Development of antibody anti-FimC-\textit{Salmonella typhi} as a detection kit model of typhoid diseases by antigen capture approach

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\textbf{ABSTRACT}

Typhoid fever is a world health problem, with 200,000 recorded deaths annually in developing countries. The discovery of new drug discovery and detection methods for typhoid is still continuing. In previous research, the 31 kilo Dalton (kDa) recombinant protein Fim-C-S typhi was successfully expressed. It was also reported that the recombinant protein Fim-C-S typhi could induce the occurrence of antibodies. This study aims to further develop anti-Fim-C-S. typhi antibodies as a detection tool. The sensitivity evaluated by western immunoblotting analysis indicated that anti-Fim-C-S. typhi antibodies can significantly recognize its antigen at a minimum level of 0.125 μg. The specificity evaluation of anti-Fim-C-S. typhi antibodies against S. typhi bacteria extract protein showed that anti-Fim-C-S. typhi antibodies could recognize S. typhi extract protein at ± 29 KDa and ± 60 kDa. In addition, anti-Fim-C-S. typhi antibody did not recognize healthy blood extract proteins. Simulation in healthy blood samples containing bacterial antigen S. typhi and recombinant antigen Fim-C-S. typhi produce bands of 29 kDa, 31 kDa and 60 kDa have been also studied. It can be concluded that anti-Fim-C-S. typhi antibodies can be used in the development of prototype detection tool. The results from this study are expected to provide a foundation to the development of a detection methods for S. typhi that are sensitive, specific, safe and simple.

\section{1. Introduction}

Typhoid fever (\textit{Typhus abdominals}) is a world health problem, recorded annually in developing countries over 200,000 people die and most are children (Crump and Mintz, 2010, Kariuki et al, 2015). A total of 258–810 people/100,000 population of Indonesia in 2007 suffered from typhoid fever (Hatta and Smits, 2007), therefore a fast and accurate detection methods are needed as well as a proper treatment are needed to reduce typhoid fever.

Typhoid fever is a systemic infection caused by \textit{Salmonella typhi}, usually through ingestion of contaminated food or water. The acute illness is characterized by prolonged fever, headache, and nausea, loss of appetite, and constipation or sometimes diarrhea. Symptoms are often non-specific and clinically non-distinguishable from other febrile illnesses. However, clinical severity varies from case to case, some might even be fatal. It occurs predominantly in association with poor sanitation and lack of clean drinking water (Radhakrishnan et al., 2018).

- The diagnosis of clinical typhoid fever is often inappropriate because there are no specific symptoms (Hatta and Smits, 2007; Andrews and Ryan, 2015). Currently, in Indonesia the most
common tool for the diagnosis of typhoid fever is serological tests which includes widal which has lower specificity, lower sensitivity and also there is no standard cut off value for the agglutination, hence widal test cannot be used as reference test for diagnosis of the typhoid fever (Septiawan et al., 2013). Another serological method used is direct ELISA, but the results are not dependable due to use of monoclonal antibodies which also gives false positive results. (Sendow et al., 2015).

These serological tests have important value in the diagnosis of typhoid fever. However; there is a huge opportunity for the development of new diagnostic methods for typhoid fever. The existing tests shows wide variation in the specificity and sensitivity, the new tests to be discovered should be more rapid, specific, sensitive and simpler which should be able to perform in the endemic areas of Indonesia. (Wijedoru et al., 2017; Olsen et al., 2004). Looking at the disadvantages that exist, many researchers developed a method of detection of typhoid disease by utilizing various types of genes and potential proteins such as Flagellar protein (a surface protein found in the Flagella section measuring 40 KDa) developed in India. In addition, in Vietnam a method of detection of typhoid disease is also developed based on the interaction of antigen Lipopolysaccharides O antigen and H Antigen with its antibodies (Deborah et al., 2001). The two results of the study stated that it still needed improvement to get optimal results, as reported by Ismail from the Malaysian research institute for effective management of typhus, a fast, accurate detection tool was needed (Ismail, 2000). In relation to the serological method, our previous studies were successful in expressing the 31 kDa S. typhi Fim-C recombinant protein (Nurjayadi et al., 2017) and produce anti-Fim-C S. typhi antibodies both in ddY mice and Wistar rats (Nurjayadi et al., 2014). Furthermore, it has also been tested for the potential anti-Fim-C antibody, which results that the antibody can significantly recognize the S. typhi Fim-C recombinant protein as its antigen. (Nurjayadi et al., 2016; Hassan; Nurasiah, 2017).

This study aims to develop anti-Fim-C-Salmonella typhi antibodies for diagnostic kit model of typhoid disease in humans. Specifically, focus to produce detection methods that are simple, cheap, rapid, specific, and sensitive to bacteria that cause typhoid fever. The usage of antibodies for the recombinant S. typhi protein is expected to improve the specificity of existing detection method.

