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Development of antibody anti-FimC-*Salmonella typhi* as a detection kit model of typhoid diseases by antigen capture approach

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ABSTRACT

Typhoid fever is a world health problem, with 200,000 recorded death 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*-FimC-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.

1. Introduction

Typhoid fever (*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 358–810 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 *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

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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; Hasan, 2014; 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 OD_{600} 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 3 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 are 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 orbitalis 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 with 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 I (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, 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 H_2SO_4 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 (Clinichek Laboratories Indonesia No.19/LAB-CL/I/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-mercaptoetanol 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* extract protein and Fim-C-*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,

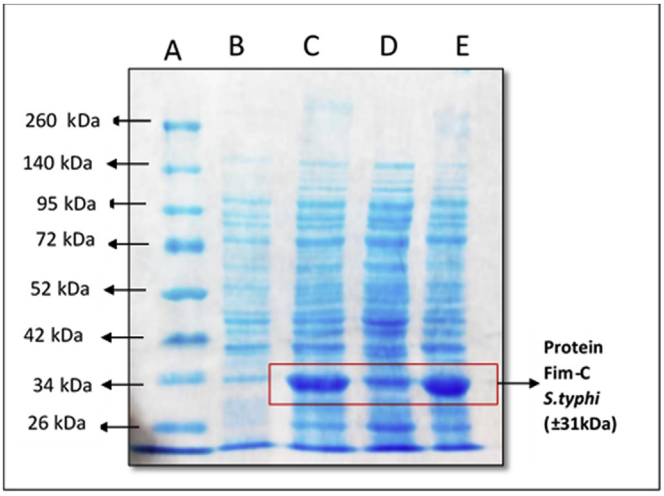

Fig. 1. Results of Fim-C-*S. typhi* Recombinant Protein Overexpression. Lane A is 10 μL (Bio-Rad) Protein Marker. Lane B is 20 μL of Fim-C protein before induction. Lane C is 20 μL of Fim-C protein after induction. Lane D is 20 μL native Fim-C protein results from overexpression dissolved in the cytoplasm with a concentration of 25 μg/mL. Lane E is 20 μL of overexpressed protein Fim-C which forms inclusion bodies with a concentration of 25 μg/mL.

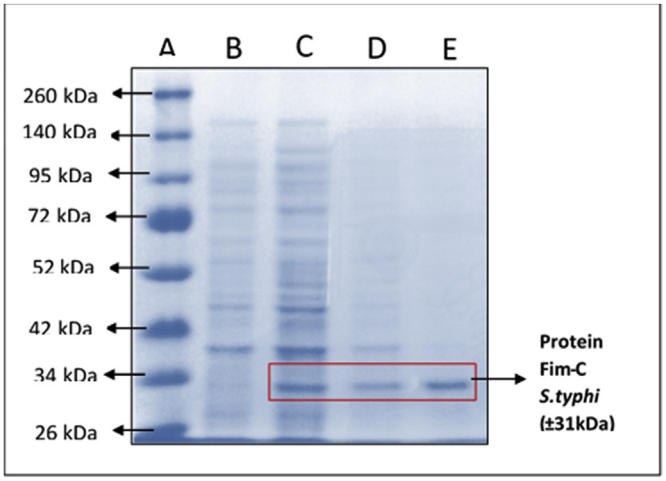

Fig. 2. Purification of Recombinant Fim-C Inclusion Bodies *S. typhi* Proteins. Lane A is 10 μL (Bio-Rad) marker protein. Lane B is B 20 μL of Fim-C recombinant protein before induction. Lane C is C 20 μL of Fim-C recombinant protein after IPTG induction. Lane D is 20 μL Fim-C recombinant protein in inclusion bodies form. Lane E is 20 μL purification results of Fim-C recombinant protein inclusion bodies in 3 μg.

25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol and 0.1% bromophenol). Comparison of volumes between protein samples with a sample buffer of 4: 1. Then the substance denaturized at 100 °C for 10 min. After that, it centrifuged with micro centrifuge at 10,000 rpm for 30 s. The protein sample is ready for electrophoresis and continuing to Western blot process to evaluate of detection models. The western Blot process similar with the previous stage (Nurjayadi et al., 2016; Nurasiah, 2017; Novagen, 2011).

