Toxicity evaluation of recombinant Fim-C Salmonella typhi Protein on ddY Mice for Typhoid Vaccine Development

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Abstract

Background: Typhoid fever is caused by Salmonella typhi infection, commonly occurs in developing countries from bad sanitation and living conditions. Vaccination against the fever has been developed by means of induction of antibody through introduction of Fim-C, one of the virulence factor proteins on the cell surface of the bacterium. Recently, we have cloned and overexpressed the protein in Escherichia coli. The 31 kDa recombinant Fim-C induced immune response upon introduction to ddY mice at a concentration of 40-60 µg/mL, indicating its potency as an anti-typhoid fever vaccine candidate.

Objective: In this study, the safety of the protein was evaluated through abnormal and acute toxicity test in ddY mice, as well as determine its lethal dose (LD50).

Methods: Forty of equal number male and female mice were recruited and observed according to the physiology, body weight and temperature, and mortality rate was performed on fourteenth days after immunization.

Results: No abnormalities were observed at 25 µg/mL while 60% mortality occurred at 125 µg/mL. The latter observation correlates with our finding that the LD50 of the recombinant Fim-C was 123.5 µg/mL.

Conclusion: Our results suggest that the recombinant Fim-C protein is safe for use as anti typhoid fever vaccine.

Background

Typhoid fever is one of the endemic diseases in developing countries, including Indonesia, with an estimated 21 million annual cases of which 1–4% death reported by WHO [1, 20]. Prevention against the fever has been the vaccination with the commercially available Ty21a (an attenuated strain of S. enterica var. Typhi) or Vi (the purified capsular polysaccharide S. enterica var. Typhi Vi antigen), administered through oral or injection, respectively [25, 26]. However, neither is adopted as the standard prevention procedure [11]. Furthermore, the two vaccines are unsuitable for children under five years old and require revaccination every three years [12]. Therefore, a more effective and efficient vaccine against typhoid fever is still in pursue.

The Fim-C (fimbriae) protein is one of the virulence factors reside on the surface or outer membrane of Salmonella protein that is responsible for the bacteria capability to penetrate the epithelial cell barrier upon infection and has significance as one of the potential targets for protective immunity [2, 24]. Therefore, the protein has been developed as a protein vaccine against typhoid fever. The protein has successfully been cloned and overexpressed in Escherichia coli BL21DE3 as inclusion bodies. After solubilization, the purified recombinant Fim-C-S. typhi protein appears to induce the mice humoral immune response against S. typhi bacterial infection as shown by generation of antibody at 40–60 µg/mL [3, 14]. Thus, the recombinant Fim-C-S.typhi inclusion bodies protein appear to demonstrate good potency as a vaccine candidate. For further development of the recombinant Fim-C-S.typhi as a
recombinant vaccine candidate, we evaluated its safety through acute toxicity test and determination of its lethal dose (LD) and LD$_{50}$. Our preliminary toxicity study shows that the recombinant Fim-C-S. typhi protein passes the abnormal toxicity test at 50 µg [13, 22], thus indicates potential safety of the recombinant protein. The toxicity test was performed against the same mice breed in order to obtain a comprehensive result. The recent results suggest that at its intended dose for use, the recombinant Fim-C-S. typhi protein is not toxic and thereby meets the safety requirement for use as a recombinant vaccine.

**Methods**

**Chemicals and reagent**

Chemicals and materials were purchased from Sigma (St. Louis, MO–USA) or Merck (Darmstadt, Germany), Bio-Rad Laboratories, Inc (USA), Thermo Fisher Scientific, except when mentioned specifically.