2. Materials and methods

2.1. Production of Fim-C-S. typhi recombinant protein

Protein production was performed following the procedure of pET system, Novagen and Thermo Scientific HisPur Ni-NTA system. Stages of protein production consist of (1) inoculum preparation, (2) overexpression of Fim-C-S. typhi protein (3) Isolation of Fim-C-S. typhi inclusion bodies protein (Novagen, 2011; QiaExpressionist, 2003).

2.1.1. Preparation of inoculum bacteria

The *Escherichia coli* BL21 (DE3) pLysS bacteria containing recombinant plasmid pET-30a-Fim-C-S. typhi from previous study is inoculated in a 20 mL liquid LB medium containing 60 µg/mL Kanamycin (LBK) antibiotics. The mixture was incubated at 37 °C and aerated at 150 rpm during overnight (16–18 h) (Novagen, 2011; Nurjayadi, 2005).

2.1.2. Overexpression of fim-C-S. typhi recombinant protein

The overexpression processes were used 5 mL of inoculum into 250 mL sterile LBK medium. The inoculum is grown at 37 °C and aerated 150 rpm for 3 h or until the condition of OD600 0.6–0.8. Erlenmeyer containing 250 mL of sterile LBK medium was then subsequently induced by addition of Isopropyl-β-D-thiogalactopyranoside (IPTG) with final concentration of 0.5 mM Incubation is continued for 4 h. The next stage of overexpression follows the pET-system or Thermo Scientific HisPur Ni-NTA system (QiaExpressionist, 2003; Nurjayadi, 2005, Verma et al., 2009).

2.1.3. Isolation of Fim-C-S. typhi recombinant protein

The Fim-C-S. typhi recombinant protein is isolated from soluble protein in the cytoplasm and the inclusion bodies (Novagen, 2011; QiaExpressionist, 2003; Nurjayadi, 2005). A total of 250 mL of induced cell was transferred to a sterilized centrifugation tube. By ultracentrifugation, the mixture was centrifuged at 8000 rpm for 30 min and 4 °C. The pellets were re-suspended by 5 mL of native equilibration buffer. Subsequently, the mixture was sonicated for 15 min at frequency of 4 Hz (at 30 s intervals), until obtaining clear mixture. During the sonication process, the cell mixture was cooled in ice. After this step, the mixture was centrifuged at 12,000 rpm for 5 min at 4 °C. The resulting supernatant is a native Fim-C-S. typhi protein and removed into a sterile Eppendorf tube. The cell pellet was re-suspended using denaturing equilibration buffer. The mixture was incubated for 30 min at 4 °C, then vortex slowly for 15 min. Subsequently, the mixture was centrifuged at 12,000 rpm for 5 min at room temperature. The resulting supernatant is a Fim-C-S. typhi recombinant protein that forms aggregates or inclusion bodies. The protein obtained was then characterized using SDS-PAGE (Nurjayadi, 2005; Bio-Rad, 2016; Deutcher, 1990).

2.2. Purification of Fim-C-S. typhi recombinant protein

Purification of the isolated protein forms the aggregate was done by using HisPur Ni-NTA Kit. The procedure used in accordance with Kit Thermo Fisher, Inc. The Ni-NTA columns are equalized with denaturing equilibration buffer, after that the Fim-C-S. typhi inclusion bodies protein is put through the column and incubated for 30 min. The column was washed three times using a 6 mL denaturing washing buffer solution. Furthermore, the elution of protein using denaturing elution buffer for three times, so obtained pure protein each 5 mL and the total protein obtained are 9 mL. The Fim-C-S. typhi protein from purification results then measured its concentration using Kit BCA and analysed its characterization using SDS-PAGE (Amersham Bioscience, 2013; Bio-Rad, 2016; Deutcher, 1990).

2.3. Preparation of animals tested for antibodies production

Animal used in this study were male rats, Wistar strains, age 6–8 weeks and weight 100–200 g. 25 Wistar rats were used for antibody production. Rats were kept in cages under constant conditions of 24 °C air temperature, 12 h light and dark cycle, 70% air humidity for a week. During the conditioning, the rats were weighed on days 0, day 3 and day 5, and its feed, cage, health, and activity were monitored. After the conditioning process, pre-immune plasma was measured from 250 µL blood, taken from the eye’s orbital sinus. Rats were grouped into 3 (three) major groups, the Normal group (KN), the experimental group (KS-1 and KS-2) and the control group (KK). The experimental group had two subgroups, the group injected with a mixture of recombinant Fim-C-S. typhi protein and Freund complete/incomplete adjuvant (KS-1) and the group injected with recombinant Fim-C-S. typhi protein (KS-2). The control group consisted of two subgroups, a group injected with Freund Complete/Incomplete Adjuvant (KK-1), and a group injected with Phosphate Buffer Saline or PBS 1x (KK-2). Each group consists of 5 (Five) rats. The formation of anti-Fim-C-S. typhi antibody is observed for 6–8 weeks. Ethical clearance for this experiment has been approved by Ethics Committee of Faculty Medicine of Universitas Indonesia No. 997a/UN2.F1/ETIK/2016 (Deutcher, 1990; Charan and Kantharia, 2013).

