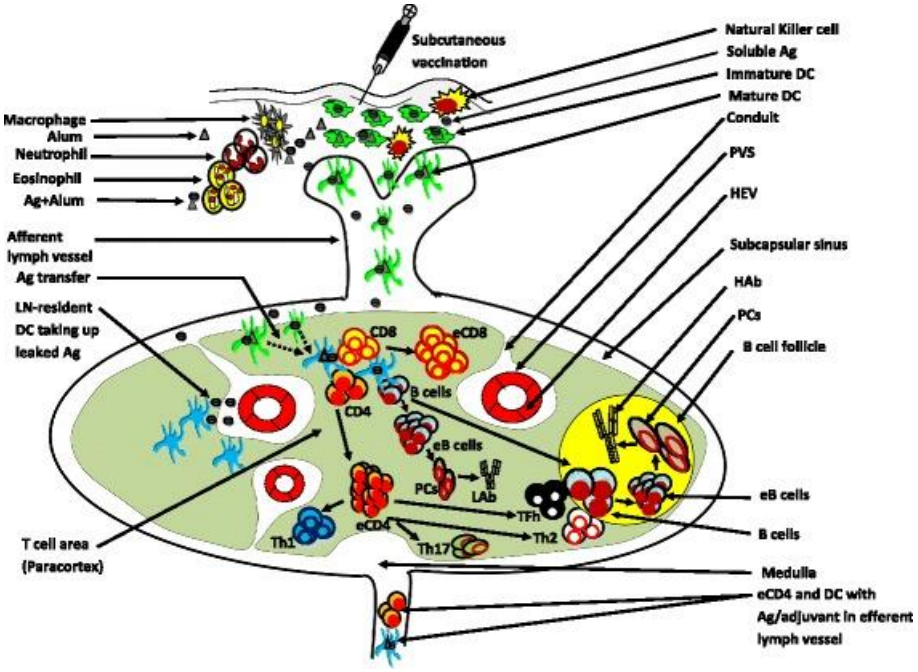

Figure 3. Model of the mechanism of induction of immune responses caused by recombinant proteins as antigens that are mixed with the alumina adjuvant. Detailed descriptions are in the text [6].

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**.

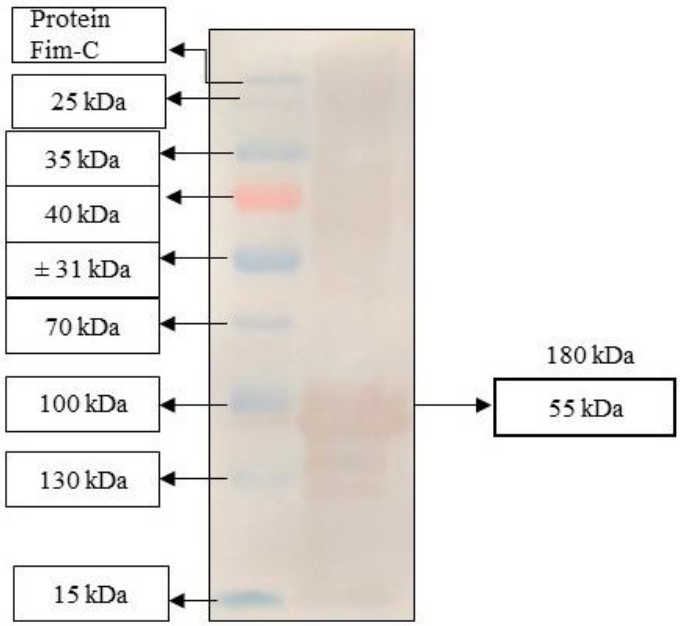

Figure 4. Results of *S. Typhi* Anti-Fim-C Antibody Characterization with Western Blot.

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.

Acknowledgment

Thanks to the Faculty of Mathematics and Natural Sciences, Universitas Negeri Jakarta which provide funding, Oral Biology Laboratory of FKG UI and LAPTIAB BPPT, Serpong which supported for the implementation of this research.

References

[1] Pakkanen S H, Kantele J M, Savolainen L E, Rombo L and Kantele A 2015 Specific and cross-reactive immune response to oral Salmonella Typhi Ty21a and parenteral Vi capsular polysaccharide typhoid vaccines administered concomitantly *Vaccine* **33** 3 451-8

[2] Nurjayadi M, Syukriani Y F and Kartika I R 2009 Laporan Penelitian Fundamental DIKTI: Studi Fungsi Protein Reference Spot Bakteri Salmonella typhi dalam Upaya Penemuan Protein Bioaktif. (Jakarta: Universitas Negeri Jakarta)

[3] Nurjayadi M, Dewi F K and Puspitasari F 2013 Laporan Akhir Penelitian Strategi Nasional: Pengembangan Potensi Molekul Bioaktif Fim-C Salmonella typhi Sebagai Kandidat Vaksin Rekombinan dalam Mencegah Penyakit Typhus pada Manusia. (Jakarta: Universitas Negeri Jakarta)
[4] Hasan U 2016 Produksi dan Karakterisasi Antibodi Anti Fim-C Salmonella Typhi pada Mencit DDY *Risenologi Jurnal* **1** 1 9-22
[5] HogenEsch H 2002 Mechanisms of stimulation of the immune response by aluminum adjuvants *Vaccine* **20** S34-S9
[6] Ghimire T R 2015 The mechanisms of action of vaccines containing aluminum adjuvants: an in vitro vs in vivo paradigm *Springerplus* **4** 1 181
[7] Shaw A R and Feinberg M B 2013 *Clinical Immunology (Fourth Edition),* ed R R Rich*, et al.* (London: Content Repository Only!) pp 1095-121
[8] Desmet C J 2014 *Biological DNA Sensor: The Impact of Nucleic Acids on Diseases and Vaccinology*: Elsevier) pp 271-312
[9] Agger E M, Rosenkrands I, Olsen A W, Hatch G, Williams A, Kritsch C, Lingnau K, Von Gabain A, Andersen C S and Korsholm K S 2006 Protective immunity to tuberculosis with Ag85B-ESAT-6 in a synthetic cationic adjuvant system IC31 *Vaccine* **24** 26 5452-60
[10] Riedl K, Riedl R, von Gabain A, Nagy E and Lingnau K 2008 The novel adjuvant IC31® strongly improves influenza vaccine-specific cellular and humoral immune responses in young adult and aged mice *Vaccine* **26** 27-28 3461-8
[11] Nurjayadi M, Apriyani D, Hasan U, Santoso I, Kurniadewi F, Kartika I R, Agustini K, Puspasari F, Natalia D and Mangunwardoyo W 2016 Immunogenicity and Specificity of Anti recombinant Protein Fim-C-Salmonella typhimurium Antibody as a Model to Develop Typhoid Vaccine *Procedia Chem.* **18** 237-45
[12] Harlow E and Lane D 1999 *Using Antibodies: A Laboratory Manual*: Cold Spring Harbor Laboratory Press)
[13] Santoso I and Nurjayadi M 2014 Laporan Akhir Penelitian Fundamental: Ekspresi dan Purifikasi Protein Fim-C Salmonella typhimurium sebagai Model Pengembangan Vaksin Rekombinan Penyakit Typhus pada Manusia. (Jakarta: Universitas Negeri Jakarta)
[14] Kool M, Fierens K and Lambrecht B N 2012 Alum adjuvant: some of the tricks of the oldest adjuvant *J. Med. Microbiol.* **61** 7 927-34
[15] QIAexpressionist™ T 2003 *A handbook for high-level expression and purification of 6xHis-tagged proteins* Qiagen USA