**Test animal**

The forty ddY mice (20 male and 20 female) aged 5–6 weeks of 17–24 gram were employed in this study (experimental animals are bought commercially at PT BioFarma Indonesia). In this study, male and female rats were used because typhoid disease can affect humans both men and women, so it is expected to obtain more complete information on the use of both sexes of the test animals. The mice were taken care in the animal laboratory of LAPTIAP, BBPT, housed in a cage under controlled environment with enough light at 20–25 $^\circ$C [22]. The male and female ddY mice were divided by two groups that are treatment group (KS1 to KS3) and control group (KN). Sampling of male and female mice for the control group and the treatment group was carried out randomly. Each group consist of five animals tested. The treatment groups-1 until group 3, immunized respectively by 25 µg, 125 µg, 250 µg of recombinant protein Fim-C S. typhi. The control group didn't have any immunized. The mice were anesthetized with Ketamine (90–150 mg/kg) then euthanasia was used using Xylazine (10 mg/kg). The animal testing procedure has been approved by the ethical committee Medical School Universitas Indonesia, protocol no. 997/UN2.F1/ETIK/2016.

**Production of recombinant Fim-C-S. typhi**

The recombinant protein production was performed according to the manufacturer instructions from Novagen and Qiagen [4, 5]. It was briefly ; (1) isolation of the genome S. typhi from Microbiology Laboratories Indonesia University by Wizard kit [16], (2) amplification of fim-C-S. Typhi gene 0.7 kb size by PCR method (3) cloning of fim-C-S. Typhi gene 0.7 kb on vector cloning pGEMT easy kit [4, 17] which constructed recombinant plasmid pGEM-fim-C-S. Typhi, (4) sub cloning gene fim-C-S. typhi from plasmid recombinant pGEM-fim-C-S.typhi to expression vector pET30A [4] to produce recombinant plasmid pET30A- fim-C-S. typhi [14, 15].

Further, the recombinant Fim-C-S. typhi protein was overexpressed in E. Coli BL21 (DE3) pLys. A total of 0.5-2% volume of bacterial inoculum of E. Coli BL21 (DE3) pLysS containing recombinant plasmid pET-
30a-fim-C-S.typhi by OD$_{600}$ from 0.6 to 0.8 is inserted in a 1000 mL Erlenmeyer flask was filled to 250 mL of Luria Broth sterile medium which contains 60 µg/mL Kanamycin. Bacterial cell cultures were incubated at 37°C, with shaking of 150 rpm for 3 hours until OD$_{600}$ was obtained at 0.6 to 0.8. Induction of recombinant protein expression was performed by adding IPTG (Isopropyl-1-thio-β-D-galactopyranoside) to a final concentration of 0.5 mM in the medium, then cell culture incubated in an incubator shaker at 37°C for 4 hours to OD$_{600}$ is 0.6 to 0.8.

The recombinant Fim-C-S. typhi inclusion bodies were recovered from the E. coli cell according to the His-Pur Spin Ni-NTA purification protocol from Thermoscientific [7] with modifications. Briefly, a total of 250 mL of induced cell was transferred to a sterile centrifugation tube. E. coli cell culture was centrifuged at ultracentrifugation at 8,000 rpm for 30 min at 4°C so that the bacterial cell extract was separated from its medium. Pellet resuspended with 4 mL Native equilibration buffer, then sonicated for 30 minutes with a sonicator at a frequency of 4 Hz (sonication process was 30 seconds on and 30 seconds off) until the mixture becomes clear. During the sonication process, the cell suspension is placed in a container of ice to prevent excess heat which can cause damage to the protein that is formed. After that the protein extracted was centrifuged at 4°C at 8000 rpm for 30 minutes to obtain a supernatant which is a protein dissolved in the cytoplasm (native protein). The centrifugation pellets will be prepared further for the isolation of proteins that make up aggregates or inclusion bodies. The pellets cell was re-suspended by 4 mL of Denaturing Equilibration Buffer solution. The mixture was incubated in ice for 1 hour and homogenized by vortex gently for 15 minutes. Subsequently the mixture was centrifuged at 8000 rpm, for 30 minutes at 4°C. The resulting supernatant is a Fim-C S. typhi protein that forms aggregates or inclusion bodies. It hereinafter referred to as the Fim-C-S. typhi recombinant protein.

