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Immunogenicity and Specificity of Anti recombinant Protein Fim-C-Salmonella typhimurium Antibody as a Model to Develop Typhoid Vaccine

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Abstract

Salmonella typhimurium, that cause typhoid fever in mice was used as a model to study typhoid fever in humans. This study is aimed to determine the immunogenicity of recombinant protein of FimC-S. typhimurium and specificity of antibody anti-recombinant protein of FimC-S. typhimurium in ddY mice. The immunogenicity studies by ELISA indicated that recombinant protein of FimC-S. typhimurium evokes strong immune response in ddY mice, particularly in experiment group. The specificity evaluated by Western Immunoblotting technique indicated that the antibody anti-recombinant protein of FimC-S. typhimurium can detect the 31 kDa of recombinant protein of FimC-S. typhimurium as an antigen. This data reported that recombinant protein of FimC-S. typhimurium is immunogenic and the antibody anti-recombinant protein of FimC-S. typhimurium is specific. The results of this study can be used as a model to develop recombinant vaccine candidate against typhoid.

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Keywords: Salmonella typhimurium; typhoid fever; recombinant protein FimC-Salmonella typhimurium antibody; recombinant vaccine

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1. Introduction

Typhoid fever, caused by Salmonella typhi \((S. \text{typhi})\) bacteria, is a major cause of illness and death, particularly among children, in developing countries. By the year 2000, it is estimated 22 million cases of typhoid fever and 200,000 deaths occurring worldwide\(^{1,4,20}\). However, to study the pathogenesis of \(S. \text{typhi}\) in human is restricted. Thus, the urgency to find a pathogen with similar symptoms should be carried out. Salmonella typhimurium \((S. \text{typhimurium})\), that cause typhoid fever in mice and has similar symptoms to typhoid fever in human, are used as a model.

Salmonella typhimurium are pathogenic, Gram negative, facultative anaerobic bacilli which have flagels and fimbriae and live in human and animal intestinal tracks. The organisms stimulate different effects in different host cells. Those cause gastroenteritis in humans, while also typhoid fever in mice. Therefore, \(S. \text{typhimurium}\) in infected mice are used as a model to study the pathogenesis of \(S. \text{typhi}\) in humans\(^{17,21}\). Typhoid fever is overcome by vaccines. Two available vaccines against typhoid, Ty21a (oral vaccine) and Vi CPS (injection vaccine), are produced from attenuated strains of \(S. \text{typhi}\). However, those vaccines provide moderate protection against typhoid, especially in hyper-endemic areas\(^{5,8}\). In Indonesia, effectiveness of protection against typhoid is 33%-53% within three years lower than other countries\(^{21}\). Therefore, it has stirred an urgency to develop better and efficient typhoid vaccines.

Recombinant protein vaccine is one of the alternatives against typhoid. There are advantages of recombinant protein vaccine, such as higher protection for patients, higher purity, more specific, and allowing the production of large quantities\(^{1,2,21}\). The recombinant subunit approaches are being used for the development of new vaccines and improvement of already existing vaccines\(^5\). Many recombinant vaccines are now being tested in preclinical and clinical trials, and some recombinant vaccines are already on the market. Some recombinant vaccine showed at Table 1.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Description</th>
<th>Application</th>
<th>Company</th>
<th>Approved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombivax</td>
<td>rHBsAg produced in (S. \text{cerevisiae}) as one component</td>
<td>Hepatitis B prevention</td>
<td>Merck</td>
<td>1986 (US)</td>
</tr>
<tr>
<td></td>
<td>Combination vaccine, containing rHBsAg produced in (S. \text{cerevisiae}) as one component</td>
<td>Vaccination of infants against (H. \text{influenzae}) type B and hepatitis B</td>
<td>Merck</td>
<td>1996 (US)</td>
</tr>
<tr>
<td>Comvax</td>
<td>rHBsAg produced in (S. \text{cerevisiae}) as one component</td>
<td>Vaccination against hepatitis B, diphtheria, tetanus, and pertussis</td>
<td>SmithKline Beecham</td>
<td>1996 (EU)</td>
</tr>
<tr>
<td>Tritanrix-HB</td>
<td>Combination vaccine, containing rHBsAg produced in (S. \text{cerevisiae}) as one component</td>
<td>Immunization against hepatitis A and B</td>
<td>SmithKline Beecham</td>
<td>1996 (EU)</td>
</tr>
<tr>
<td>Twinrix, adult and pediatric forms</td>
<td>Combination vaccine, containing rHBsAg produced in (S. \text{cerevisiae}) as one component</td>
<td>Immunization against diphtheria, tetanus, and hepatitis B</td>
<td>Pasteur</td>
<td>2004 (EU)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Merieux</td>
<td>1998 (EU)</td>
</tr>
<tr>
<td>Primavax</td>
<td>Combination vaccine, containing rHBsAg produced in (S. \text{cerevisiae}) as one component</td>
<td>Immunization against (H. \text{influenzae}) type B and hepatitis B</td>
<td>Pasteur</td>
<td>1999 (EU)</td>
</tr>
<tr>
<td>Procomvax</td>
<td>Combination vaccine, containing rHBsAg produced in (S. \text{cerevisiae}) as one component</td>
<td>Lyme disease vaccine</td>
<td>SmithKline Beecham</td>
<td>1998 (US)</td>
</tr>
</tbody>
</table>