2.4. Production of anti-fim-C-S. typhi antibodies

A total of 50–100 µg of Fim-C-S. typhi protein in form of inclusion
bodies (denaturing form) was dissolved in PBS with total volume of 100 μL. Then, Freund’s complete adjuvant (FCA) was added with 1:1 ratio. The mixture was homogenized using vortex until a white emulsion was formed. Immunization processes were performed at the back of rats near the front of the head subcutaneously as much as 2–5 points for one injection. The first immunization was performed with 50 μg of Fim C–S. typhi recombinant protein antigen mixed with Freund’s complete Adjuvant (FCA). The injection dosage is adjusted for units per 200 g of rat weight. One week after the first injection, the blood is withdrawn from the sinus orbitals and then prepared for serum. Blood was incubated at 37 °C for 30–60 min until visible separation between serum and platelet. Centrifugation is carried out for 10 min at a rate of 5,000 g at 4 °C. The serum liquids are taken and put in Eppendorf. Then the serum is stored at 20 °C. One day after blood collection from the first injection (8th day) booster dose was given with 75 μg Fim C-S. typhi recombinant protein mixed with Freund’s incomplete Adjuvant (FIA) in comparison same to FCA. The third booster was done with 100 μg Fim-C-S. typhi recombinant protein mixed with FIA after one week of the second injection. On day 37, the final bleeding is done. An amount of 5–6 mL of blood is taken from the eye’s orbital sinus, and inserted into a sterile Eppendorf tube. Separation of blood serum is carried out by standard procedures (Jennings, 1995).

2.5. Analysis of anti-fim-C-S. typhi antibodies production by ELISA

Analysis of the amount of antibody formation or production against Fim-C-S. typhi s was carried out from 0th day (serum pre-immune, before injection with Fim-C-S. typhi protein as antigen) until week 5 by ELISA technique. Antigen (30–300 ng Fim-C-S. typhi recombinant protein in 50 μL phosphate salt buffer, PBS 1x, per well) was incubated in a microtiter plate well at room temperature overnight. Each well was washed three times with PBS 1x. After washing, 150 μL of 5% blotto (5 g of skim milk in 100 mL PBS 1x) was added to each well, then the microtiter plate was incubated at 37 °C for 1 h. After incubation, plates were again washer for 3 times with the washing buffer. The 50 μL serum Wistar rat (derived from bleed 1 (day 0/pre-immune serum) until bleed 4 (week 5), with 100x and 300x dilutions added to each well in accordance with the prepared ELISA design, incubated at 37 °C for 1 h. Microtiter plate well was washed again with washing buffer for three times. After washing, 5000x dilution of 50 μL secondary antibody was added into the well and then incubated at 37 °C for 1 h. After incubation, washed again with washing buffer for three times. A 100 μL substrate of TMB substrate (3, 3′, 5′-Tetramethylbenzidine) was added to each well, then incubated at 37 °C for 1 h until blue color was produced. Then the reaction was stopped with 2M H2SO4 and yellow color were produced. Furthermore, an absorbance reading was done by ELISA-Reader at 450 nm wavelength (Nurjayadi et al., 2016; Sambrook and Maniatis, 1989).

2.6. Antibodies sensitivity analysis with western blot

The initial step of Western blot is the separation of pure Fim-C-S. typhi protein by polyacrylamide gel electrophoresis. The protein that has been electrophoresed is transferred to the membrane. After transfer, nitrocellulose membranes were submerged in 5% blotto in 1x PBS buffer for 30 min at room temperature. Fim-C-S.typhi anti bodies were added to the blocking solution (100x dilution), and then incubated again for 1 h. The membrane is then washed with TBS buffer for three times, 5 min each, at room temperature. The membrane is then submerged once more in a blocking solution, and secondary antibodies were added (HRP anti IgG-Mouse diluted 5000x) (Thermo Scientific, 2014). The process continues with washing similar to the previous step. Membrane staining was carried out by inserting the membrane into the DAB substrate solution with a 1x dilution concentration, until a brown protein band was seen. Variations in protein concentration used to be tested by Western blot were 1 μg, 0.5 μg, 0.25 μg, 0.125 μg and 0.0625 μg (Nurjayadi et al., 2016; Harlow, and Lane, 1988; Jennings, 1995; Bio-Rad, 2014).