Purification of the isolated proteins was performed using His-Pur Ni-NTA Spin Purification Kit from Thermo Scientific. The procedure was used in accordance with His-Pur Spin Ni-NTA Purification Kit protocol from Thermo Scientific [6].

**Analysis and characterization of recombinant Fim-C S. typhi**

Expression, isolation, and purification of the recombinant Fim-C-S. typhi protein was monitored on an SDS PAGE analysis according to the kit manufacturer manual [4, 5, 6, 7, 8, 14]. The identity of Fim-C-S. typhi was confirmed using primary antibody anti-Fim-C-S.typhi produced by ddY mice, with DAB as the substrate and anti IgG-mice-HRP diluted 5000 times as secondary antibody [14, 21], on a Western blot analysis carried out according to the kit manufacturer protocol [9]. The protein concentration was determined with the bicinchoninic acid (BCA) assay kit according to the manufacturer instruction [7], using bovine serum albumin (BSA) as the standard [22].

**Toxicity study**

Prior to the testing, the mice were acclimatized for seven days with drink ad libitum. The mice were treated according to the WHO guidance during the acute toxicity test and divided into four groups that
represent the treatment group of 25 µg, 125 µg, 250 µg, and the control [10,19,22]. The mice were monitored for 14 days for the changes in their body weight and temperature, death, and physiological attributes, which were the central nerves (sedated, motoric, convulsion, tremor), autonomous nerve (eyelid, saliva, urination), respiration, gastrointestinal tract, and fur. The mice were sacrificed one week after immunization to obtain the organs (liver, kidney, and spleen) for macroscopic observation. The LD$_{50}$ was calculated from a linear curve representing the administered dose and number of death casualties [10].

**Statistical analysis**

The statistical significances were evaluated using one-way ANOVA test with SPSS 21.0 (SPSS, Inc., Chicago, IL) and defined as a P-value < 0.01. It is used for comparison of the weight, temperature, and organ mass change between test and control groups. [23].

**Results**

Production of Recombinant protein Fim-C Salmonella typhi

In this study, protein samples of Fim-C–S. typhi have been successfully produced through 250-mL protein overexpression process of E. coli BL21 (DE3) pLysS bacterial culture containing pET-30a-Fim-C- S. typhi recombinant plasmid yielding 2,0-gram pellet of bacterial cell. Isolation of overexpressed cells produced 5 mL protein extract that forms aggregates (Inclusion Bodies). The recombinant protein purification process of Fim-C-S. typhi was done by IMAC method using columns containing Ni-NTA resins to produce pure Fim-C Inclusion Bodies S. typhi protein with refining concentration of the first purification (P1) of 271.66 µg/mL and the second purification (P2) of 198.26 µg/mL with yield of 38%. Pure purified proteins were characterized by SDS-PAGE electrophoresis shown in Fig. 1.

Activity and Specificity of Recombinant protein Fim-C Salmonella typhi

The result of characterization activity and specificity of recombinant S. typhi protein was qualitatively analyzed using Western blot. The results are indicating that in Lane B and Lane C (Figure.2) there was brown band with high intensity on ± 31 kDa protein molecular mass.