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 188,588 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 (*anti-body 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,

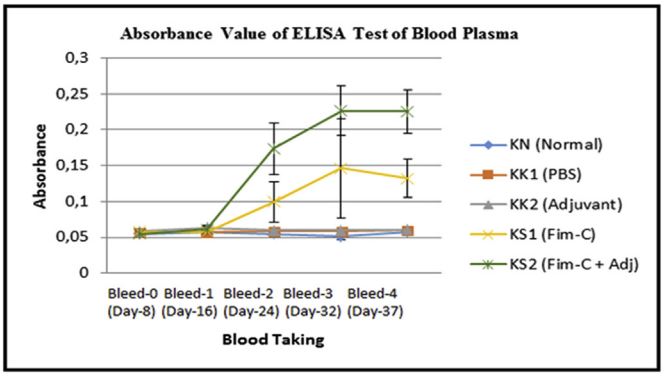

Fig. 3. Graph Analysis of *anti*-Fim-C-*S. typhi* inclusion bodies antibody formation in the treatment and control groups. A green line shows the absorbance value of the group injected with Fim-C-*S.typhi* recombinant protein diluted in PBS 1x and Adjuvant FCA/FIA (KS-1). A yellow lines value absorbance of groups injected with Fim-C-*S.typhi* recombinant Protein diluted in PBS 1x (KS-2). A Gray lines show the absorbance value of groups injected with Adjuvant FCA/FIA diluted in PBS1x for control-1 group (KK-1). The orange line shows the value of the absorbance of the group injected with PBS1x only for control-2 (KK-2). The blue line shows the value of the absorbance of the non-injected group (KN). The X axis shows the development of immunization results on each 0–4 bleed. The Y axis shows the absorbance value of the reading ELISA reader. ELISA is carried out at 100 ng antigen concentration, dilution of primary antibodies 1/100 and secondary antibodies HRP anti IgG-Mouse 1/5000. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

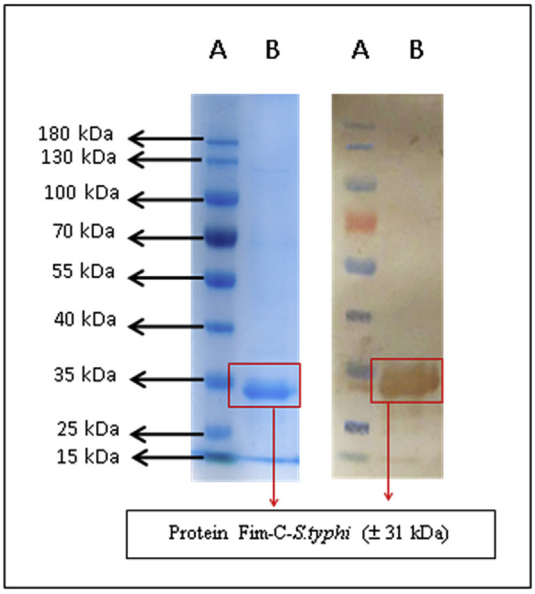

Fig. 4. Characterization of *anti*-Fim-C-*S. typhi* inclusion bodies antibody formation. Line A: 5 µL protein marker *thermo scientific*. Line B: Fim-C-*S.typhi* recombinant protein 20 µL with concentration 3 µg/mL.

2005).

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).

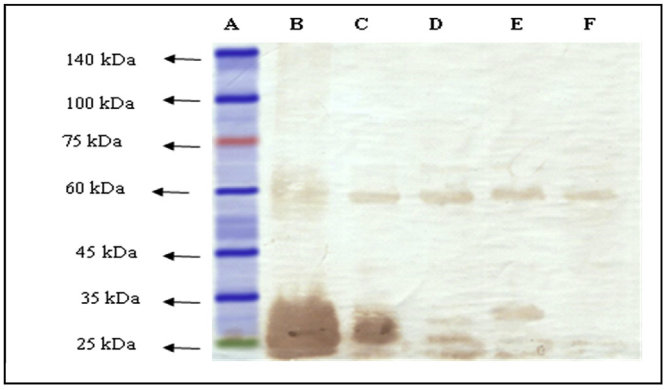

Fig. 5. Western blot result of specificity test t of antibody *anti*-Fim-C-*S.typhi*. Line A Protein Marker 10 µL (Biorad). Line B. Recombinant Fim-C-*S.typhi* Protein; Line C. *S. typhi* Extract Protein; Line D. *S. typhimurium* extract Protein; Line. E. *E. coli* extract Protein; Line F. *Shigella* Extract Protein.