2.7. Evaluation of detection anti-fim-C-S. typhi antibodies to healthy people blood, typhoid patient’s blood and positive control by antigen capture

The specificity of anti-Fim-C-S. typhi antibody is determined by whether or not there is a cross reaction using the sample, and control as a comparison. The sample used was protein isolated from typhoid patient’s blood. While the controls used are purified recombinant Fim-C-S. typhi protein, protein extract of S. typhi bacteria from pure culture, and healthy people blood extracted protein (Clinicheck Laboratories Indonesia No.19/LAB-CL/1/2019). Stages of this experiment are consisting of: (1) protein isolation of S. typhi bacteria from pure culture; (2) isolation of blood protein (healthy blood people and typhoid blood patient); (3) preparing protein sample for detecting model; and (4) Western blot analysis. (Nurjayadi et al., 2016; Harlow and Lane, 1988; Jennings, 1995; Bio-Rad, 2014).

2.7.1. Protein isolation of S. typhi bacteria from pure culture

Cultivate S. typhi bacteria as much as 10 μL inoculated into 10 mL sterile liquid LB media. Each mixture was then incubated at 37 °C and aerated at a speed of 150 rpm for 16–18 h. The resulting mixture was then centrifuged at a speed of 5000 rpm at 4 °C for 30 min. The supernatant is decanted and discarded. Bacterial cell pellets produced were re-suspended with 5 mL PBS 1x. Then centrifuged at 5000 rpm at 4 °C for 5 min. The washing and centrifugation process is repeated twice. After that, pellets were re-suspended with 2 mL PBS 1x. Then sonication was carried out by means of a sonicator at the frequency position 4 Hz (sonication process 30 s on and 30 s off) for 15 min. During the sonication process, the cell mixture is incubated in ice. The mixture was centrifuged at 12,000 rpm, for 10 min at 4 °C with ultracentrifugation. Then the pellets and supernatants are separated. The protein dissolved in the cytoplasm contained in the supernatant is stored as an extract of the pure bacterial protein S. typhi at a temperature of −20 °C for SDS-PAGE analysis. While the pellet is removed (Nurjayadi et al., 2016; Nurasiah, 2017; Novagen, 2011; Sambrook and Maniatis, 1989).

2.7.2. Isolation of blood Protein (healthy blood people and typhoid blood patient)

A total of 500 μL of each blood sample (Healthy blood and typhoid blood) was re-suspended with 500 μL TE pH 8 buffers (10 mM Tris-Cl, 1 mM EDTA). Each mixture is homogenized with a vortex device. Then centrifuged at 10,000 rpm for 2 min with micro centrifuge Eppendorf. A total of 500 μL of supernatant was decanted. Then each mixture was added 500 μL TE buffer pH 8. The mixture was homogenized again with a vortex and centrifuged at a speed of 10,000 rpm for 2 min with Eppendorf micro centrifuge. This process is repeated 8–10 times until the red color of the blood is lost. After each mixture is clear, the supernatant is removed. While the resulting pellets were dissolved in 100 μL 5x sample buffer (60 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol and 0.1% bromphenolblue) and added 10 μL EDTA. Then each mixture was heated at 100 °C for 10 min. After that, centrifugation was carried out at 10,000 rpm for 5 min with Eppendorf micro centrifuge. Supernatant was stored as healthy blood protein extract and typhoid blood protein extract at −20 °C for Western Blot analysis (Nurjayadi et al., 2016; Nurasiah, 2017; Novagen, 2011).

2.7.3. Preparing Protein sample for detecting models by antigen capture

Protein samples that are (1) purified Fim-C-S. typhi recombinant protein; (2) S. typhi pure bacterial protein extracts; (3) healthy blood protein extracted; (4) typhoid blood protein extracted; (5) healthy blood samples contaminated with S. typhi extract protein, and healthy blood samples contaminated with S. typhi recombinant protein. Each of 20 μL sample was put in a 1.5 mL micro tube then 5x sample buffer was added (60 mM Tris-HCl pH 6.8,
3. Results

3.1. Production of Fim-C-S. typhi recombinant protein

E. coli culture BL21 (DE3) pLysS containing pET-30a-Fim-C-S. typhi with a volume of 250 mL produces a mass of pellets of 2.7 g. Furthermore, the resulting pellets were isolation according to the Qiaprep/Thermo scientific procedure and produced 5 (five) mL protein extract of Fim-C inclusion bodies Salmonella typhi. Determination of concentration with BCA assay shows a result of 3508, 1 μg/mL. SDS-PAGE results from Fim-C S. typhi protein overexpression is shown in Fig. 1.

3.2. Purification of Fim-C-S. typhi recombinant protein

Fim-C-S. typhi recombinant protein is purified with His-Pur NiNTA (QiaExpressionist, 2003). Purification 1 (P1) was carried out on 3 mL Fim-C-S. typhi recombinant extract protein in a concentration of 3508.1 μg/mL. This purification processes produce 9 mL of pure protein in a concentration of 255,818 μg/mL. While the purification 2 (P2) was 2 mL with a concentration of 3508.1 μg/mL produced 9 mL of pure protein in a concentration of 188,528 μg/mL. Based on the results of this purification, protein Fim-C inclusion bodies obtained Salmonella typhi with a randement of 22.8%. Characterization of SDS-PAGE from purification results is shown in Fig. 2.