Acute Toxicity Test of Recombinant protein Fim-C Salmonella typhi

**Physiological Health Analysis of ddY Mice**

The evaluation results on the physiological health of the mice following the acute toxicity test are shown in Table 1.
Table 1
Observation data of physiological health of ddY mice.

| Observation | KS 1 (25 ug Fim-C S. typhi) | KS 2 (125 ug Fim-C S. typhi) | KS 3 (250 ug Fim-C S. typhi) | KN (Normal) |
|-------------|-----------------------------|-----------------------------|-----------------------------|-------------|
|             | Male | Female | Male | Female | Male | Female | Male | Female |
| Central Nervous System | - | - | + | + | + | + | - | - |
| Sedation     | - | - | + | + | + | + | - | - |
| Tremor       | - | - | + | + | + | + | - | - |
| Convulsion   | ++ | ++ | - | - | - | - | ++ | ++ |
| Motor Activity| + | + | +/- | +/- | +/- | +/- | + | + |
| Autonomic Nervous System | - | - | - | - | - | - | - | - |
| Urination    | - | - | - | - | - | - | - | - |
| Salivation   | - | - | + | + | + | + | - | - |
| Openin g Eyelid| + | + | +/- | +/- | +/- | +/- | + | + |
| Respiratory rate| + | + | +/- | +/- | +/- | +/- | + | + |
| Digestive System | - | - | - | - | - | - | - | - |
| Constipation | - | - | - | - | - | - | - | - |
| Bloody Feces | - | - | - | - | - | - | - | - |
| Diarrhea     | - | - | - | - | - | - | - | - |
| Standing Fur | - | - | + | + | + | + | - | - |
| Mortality    | - | - | + | + | ++ | ++ | - | - |

Analysis of Weight Change in ddY Mice

Graph of body weight evaluation of male and female ddY mice during the acute toxicity test is shown in Fig. 3. Specifically, evaluation of weight changes of animal test before and post injection of Fim-C.
antigen S. Typhi is presented in Fig. 4.

**Analysis of Changes in Body Temperature of ddY Mice**

The evaluation of body temperature during the observation shows in the graph of Fig. 5. The results show that average body temperature of ddY mice of fluctuations (not constant). Specifically, evaluation of body temperature changes before and after injection of the Fim-C S. typhi protein is presented in Fig. 6.

**Analysis of Mass Organ Changes in ddY Mice**

Macroscopic observations on mass organs of male and female ddY mice are shown in Table 2. The comparison of spleen mass organ male and female animal test is shown in Fig. 7. The evaluation result of acute toxicity test with variation doses of Fim-C-S. typhi of 25 µg, 125 µg, and 250 µg in detail is shown in Table 3.

### Table 2

**Mass of liver, spleen, and kidney of ddY mice**

| Mice Groups | Liver (g)          | Spleen (g)       | Kidney (g)        |
|-------------|--------------------|------------------|-------------------|
| Fim-C 25 αg | 1,542 ± 0,147      | 0,202 ± 0,030    | 0,416 ± 0,052     |
| Male        |                    |                  |                   |
| Female      | 1,200 ± 0,288      | 0,174 ± 0,033    | 0,264 ± 0,043     |
| Fim-C 125 αg| 1,500 ± 0,567      | 0,146 ± 0,097    | 0,410 ± 0,090     |
| Male        |                    |                  |                   |
| Female      | 0,890 ± 0,507      | 0,226 ± 0,353    | 0,25 ± 0,059      |
| Fim-C 250 αg| 1,609 ± 0,147      | 0,108 ± 0,030    | 0,280 ± 0,074     |
| Male        |                    |                  |                   |
| Female      | 1,292 ± 0,727      | 0,208 ± 0,252    | 0,248 ± 0,055     |
| Control Group| 1,958 ± 0,491      | 0,218 ± 0,063    | 0,432 ± 0,039     |
| (Normal) Male|                  |                  |                   |
| Female      | 1,032 ± 0,075      | 0,236 ± 0,024    | 0,290 ± 0,034     |
Table 3
Results of evaluation of acute toxicity test (LD50)

| LD₅₀ Fim-C Protein Dose (µg/mL) | Number of Mice | Number of Mortality Case | Mortality Rate |
|---------------------------------|----------------|--------------------------|---------------|
| 0                               | 10             | 0                        | 0%            |
| 25                              | 10             | 0                        | 0%            |
| 125                             | 10             | 6                        | 60%           |
| 250                             | 10             | 10                       | 100%          |

Discussion

Production of Recombinant protein Fim-C Salmonella typhi

The characterization of production and purification recombinant protein Fim-C Salmonella typhi show the presence of a single band on Lane E on Fig. 1 with ± 31 kDa molecular weight. These results concluded that the process of overexpression and purification of the Fim-C Inclusion Bodies S. typhi protein has been successfully performed [14, 15, 27].