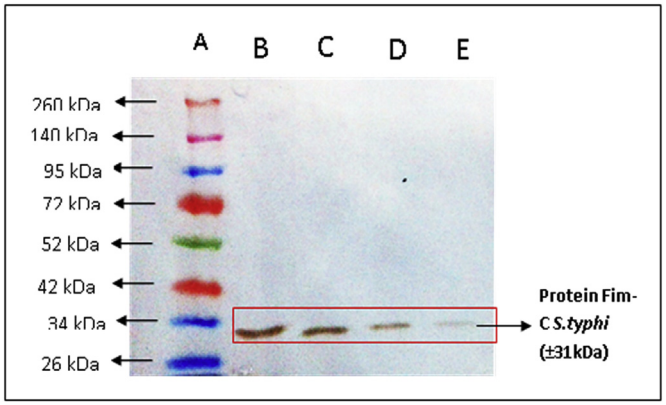

Fig. 6. Western blot result of sensitivity test of antibody *anti*-Fim-C-*S.typhi*. Lane A is 10 µL (Bio-Rad) Protein marker. Lane B is 1 µg/20 µL Fim-C-*S. typhi* recombinant protein; Lane C is 0.5 µg/20 µL Fim-C-*S. typhi* recombinant protein; Lane D is 0.25 µg/20 µL Fim-C-*S. typhi* recombinant protein. Lane E is 0.125 µg/20 µL Fim-C-*S. typhi* recombinant protein.

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. Literary 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

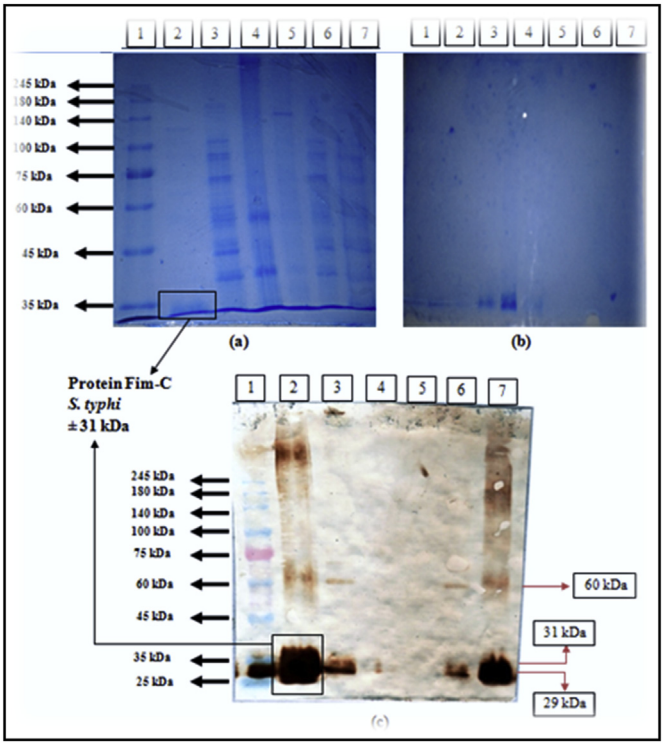

Fig. 7. The Results of Western Immunoblotting *Prototype of detection kit*. **(a)** Results protein electrophoresis gel with several sample before transferring to the membrane. **(b).** Results protein electrophoresis gel (from a) after the protein is transferred to the membrane **(c)** The nitrocellulose membrane produced by Western blot. Lane 1 is 5 µL protein marker SMOBIO; Lane 2 is 20 µL Fim-C-*S. typhi* recombinant protein in 18.3 µg/mL; Lane 3 is pure bacterial *S. typhi* 20 µL protein in 46.3 µg/mL; Lane 4 is healty human blood protein 20 µL; Lane 5 is 20 µL typhoid patient blood protein. Lane 6 is healty human blood protein 10 µL plus pure bacterial protein *S. typhi* 10 µL. Lane 7 is healty human blood protein 10 µL plus of pure bacterial protein *S. typhi* 10 µL plus 5 µL of Fim-C-*S. typhi* recombinant protein.