Table 1. Selected examples of recombinant vaccines currently approved in the US or EU

The previous research in 2007-2009, we found thirteen potential \(S. \text{typhi}\) protein, one of them is Fim-C \(S. \text{typhi}\), using 2D and MALDI TOFF\(^{11}\). In parallel we also study Fim-C \(S. \text{typhimurium}\) protein as a model. The Fim-C \(S. \text{typhimurium}\) protein as well known outer membrane protein. References analysis indicated that the outer membrane proteins of \(Salmonella\) have strong immunogenic potential, this characteristic is supporting for development of vaccine. The use of recombinant proteins allows the targeting of immune responses focused against few protective antigens was studied by Nascimento and Leite [2012]. There are a variety of expression systems with different advantages for recombinant protein, such as allowing the production of large quantities of proteins depending on the required characteristics, a high purity, specific and easy to handling\(^1\). In parallel research, we have been developing of recombinant protein of Fim-C-\(S. \text{typhi}\) as vaccine candidate against typhoid\(^{11}\). Earlier study carried out in our
laboratory, we have done isolated, cloned, expressed of fim-C- S. typhimurium gene to be recombinant protein of fim-C- S. typhimurium, and purified and characterize of recombinant protein of Fim-C-S. typhimurium. This study is aimed to determine the immunogenicity of recombinant protein of Fim-C-S. typhimurium and specificity of anti recombinant protein of Fim-C-S. typhimurium antibody in ddY mice. The research is very important as model/basic on developing recombinant typhoid vaccine for human.

2. Methods

2.1. Antigen

Antigen used in this experiment is recombinant protein Fim-C-S. typhimurium in the form inclusion bodies. Antigen of Fim-C-S. typhimurium was produced in the previous research using recombinant DNA technology. The procedure for production of recombinant protein of Fim-C-S. typhimurium as follow: (1) isolation of the genome S. typhimurium LT2 by Wizard kit, (2) amplification of fim-C-S. typhimurium gene 0.7 kb size by PCR method, (3) cloning gene of fim-C-S. typhimurium 0.7 kb on vector cloning pGMeasy kit, which constructed recombinant plasmid pGEM-fim-C-S. typhimurium, (4) subcloning gene fim-C-S. typhimurium to plasmid recombinant pGEM-fim-C-S. typhimurium to expression vector pET30A [pET system, Invitrogen, 2012] to produced plasmid recombinant pET30A-fim-C-S. typhimurium, (5) Expression of recombinant protein of Fim-C-S. typhimurium, (6) isolation and purification of recombinant protein of Fim-C-S. typhimurium in inclusion bodies form by Hispur kit.

Preparation of antigen for immunization as follow as: the amount of 20-60 µg recombinant protein Fim-C-S. typhimurium in inclusion bodies form resulted on the purification dissolved with buffer PBS 1x on Eppendorf 1,5 mL the total volume was 100-500 µL. Addition with Freund’s complete adjuvant (FCA) or Freund’s incomplete adjuvant (FIA) with equal comparation 1:1. Homogenation by vortexing up to the white color appear.