Activity and Specificity of Recombinant protein Fim-C Salmonella typhi

Developing band with brown color was the results of oxidation reaction between peroxide's substances (H₂O₂), with catalyzed by Horse Radish Peroxidase (HRP) enzyme. The Horse Radish Peroxidase (HRP) enzyme changed DAB (3, 3'-Diaminobenzidine or 3,3',4,4'-Biphenyltetramine) substrate to Quinone Iminium substance, which is radical properties. Afterward, the Quinone Iminium substance induced the occurrence of polymerization reaction to form polymer substance with bigger molecular size and produce precipitate with brown color [9, 14, 18, 21]. The condition of antigen-antibody interaction on Fig. 2, was respectively as follows (1) The concentration of antigen is 3 µg, (2) the 100-time dilution of anti-recombinant protein of Fim-C S. typhi antibody, and (3) the 5000-time dilution of secondary antibody anti-mice labeled with the Horse Radish Peroxidase (HRP) enzyme. This result proves that Fim-C-S. typhi antigen has successfully identified its specific Anti Fim-C-S. typhi antibody.

Acute Toxicity Test of Recombinant protein Fim-C S. typhi

Physiological Health Analysis of ddY Mice

The clinical observation until day 14 of post-acute toxicity test showed that giving recombinant protein Fim-C S. typhi with 25 µg concentration did not show any physiological disorder to central nervous system of ddY mice. It is such as the influence of analgesia and decrease of cognitive sharpness
(sedation), trembling or seizure (tremors and convulsions), and motor activity (the mice remain active hanging on the roof of the cage and often sniff around the cage/have a high curiosity).

The male and female ddY mice immunized with a dose of 125 µg protein Fim-C-S. typhi experience symptoms such as sedation, convulsions and tremors, eyelid opening and abnormal breathing rates as well as tail standing in some of the mice. Three hours’ post-injection of antigen Fim-C-S. typhi with 125 µg concentration, three males and females in Sample Group 2 (KS-2) experienced mortality and two mice of each group still can survive until 14 days of the intensive observation period.

The male and female ddY mice immunized with a dose of 250 µg protein Fim-C-S. typhi experience symptoms such as sedation, convulsions, tremor, eyelid opening and abnormal breathing rate as well as tail standing in some of the mice. One-hour post-injection of Fim-C-S. typhi 250 µg concentration, three males and females ddY mice from Sample Group experienced mortality.

**Analysis of Weight Change in ddY Mice**

The evaluation results of body weight measurement of ddY mice in the control group and sample groups in general experienced weight gain. This indicates that either mice feed pellet or Fim-C-S. typhi protein test had the same effect to weight gain of mice. We can conclude that the giving Fim-C-S. typhi didn’t give significant change of appetite the animal test.

The evaluation results indicate that the weight is gain post immunization. The blue line shows the weight before treatment, and the red line indicated weight after treatment.

**Analysis of Changes in Body Temperature of ddY Mice**

The mice on sample groups (KS) experienced increased body temperature on the first day-after injection of Fim-C-S. typhi recombinant protein. It is caused by the body’s response to the entry of foreign substance (Fim-C protein) into the mice body. The Fim-C protein will act as an antigen affecting the immune system and stimulating the leukocytes to release interleukins that will directly the set point thermoregulators in the hypothalamus [10, 19]. The body temperature of the mice ranges from 34.9–35.2 °C with an average body temperature change (ΔT) of 0.1-0.3°C. Increase in body temperature of mice is still considered normal if it is not more than 1°C.