3.3. Monitoring of animals tested in antibodies production

Wistar rat health monitoring is done by weighing, observing diet, and observing physical conditions. While monitoring the condition of maintenance space is done by measuring room temperature, air circulation, cleanliness and humidity of the room. Weighting results showed that each rat in each group experienced an increase which indicated that mice could adapt well to their new environment. After conditioning, the mouse is taken their blood in a mount 250 μL from sinus orbitalis eyes as pre-immune serum. The results of pre-immune serum from the eye sinus orbitalis produce 0, 2–0, 5 mL of blood, the serum produced is 0, 1–0, 2 mL of serum. The results are stored at −20 °C for further purposes (Harlow and Lane, 1988; Jennings, 1995).

3.4. Production of anti-fim-C-S. typhi antibodies

The antigen used in this study was compiled into four types according to the test animal group. The four types of antigens are (1) Fim-C-S. typhi recombinant protein diluted in PBS 1x and Adjuvant FCA/FIA for the 1st-test group (KS-1), (2) Fim-C-S. typhi recombinant Protein diluted in PBS 1x for the 2nd-test group (KS-2), (3) Adjuvant FCA/FIA diluted in PBS1x for control-1 group (KK-1), (4) PBS1x only for control-2 group (KK-2). In addition to the four groups, there is one group of test animals that are not injected with any antigen called the normal group (KN). The injection process is carried out in rat subcutaneously. The 1st injection was of a dose of 50 μg/mL, the 2nd injection of a dose of 75 μg/mL, the 3rd injection of a dose of 100 μg/mL. The formation of antibodies is then monitored by the Enzyme Link Immunosorbent Assay (ELISA) technique (Novagen, 2011; Harlow and Lane, 1988; Jennings, 1995). The total volume of Anti-Fim-C-S. typhi antibody from terminal bleeding from each rat are 4–5 mL.

3.5. Analysis of antibodies anti-fim-C-S. typhi Production by ELISA

The amount of Fim-C-S. typhi recombinant protein as the antigen used in the ELISA analysis is 100 ng, with the primary antibody (antibody anti-Fim-C-S. typhi) dilution of 100x and secondary antibody (HRP anti IgG-Mouse) dilution of 5000x. ELISA analysis results show in Fig. 3.

3.6. Analysis of antibodies specificity and sensitivity with western immunoblotting

The protein used for Western Blot analysis is the result of Purification 1 (P1). Characterization using Western blot is useful to determine the specificity and sensitivity of antibodies from Fim-C-S. typhi recombinant protein. Figs. 4–6 respectively shows the specificity and sensitivity results of anti-Fim-C-S. typhi antibodies. (Nurjayadi, et al., 2019; Biocatalysis and Agricultural Biotechnology 19 (2019) 101157.)
3.7. Prototype of detection kit for typhoid patients with anti-Fim-C-S. typhi antibodies by Antigen Capture

The evaluation results of detection anti-Fim-C-S. typhi antibodies to healthy people blood, typhoid patient’s blood, and the positive control as a prototype by Antigen Capture of typhoid fever detection by Western Immunoblotting methods are presented in Fig. 7 and Fig. 8 (Deutcher, 1990; Bio-Rad, 2014; Radhakrishnan et al., 2018).

4. Discussion

The discussion sequence is presented in 6 points which include (1) Production of Fim-C-S. typhi recombinant protein; (2) Purification of Fim-C-S. typhi recombinant protein; (3) Monitoring of animals tested in antibody production; (4) Analysis of antibody anti-Fim-C-S. typhi Production by ELISA (5) Analysis of antibody specificity and sensitivity with Western Immunoblotting; (6) Prototype of detection kit for typhoid patients with anti-fim-C-S. typhi antibodies by Antigen Capture.

4.1. Production of Fim-C-S. typhi recombinant protein

Based on Fig. 1, the presence of high-intensity protein bands in ± 31 kDa molecular mass in Lane C shows that the overexpression process of the fim-C gene in the pET-30a-fim-C-S. typhi plasmid into Fim-C protein has been successfully carried out in E. coli BL21 (DE3) pLysS host cells. Literacy analysis showed that overexpression occurred because the added IPTG as inducer. Fim-C-S. typhi protein formation by blocking the repressor in the operator area which is found in E. coli BL21 (DE3) pLysS bacteria so that the RNA polymerase enzyme in E. coli is active to express (transcribe and translate) the RNA polymerase T7 gene into T7 polymerase protein. The T7 polymerase protein derived from the E. coli host cell interacts with the bacteriophage-T7 promoter found in the pET-30a-fim-C-S. typhi recombinant plasmid. This interaction stimulates the expression of the fim-C gene into excessive production of Fim-C-S. typhi recombinant protein.
amounts of Fim-C protein, and can be identified by SDS PAGE producing thicker bands (Novagen, 2011; QiaExpressionist, 2003; Amersham Bioscience, 2013).