2.2. Animal Tested

Production of antibody anti recombinant protein of Fim-C-S. typhimurium was conducted in ddY strains, 6-3 weeks old, 17-24 gr male mice. Antibody of anti recombinant protein of Fim-C-S. typhimurium was obtained from 36 mice that have been categorized into two major groups, experiment group and control group. Each of the experiment groups were divided into two sub groups, the group that was immunized by mixed Fim-C S. typhimurium recombinant protein and Freund complete/incomplete Adjuvant (KP-1) and the group that was immunized only by Fim-C protein dilution in PBS 1x (KP-2). The control groups divided into two sub groups, the first group that was immunized by Freund Complete/Incomplete Adjuvant (KS-1) and the other group that was immunized by Phosphate Buffer Saline or PBS 1x (KS-2). Each of the groups consisted of 8 mice. Four other mice were treated as control in each group. They were categorized into normal group (KN), without immunized.

2.3. Immunization

Immunization was conducted as many as four times in day 1, day 9, day 17, and day 25. Each immunization was performed in 1-2 injections using 20 µg (dose 1), 40 µg (dose 2) and 60 µg (dose 3 and 4) on the dorsal near head for experiment groups.

2.4. Test Bleeds

Test bleeds were done from mice’s eyes sinus orbitalis. Pre-immune serum was taken before the treatment began (day 0). Continuously, the test bleed was taken on day 8 (test bleed 1), day 16 (test bleed 2), day 24 (test bleed 3), and day 32 (test bleed 4). 500 µL bloods from eyes sinus orbitalis was incubated at 37°C for 1 hour and was centrifuged at 5000 g for 10 minutes at 4°C. The serum was removed from the cell pellet and inserted to sterile Eppendorf tube. The serum then was frozen at -20°C. The yield was approximately 100-200 µL.
2.5. *Enzyme Linked Immuno Sorbet Assay (ELISA)*

As much as 100 ng and 300 ng of Fim-C *S. typhimurium* recombinant protein were used as antigen in ELISA. Antibody of anti recombinant protein of Fim-C-S. *typhimurium* in ELISA was diluted 100 times. Amount of 50 µL antigen diluted in PBS was incubated in each well micro titer plate at room temperature. Each well was washed three times with PBS contained by 1 mM MgCl₂ and 0.05% (v/v) Tween-20. As much as 150 µL 5% *blooto* (5 g skimmed milk in 100 mL PBS) was added in each well, and then micro titer plate was incubated at 37°C for one hour. Micro titer plate was washed three times with PBS. The 50 µL mouse serum from the fourth test bleeds with 1000 times dilution concentration was added in each well and incubated at 37°C for one hour. The plate was washed three times with PBS. The 50 µL labeled secondary antibody (anti IgG-mice-HRP diluted 5000 times) was added to plate, and then incubated at 37°C for one and a half hour. Micro titer plate well was washed with washing buffer for three times. 50 µL substrate solution TMB (3,3',5,5'-tetramethylbenzidine, Thermo Scientific) was added in each well and incubated at 37°C for one hour. The color formation reaction was stopped by adding 50 µL H₂SO₄. After the incubation, it was measured the absorbance value by ELISA-Reader in 450 nm wave length. The result was the absorbance value in each well.

2.6. **Specificity Test by Western Immunoblotting.**

Serological test antibody of anti recombinant protein of Fim-C-S. *typhimurium* against antigen of recombinant protein Fim-C-S. *typhimurium* was performed by Western immunoblotting method. In the previous research characterization of 31 kDa Fim-C S. *typhimurium* protein was performed. The result of protein separating by SDS-PAGE gel was transferred to nitrocellulose membrane using Western immunoblotting apparatus. Protein transfer process was taken at 4°C 200 V in 2-3 hours. Membrane was blocked with skimmed milk to avoid interaction between primary and secondary antibody. Primary antibody added to membrane was the serum from the fourth test bleed with 1000 times dilution concentration.

3. Results and discussion

3.1. **Immunogenicity Test of Recombinant Protein of Fim-C-S. typhimurium as Antigen**

Immunogenicity is the ability of a particular substance, such as an antigen or epitope, to provoke an immune response in the body of a human or animal. In other words, immunogenicity is the ability to induce a humoral and/or cell mediated immune response. To evaluate the immunogenicity of recombinant protein of Fim-C S. *typhimurium*, the pure recombinant protein described above was prepared for immunization experiment. The ddY mice were immunized with the antigen formulated with Freund's complete/incomplete adjuvants. Immunization of mice with increasing protein doses (20 µg, 40 µg, 60 µg, and 60 µg) indicated that a 60 µg dose gave the highest anti-Fim-C-S. *typhimurium* antibody response (Table 2 and Fig. 2).