The result of body temperature changes evaluation of ddY mice was proved through statistical data processing by one-way ANOVA. It showed the significance level $P > 0.01$, where the null hypothesis (Ho) is accepted, and Alternative Hypothesis (Ha) are rejected. The results showed that there was no significant difference in temperature changes (ΔT) between sample and control groups of mice (KS-1, KS-2, and KN). Sequentially P-value of the male and female mice in sample group 1 (KS-1) was 0.836 and 0.917, while in sample group 2 (KS-2) was 0.908 and 0.779 [23]. The results of statistical data processing with one-way ANOVA conclude that Fim-C-S. typhi recombinant protein with concentration of 25 micrograms (µg) and 125 micrograms (µg) had no significant effect on temperature changes of the both ddY mice.
Analysis of Mass Organ Changes in ddY Mice

Based on the organ mass data in Table 2, the statistical data was processed using one-way ANOVA of kidney, heart and spleen have results below:

a. Kidney. The statistical data processing showed significance level $P > 0.01$, which means no significant effect of Fim-C- S. typhi protein on the changes in kidney mass of ddY mice.

b. Heart. The statistical data processing showed the significance level of $P > 0.01$, which means there is no significant effect of Fim-C-S. typhi protein on changes in liver mass of ddY mice.

c. Spleen. The results showed that spleen in the mice sample group that has been immunized with the Fim-C-S. typhi protein works harder to produce antibodies in response to the presence of Fim-C-S. typhi recombinant proteins in the body. The results of statistical data processing of spleen organ in male and female ddY mice with a one-way ANOVA show significance level for sample group 2 and 3 (KS-2 and KS-3) of $P < 0.01$, while sample group 1 (KS-1) shows the significance level of $P > 0.01$. Therefore, sample group 2 and 3 are applied null hypothesis (Ho) refused and alternative hypothesis (Ha) accepted, which means there is a significant effect of giving Fim-C-S. typhi recombinant protein with 125 µg and 250 µg concentration to change spleen organ masses in both ddY mice. Meanwhile, sample group (KS-1) that was giving Fim-C-S. typhi recombinant protein with 25 µg concentration did not give significant effect to change spleen organ mass of male and female ddY mice.

Based on Table 3, mortality case was found in groups of mice immunized with Fim-C-S. typhi protein concentrations of 125 and 250 µg/mL; mortality rates in each group are 60% and 100%. Based on the acute toxicity test of recombinant protein of Fim-C S. typhi in Table 3, a curve of LD50 dosage calculation depicting the relationship between doses of Fim-C S. typhi protein with the mortality rate of ddY mice is shown in Fig. 8. The linearity that describes the relationship between dosages of Fim-C-S. typhi protein with mortality of ddY mice yielded the equation of the line $y = 0.4258x - 2.5806$ with regression value equal to 0.9758. From the equation of the lines, it can be determined LD50 dose by converting the value of x so that obtained LD50 dose (dose that can cause mortality) capable of 123.486 µg/mL. The results of the evaluation of acute toxicity test also showed that the safe dose range of Fim-C S. typhi recombinant protein for male and female ddY mice is at range 25–120 µg/mL.

Conclusion

The results of acute toxicity test showed that the dose of LD$_{50}$ of Fim-C-S. typhi recombinant protein is 123.486 µg/mL. This is evidenced by the mortality rate in sample group 2 (KS-2) after being immunized with a dose of 125 µg is 60% and in sample group 3 (KS-3) is 100%. The result of research is reinforced by statistical data processing by using one-way ANOVA which shows that by giving Fim-C S. typhi protein with 125 µg and 250 µg concentration had the significant effect ($P < 0, 01$). The evaluation of the acute toxicity test showed that safe dose range of Fim-C S. typhi protein for male and female ddY mice ranged at 25 to 120 µg/mL. Based on the results, we found that the recombinant Fim-C protein is safe for use as anti-typhoid fever vaccine and can be developed for another tested.
Abbreviations