Calculations of molecular mass of Fim-C-S.typhi recombinant protein using the DNAstar program specifically EditSeq shows that the molecular mass of the Fim-C-S. typhi recombinant protein containing 6 (six) histidine amino acids at the 5 ‘end and 10 amino acids sequence identified by factor Xa is around 31 kDa (Thermo Scientific, 2016). So the results obtained based on experiments have a match with the results of theoretical calculations.

4.2. Purification of Fim-C-S. typhi recombinant protein

The purification of Fim-C-S. typhi recombinant protein in this study used immobilized metal-affinity chromatography (IMAC) system (Fig. 9). IMAC is based on the interactions between a transition metal ion (Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}) immobilized on a matrix and specific amino acid side chains. Histidine is the amino acid that exhibits the strongest interaction with immobilized metal ion matrices, as electron donor groups on the histidine imidazole ring readily form coordination bonds with the immobilized transition metal. Peptides containing sequences of consecutive histidine residues are efficiently retained on IMAC column matrices. Following washing of the matrix material, peptides containing poly histidine sequences can be easily eluted by either adjusting the pH of the column buffer or adding free imidazole to the column buffer (Bornhorst and Falke, 2000, Crowe, et al, 2016; Radhakrishnan et al., 2018).

The 6xHis/Ni-NTA system is a fast and versatile tool for the affinity purification of recombinant proteins and antigenic peptides. It is based on the high-affinity binding of six consecutive histidine residues (the 6xHis tag) to immobilized nickel ions, giving a highly selective interaction that allows purification of tagged proteins or protein complexes from ~1% to > 95% homogeneity in just one step. The tight association between the tag and the resin allows contaminants to be easily washed away under stringent conditions, yet the bound proteins can be gently eluted by competition with imidazole, or a slight reduction in pH. Moreover, because the interaction is independent of the tertiary structure of the tag, 6xHis labeled proteins can be purified even under the strongly denaturing conditions required to solubilize inclusion bodies (Bornhorst and Falke, 2000).

The resulting Fim-C-S. typhi protein is a recombinant protein that has been bound with 6 histidine residues in the terminal N and this protein is often referred to as the His-Tag protein. This His-Tag presence facilitates the process of protein purification based on the selective affinity of proteins with poly histidine against absorbents equipped with metal chelating. Histidine forms a coordinating bond with Ni-NTA resin so that only Fim-C-S. typhi recombinant protein is bound to Ni-NTA resin (Novagen, 2011; QiaExpressionist, 2003; Bornhorst and Falke, 2000; Radhakrishnan et al., 2018). The purification process consists of three stages. The first stage is the protein binding stage with Ni-NTA resin. Then 30 min of incubation were carried out to strengthen the binding between resin and Inclusion Bodies Fim-C-S. typhi recombinant protein. The second stage is the washing stage. This stage is carried out to eliminate non-target proteins so that more specific target proteins can be obtained. The third stage is the elution stage. The elution stage is the target protein release stage which contains histidine residues from Ni-NTA resin using higher concentrations of imidazole. So that the Ni-NTA resin bond with poly histidine on recombinant protein can be released, and the eluent produced is recombinant Fim-C- S. typhi recombinant protein in a pure form, which is represented by the 31 kDa.
4.3. monitoring of animals tested in antibodies production

As we describe at the research result, we prepare the animals test following the standard procedure which paid attention to animal welfare. All the animals tested are healthy and active before the experiment began, that it showed by increasing weight, motion and kinds of activities in their cage. There is no experimental animal that looks stressed and pain (Harlow and Lane, 1988; Jennings, 1995). At the beginning of the experiment were taken pre-immune serum. The goal is as a negative control of the formation of anti-Fim-C-S. typhi antibodies, this is done to ensure that there is no interaction between the protein Fim-C antigen and mouse antibodies before immunization/antigen injection (Harlow and Lane, 1988).

4.4. Analysis of antibodies anti-fim-C-S. typhi Production by ELISA

Based on Fig. 3 shows that the KS-1 treatment group which was injected with Fim-C-S. typhi recombinant protein as antigen emulsified with the FCA/FIA adjuvant in a PBS 1x buffer (green line) gave the highest absorbance value compared to the other groups. The KS-2 treatment group injected with Fim-C-S. typhi recombinant protein antigen dissolved in the PBS 1x buffer (yellow line) also gave a good absorbance value. The results from both groups showed that Fim-C-S. typhi recombinant protein can produce specific antibodies. Meanwhile, KK-1, KK-2 and KN as the control group have very low or not significant absorbance value. Based on the data it can be concluded at the control group it cannot produce anti-Fim-C-S. typhi antibodies (Nurjayadi, 2005; Bio-Rad, 2014). As we know the function of adjuvants is to stimulate the formation of antibodies and maintain the release of antigen proteins slowly from fast catabolism, so that proteins can stimulate the formation of desired specific antibodies (Novagen, 2011; Nurjayadi, 2005; Amersham Bioscience, 2013).