To compare the immunogenicity of recombinant protein of Fim-C S. *typhimurium*, in this research the ddY mice divided in two groups, they are experiment and control group as describe above. The antibody response showed with increasing absorbance value from ELISA process. As we known the increasing absorbance value shows interaction between recombinant protein of Fim-C S. *typhimurium* as antigen and antibody anti recombinant protein of Fim-C S. *typhimurium*. Those interaction showed with the link of the antigen, primary antibody (anti recombinant protein of Fim-C-S. *typhimurium* antibody) and secondary antibody anti-mice conjugated with Horse Radish Peroxidase (HRP) enzyme. Therefore HRP bonded on secondary antibody will oxidize the substrate TMB (3,3',5,5'-Tetramethylbenzidine) became 3,3',5,5'-tetramethylbenzidine diimine.

The resulting diimine causes the solution to take on a blue color, and this color change can be observed on a spectrophotometer at a wavelength of 650 nm. The reaction can be stopped by addition of acid or another stopper reagent. Using sulfuric acid turns TMB to be yellow. The color may be read at 450 nm and a higher antibody titre produced the thicker colour appear. The oxidation reaction of changing 3,3',5,5'-tetramethylbenzidine became 3,3',5,5'-tetramethylbenzidine diimine showed at Fig.
Antigen concentrations in ELISA test were 100 ng and 300 ng. In the experiment group, each of test bleed produced a higher absorbance in 300 ng antigen concentration. The higher antigen concentration bound higher amount of antibody. Antigen-antibody interaction, or antigen-antibody reaction, it is a specific chemical interaction between antibodies produced by B cells of the white blood cells and antigens during immune reaction. It is the fundamental reaction in the body by which the body is protected from complex foreign molecules, such as pathogens and their chemical toxins. In the blood, the antigens are specifically and with high affinity bound by antibodies to form an antigen-antibody complex. The immune complex is then transported to cellular systems where it can be destroyed or deactivated.

Table 2 and Fig. 2 show the amount of antibody titer in each test bleed from experiment and control groups. Antibody titer in experiment group 1 (KP-1) and experiment group 2 (KP-2) increased in every test bleeds, that showed the increasing antibody response against the antigen. Antibody titer of control group 1 (KS-1, immunized by adjuvant) and control group 2 (KS-2, immunized by PBS) continuously did not perform the increasing antibody response. Those results showed that Fim-C S. typhimurium protein has satisfying as immunogenicity.

The increasing of antibody titer of experiment group 1 (immunized by antigen Fim-C S. typhimurium +CA/FIA adjuvants (KP-1)) was higher than antibody titer of experiment group 2 (immunized by antigen Fim-C S. typhimurium + PBS (KP-2)). Those proved that adjuvants can stimulate strong, prolonged responses and protect the antigen from releasing faster. As we known adjuvants are nonspecific stimulators immune response. The judicious use adjuvants are essential to induce a strong antibody response to soluble antigens. Perrie et al, 2008 also reported the use of adjuvant systems have proven to enhance the immunogenicity of these sub-unit vaccines through protection (i.e. preventing degradation of the antigen in vivo) and enhanced targeting of these antigens to professional antigen-presenting cells. However, the experiment group 2 (KP-2) also showed antibody response with the increasing of antibody titer in each test bleed. That’s means the recombinant protein of Fim-C-S. typhimurium have a good immunogenicity, because those protein can induce respon immune without adding the adjuvant. So it’s very potential as vaccine candidate.
The comparison between experiment group 1 (KP-1) and control group 1 (KS-1) showed significant difference to give response immune. FCA/FIA Adjuvant given alone to the control groups did not affect the production of antibody. Similar results given by the experiment group 2 (KP-2) and control group 2 (KS-2). The result showed that only recombinant protein of Fim-C S. typhimurium antigen can induced the increasing of antibody titer. The result also showed that Fim-C S. typhimurium antigen can respond to specific antibody, while PBS as a solvent and adjuvant alone does not have immunogenic properties. Compare with other recombinant protein that showed in Table 1, mostly the recombinant protein with conjugate, however recombinant protein of Fim. C S. typhimurium even without conjugate can produce a good immune respond. That showed those protein have good characteristic as antigen and vaccine candidate.