We used some abbreviation in this manuscript, that are S. typhi (Salmonella typhi); E.coli (Escherichia coli); Fim-C (fimbriae C); LD_{50} (Lethal Dose 50); KS-1 (treatment Group 1); KS-2 (Treatment Group 2); KS-3 (Treatment Group 3); KN (Normal Group); SDS PAGE (Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis); OD (optical Density); DAB (3, 3'-Diaminobenzidine or 3,3',4,4'-Biphenyltetramine); HRP (Horseradish Peroxidase) enzyme; BCA (Bicinchoninic Acid) assay kit; ANOVA (Analysis of Variance); SPSS (Statistical Program).

Declarations

Ethics and Consent to participate

The animal testing procedure has been approved by the ethical committee of Medicine Faculty Universitas Indonesia with protocol No. 997/UN2.F1/ETIK/2016.

Consent for publication

I would like to propose waiver publication budget, because the funding is not enough for pay budget for open access publication. I have email to the editorial Team and filed in the questioner for the detail reason.

Availability of data and material

All data generated or analyzed during this study are included in this published article (and its additional files). The document that already publish related with this article can be accessed on https://doi.org/10.1016/j.proche.2016.01.037;

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Competing interests

The authors declare that no competing interests.

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Author’s Contributions

Conceptualization and design: MN, and WM; Development of methodology: IRK and KA; Material support: GR, GA and NIK; Analysis and interpretation of data: MN and KA; Writing: MN, GR, GA and NIK; Revision: MN, NIK and WM. All authors read and approved the final manuscript.

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Figures

![Figure 1](image_url)

**Figure 1**

Result of characterization of pure protein Fim-C S. typhi. Lane A 10 μL protein marker (Bio Rad). Lane B is 20 μL Fim-C protein before induced. Lane C is 20 μL Fim-C protein after induced. Lane D is 20 μL Fim-C protein inclusion bodies prior to purification with concentration of 3 μg/mL. Lane E is 20 μL Fim-C protein inclusion bodies purified with concentrations of 3 μg/mL.
Figure 2

Result of characterization of Fim-C S. typhi protein with Western Blot. Lane A indicates 10 μL marker protein (Bio Rad). Lane B showed 20 μL Fim-C inclusion bodies prior to purification with concentration of 3 μg/mL. Lane C showed Fim-C inclusion bodies purified with concentrations of 3 μg/mL.
Figure 3

Graph of ddY mice weight measurement. The blue line indicates the body weight of the ddY mice in Sample Group 1 (KS 1). The red line indicates the body weight of the ddY mice in Sample Group 2 (KS 2). The green line shows the weight of ddY mice in Normal Group (KN).

Figure 4

Graph of weight change in ddY mice. A. Female mice evaluation, and B. Male mice weight evaluation. Evaluation results indicate weight gain post immunization. The blue line shows weight before treatment (H0) and the red line indicate weight after treatment (H1).
Figure 5

Graph of body temperature measurement of acute toxicity test of male and female mice. Blue line indicates ΔT of ddY mice in sample group 1 (KS1). Red line indicates ΔT of ddY mice in sample group 2 (KS2). Green line indicates ΔT of ddY mice in normal group (KN).

Figure 6

Graph of body temperature changes of ddY mice day-0 and day-14. The results of body temperature evaluation showed insignificant increase in body temperature on day 0 to day 14.
**Figure 7**

Graph of the spleen organ mass of ddY mice. (A) The average spleen organ mass of male ddY mice, and (B) The average spleen organ mass of female ddY mice.

**Figure 8**

Graph of acute toxicity test of LD50. The curve indicates linear relationship between LD50 proteins Fim-C S. typhi doses with mortality rate.

**Supplementary Files**

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