In the ELISA test the formation of color is the result oxidation of the TMB substrate reaction (3.3′, 5,5′-Tetramethylbenzidine) by the Horse Radish Peroxidase enzyme which is conjugated to the anti-IgG mouse antibody or secondary antibody. Peroxidase catalyzes H2O2 through an oxidation reaction. The reactions that occur can produce products that equilibrate with radical cations. Addition of equimolar hydrogen peroxide produces a yellow di-imine compound, which is a stable product at acidic pH. The yellow color formed is then read at a wavelength of 450 nm. The color intensity formed is equivalent to the increase in the primary antibody titer produced, so that the increase in absorbance from the ELISA results shows that there is an interaction between the protein antigens (Fim-C-S. typhi recombinant protein) with antibodies produced by Wistar rat.

The increasing antibodies’ titers is in accordance with the antibody formation mechanism which states that when the body is infected by foreign substances or antigens, the body forms a memory B cell. These cells are antigen-specific, if the same antigen is repeated, then with the presence of memory B cells, the body form’s antibodies to the antigen. The further often the antigen is inserted into the body, the more the IgG is formed. This increase in the amount of IgG was detected using anti-IgG mouse secondary antibodies, which were reflected in the increase in the absorbance value of the ELISA test results (Nurjayadi, 2005).

4.5. Analysis of specificity and sensitivity anti-fim-C-S. typhi antibodies with western immunoblotting

The aim’s analysis using Western Blot technique is to analyze particular proteins in the sample and to prove whether the antibodies produced in vivo by Wistar rat are anti-Fim-C-S. typhi antibodies. Western Blot used in this research are characterized by the formation of brown color that appears on the membrane due to the occurrence of specific antigen (Fim-C-S. typhi recombinant protein) and antibody (Anti Fim-C-S. typhi recombinant protein antibodies) interactions. The brown formation color on the nitrocellulose membrane shows the occurrence of oxidation reactions of DAB substrates (3, 3′-Diaminobenzidine or 3, 3′, 4, 4′-Biphenyltetramine) forming radical Quinone Iminium compounds and inducing heavy compound formation larger molecules through polymerization reactions. The reaction mechanism of the brown deposits’ formation is showed in Fig. 10.

At Fig. 4, we can see the formation of brown color on membrane nitrocellulose have same size with SDS-PAGE from results of purification from previous step. These result shows that the antibodies used as primary antibodies that originating from Wistar rat can significantly recognize recombinant Fim-C-S. typhi proteins used as antigens. So that it can be concluded that antibodies formed in Wistar rat is anti recombinant Fim-C-S. typhi proteins antibodies.

Besides Fim-C-S. typhi recombinant protein specificity tests were also carried out on several bacterial extracts namely S. typhi, S. typhimurium, E. coli and Shigella. This analysis aims to determine whether anti-Fim-C-S. typhi antibodies can recognize other antigens. Based on the results of Western immunoblotting in Fig. 5, it shows that anti-Fim-C-S. typhi antibodies can specifically interact with Fim-C-S. typhi recombinant protein as an antigen which is also used as a positive control at 31 kDa. In addition, data was as well obtained that anti Fim-C-S. typhi antibodies can interact with pure bacterial protein extracts (S. typhi, S. typhimurium, E. coli, and Shigella) which are characterized by the appearance of brown protein bands at different molecular weight sizes.
The results showed that the Fim-C-2039 Da (Nurjayadi, 2005; Josephy et al., 2018). The experimental pET-30a expression vector used in the cloning process, there are six measuring 29 KDa is thought to be a Fim-C-4.6. Prototype of detection kit for typhoid patients with anti-antibodies by antigen capture recombinant is 0.125 μg.

The S. typhi bacteria extract protein was identified by the antibodies' anti Fim-C-S. typhi at ± 29 kDa and 60 KDa. The appearance of a band measuring 29 KDa is thought to be a Fim-C-S. typhi protein before experiencing recombination or wild type. It can be explained that in the pET-30a expression vector used in the cloning process, there are six amino acids histidine making up His. Tag and Xa factor. If the two parts are calculated based on the amino acid constituent mass, the value is 2039 Da (Nurjayadi, 2005; Josephy et al., 2018). The experimental results showed that the Fim-C-S. typhi recombinant protein measures 31 kDa. So that if the size is reduced by 2 kDa it will correspond to the 29 kDa band recognized by Anti-Fim-C-S. typhi antibodies from S. typhi extracts protein.