![Graph](image)

Fig. 2. The comparison analysis of antibody titer in all groups. The blue line shows the increasing of antibody in experiment group 1 (KP-1). The red line shows the increasing of antibody in experiment group 2 (KP-2). The green line shows antibody titer in control group 1 (KS-1). The purple line shows antibody titer in control group 2 (KS-2). The comparison was performed in 300 ng antigen concentration and 100 times dilution of antibody anti recombinant protein of Fim-C S. typhimurium.

3.2. Specificity test of anti-Fim-C S. typhimurium antibody

The results of specificity test of anti Fim-C S. typhimurium antibody by Western Immunoblotting showed interaction between Fim-C antigen and anti-Fim-C S. typhimurium antibody. The interaction was made by the formation of band with brown color on 31 KDa nitrocellulose membrane, same size as antigen Fim-C S. typhimurium protein.

The band with brown color was the results of oxidation reaction with peroxides substances (H₂O₂), which 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 Imine substance which is radical properties.

Afterward, the Quinone Imine substance induced the occurrence of polymerization reaction to form polymer substance with bigger molecular size and produce precipitate with brown color. The reaction mechanism to produce precipitate in brown color with western Immunoblotting was showed at Fig. 3. The specificity test reported in this research was conducted for experiment group and control group. The condition of antigen-antibody interaction was respectively as follow (1) 3 μg antigen concentration, (2) 100 times dilution of anti recombinant protein of Fim-C S. typhimurium antibody, and (3) 5000 times dilution of secondary antibody anti-mice.
The Western Immunoblotting showed that the experiment group-1 (KP-1) and experiment group-2 (KP-2) produced antibody respond against antigen in nitrocellulose membrane. Meanwhile control group 1 and control group 2 did not produce the antibody. The results of testing were shown in Fig. 4.
The result showed that recombinant protein of Fim-C S. typhimurium antigens can induce the formation of anti recombinant protein of Fim-C specific antibodies, proved by the brown band in membrane nitrocellulose. Meanwhile the control group which not gets immunized by Fim-C antigen gave negative results. Based on the results we can describe that’s only experiment group 1 and 2 which produce the immune response. As we know there are several types of antibodies and antigens, and each antibody is capable of binding only to a specific antigen. The specificity of the binding is due to specific chemical constitution of each antibody. The antigenic determinant or epitope is recognized by the paratope of antibody, situated at the variable region of the polypeptide chain. The variable region in turn has hyper-variable regions which are unique amino acid sequences in each antibody. Antigens are bound to antibodies through weak and noncovalent bonds such as electrostatic interactions, hydrogen bonds, Van der Waals forces, and hydrophobic interactions\(^{18}\).

The use of adjuvant systems have proven to enhance the immunogenicity of these sub-unit vaccines through protection (i.e. preventing degradation of the antigen in vivo) and enhanced targeting of these antigens to professional antigen-presenting cells. Understanding of the immunological implications of the related disease will enable validation for the design and development of potential adjuvant systems. Novel adjuvant research involves the combination of both pharmaceutical analysis accompanied by detailed immunological investigations, whereby, pharmaceutically designed adjuvants are driven by an increased understanding of mechanisms of adjuvant activity, largely facilitated by description of highly specific innate immune recognition of components usually associated with the presence of invading bacteria or virus. The majority of pharmaceutical based adjuvants currently being investigated are particulate based delivery systems, such as liposome formulations\(^{31}\). In this research we used the FCA/FIA, in further research we will develop for other safely adjuvant for human.

4. Conclusion

The recombinant protein of Fim-C-S. typhimurium as antigen can induce the production of anti recombinant protein of Fim-C-S. typhimurium antibodies in ddY mice. The serological test with Western Immunoblotting showed that recombinant protein of Fim-C S. typhimurium as antigen can interact with specific anti recombinant protein of Fim-C S. typhimurium antibodies. The recombinant protein of Fim-C S. typhimurium has good immunogenicity and its antibodies are specific. Therefore, it is a significant model in the development of typhoid fever vaccine candidate.

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