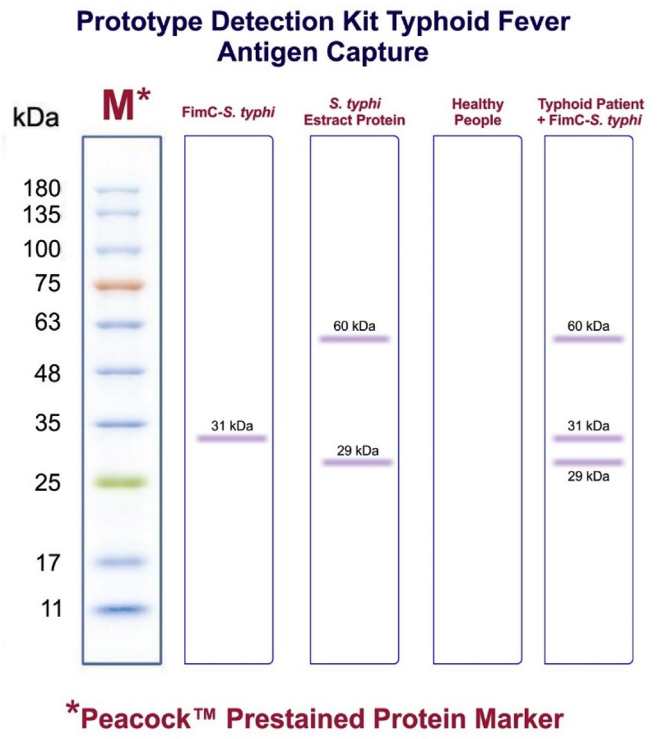

Fig. 8. The Model of prototype detection kit of typhoid disease by Antigen Capture. Line 1. M is protein marker, Line 2. Fim-C-*S. typhi*-recombinant protein, Line 3. *S. typhi* Extract protein, Line 4. Healthy people blood, Line.5. Patient typhoid blood and Fim-C-*S. typhi*-recombinant protein.

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 resins 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

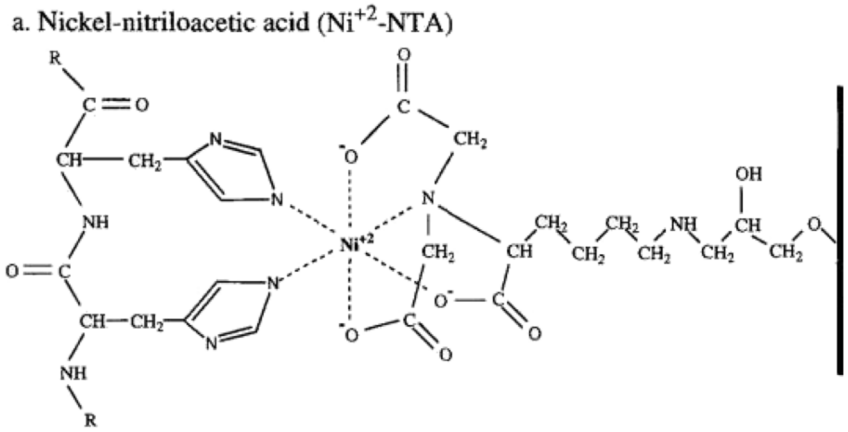

Fig. 9. Models of the interactions between the polyhistidine affinity tag and two immobilized metal affinity chromatography matrices, (a) The nickel–nitrilotriacetic acid matrix (Ni^{+2}–NTA) (Bornhorst and Falke, 2000; Radhakrishnan et al., 2018).

single band on SDS PAGE electrophoresis (Amersham Bioscience, 2013; Bio-Rad, 2016).

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 H_2O_2 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 brow 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.

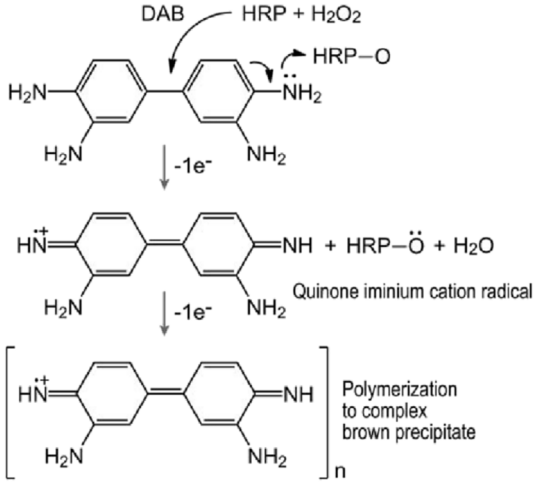

Fig. 10. Transformation of DAB Substrate to precipitate with brown color by Western Immunoblotting. DAB (3, 3′-Diaminobenzidine or 3, 3′, 4, 4′-Biphenyl tetraamines) donated electron to HRP which was catalyzed from oxidation reaction of peroxides (H_2O_2) (Bio-Rad, Laboratories. Inc, 2012; Nurjayadi et al., 2016). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 paratop 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, safe and simple.

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