In the S. typhimurium (lane D) bacterial extract protein, anti-Fim-C antibodies can recognize two bands of different sizes namely ± 28 kDa and ± 60 kDa. In the extract of bacterial protein E. coli (lane E), anti Fim-C antibodies can recognize protein bands measuring ± 34 kDa and ± 60 kDa, then on antigens extract of bacterial protein Shigella (lane F), anti Fim C-antibodies only recognizes ± 60 kDa protein band. The detection of a 60 kDa band by anti-Fim-C-S. typhi antibody was assumed that the other constituent protein from S. typhi bacteria had one epitope that was homologous with the Fim-C-S. typhi protein so that it can be recognized by one of the paratope from anti-Fim-C-S. typhi antibody and is thought to be a surface protein. In line with what Toobak et al. (2013) stated, that the outer membrane protein (Omp) has good antigenicity and is easy to interact with specific antibodies (Novagen, 2011; Nurjayadi, 2005; Toobak et al., 2013).

The sensitivity evaluation aims to get information of minimum level of anti-Fim-C-S.typhi can recognize Fim-C-S. typhi recombinant as antigen. Fig. 6 shows that various concentrations of Fim-C-S. typhi recombinant protein can be recognized by anti-Fim-C-S. typhi antibodies, indicated by Western blot brown bands at a molecular size of ± 31 kDa. Based on the results of a Western blot, it can be concluded that the recombinant Fim-C protein at the smallest concentration of 0.125 μg can still be detected with anti-Fim-C-S. typhi antibody, or it can be said that the lowest detection level of anti-Fim-C antibody to Fim-C protein recombinant is 0.125 μg.

4.6. Prototype of detection kit for typhoid patients with anti-fim-C-S. typhi antibodies by antigen capture

Fig. 7 shows that anti-Fim-C-S. typhi antibodies besides detecting a 31 kDa protein band as a positive control (Fim-C-S. typhi recombinant protein) (Lane 2), it can also recognize 29 kDa and 60 kDa protein bands derived from crude extracts of S. typhi protein (Lane 3). In healthy people blood protein (Lane 4) no brown protein bands appear. This is due to a healthy people blood sample with no antigen that can be recognized by anti-Fim-C-S. typhi antibodies, so there is no interaction between antigens in the sample with specific antibodies. As it is known anti-Fim-C-S. typhi antibody has the fimbriae antigens, the recognition of blood protein compilers is healthy people probably because in the blood component, there is no receptor protein, which functions to catch S. typhi bacteria such as the intestine which has a complex mechanism and involves various receptor components owned by host cells (Josephy et al., 2018). In the blood sample of typhus patients, there is no clear band on the size of 29 kDa and 60 kDa (Lane 5), this can be confirmed by the results of SDS PAGE electrophoresis as duplex indicating that the protein in the lane has fewer bands, so it is assumed that the protein sample in that lane is at a minimum detection level. To ensure the ability of anti-Fim-C antibodies in detecting S. typhi bacteria, a simulation was then carried out by adding extracts of S. typhi bacteria on healthy blood people (Lane 6) turned out to have a brown band which was the same as S. typhi (Lane 3) extract bacterial antigen, which was at a size of 29 kDa and 60 kDa.

- In healthy people blood samples contaminated with S. typhi extract bacterial antigens and Fim-C-S. typhi recombinant antigen (Lane 7) produced 31 kDa and 60 kDa brown protein bands with the highest intensity. In this sample, there should also be a 29 kDa protein band. However, because the concentration of recombinant Fim-C-S. typhi protein used is too high, the band that appears at a size of 31 kDa is stacked with a band that should as well appear at a size of 29 kDa. Lane 7 is a prototype that will be used as a detection tool for typhoid fever. The detection of a 31 kDa protein band belonging to the Fim-C-S. typhi recombinant protein, which is a specific antigen from anti-Fim-C-S. typhi antibody is then used as a positive control that shows the tool is valid or can running well. Meanwhile, the detection of protein bands measuring 29 kDa and 60 kDa, which belong to the bacterial extract of S. typhi shows positive result indicating that a person has typhoid fever. Therefore, it can be concluded that if a person is positively typhoid fever, three colored bands will appear in the detection device which are 29 kDa, 31 kDa, and 60 kDa. Then, if the person is negative with typhoid fever, only one control band of 31 kDa will appear. However, if the control band does not appear, the test is declared invalid. The model of the detection present at Fig. 8.

5. Conclusion

The development anti-Fim-C-S. typhi antibodies can be made prototype detection kits by antigen capture has been carried out. The anti-Fim-C-S. typhi antibodies precisely recognize S. typhi bacteria that have Fim-C protein and Fim-C-S. typhi recombinant protein as antigens. Increased sensitivity of anti-Fim-C-S. typhi antibodies still needed to be developed by coupling techniques with compounds that can increase its sensitivity. The results of this study are expected to provide a foundation in the development of S. typhi detection methods that are sensitive, specific, and simple.

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**References**


