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The Response of Anti-Peptide Serum Antibodies ATLGATLNRLDFNVNNK Derived from *S. Flexnneri* Protein Sub-Unit Pili toward of Activity the Hemagglutination and Protection from Leaking Solution Enterocyte in Mice

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Abstract

***The purpose** of this study is whether the antibodies produced from injections of ATLGATLNRLDFNVNNLK (A-K) amino acid found in Bacillus thuringiensis are protective against Shigella infection.*

***The methods** first step is the intraperitoneal injection of A-K amino acid. Furthermore, the antibodies produced sequentially were carried out with an anti hemagglutination test on the hemagglutinin pili subunit 49.8 kDa S. flexneri, dot blot examination, western blot examination and diarrhea effectiveness test in mice using the MILL method.*

***The results** obtained that the anti-body A-Kamino acid inhibited the pili subunit 49.8 kDa S. flexneri. By using the dot blot method found that the effective concentration of 10^{-5} as the basis for further examination, western blotting test and the effectiveness of diarrhea in mice.*

***The conclusion** of this study is that the results of immunization protection with the A-K amino acids still require continuation if given orally.*

Keywords: *Shigella flexneri, protein hemaglutinin 49,8 kDa, (A-K) amino acid.*

Introduction

Cases of diarrhea in the world are found in around 80 million cases which cause 700,000 deaths per year. About 60% of diarrhea attacks children under the age of five [**1**]. In Indonesia diarrhea is the number three cause of death for neonates. The study in Mampang, South Jakarta, resulted from Shigellosis surveillance in 612 children aged 0-12 years who suffered from diarrhea during the years 2005 to 2007, the results showed 9.3% found Shigella spp. and it turned out that 63.2% (36/57) was S. flexneri [**2].**

The most effective eradication of infectious diseases is to vaccinate. Until now, several types of candidate vaccines have been found to treat Shigellosis [**1**]. An example is polysaccharide conjugate vaccines (PCV) which was carried out in the A Phase III stage: S flexneri 2a and S sonnei O conjugate vaccines antigen has been tested in a group of children in Israel [**3].** Extensive field studies conducted on Live attenuated vaccines in China showed a protective coverage of 61-65% against S. flexneri 2a and 57-72% for S. sonnei. Also found to be protective efficacy against heterologous Shigella species [**4].**

Other Shigellosis vaccines that have been studied were A formalin-inactivated S sonnei vaccine (SsWC) by Johns Hopkins University in Baltimore, MD, USA

where the preparations were in the form of oral, killed, whole-cell vaccine. This research has arrived in Phase I by using volunteers [**5]**. The same type of vaccine was also developed by the named Antex (USA) (ActivaxTM) which is a vaccine combo and contains antigens from Campylobacter, Shigella and ETEC. This vaccine immediately goes to the clinical trial stage.

A new vaccine formulation called invaplex, which contains IpaB, IpaC, and LPS from *S flexneri* and *S sonnei*, has been investigated and appears to be promising after trials on rabbits [5,**6]**.

The results of our study in order to participate in developing the Shigellosis vaccine have found the hemagglutinin sub unit pili *S.dysenteriae* protein with a molecular weight of 49.8 kDa and sub unit pili S.dysenteriae protein with a molecular weight of 7.9 kDa which acts as a sub-unit agglutination inhibitor of the hemagglutinin protein pili S.dysenteriae with a molecular weight of 49.8 kDa. These two proteins are molecule adhesin in mice enterocyte [7]**.**

The results of the examination using the immunocyto-chemical method add to the evidence that the hemagglutinin protein sub unit pili *S. dysenteriae* with a molecular weight of 49.8 kDa is an adhesin molecule. As a receptor in this study were mouse cell enterocytes [**8].**

Furthermore, it was also found that the antibodies of the sub unit pili *S. dysenteriae* protein with molecular weights of 49.8 kDa and 7.9 kDa cross-reacted with the Pili sub-unit protein molecular weight of 49.8 kDa and other 7.9 kDa Shigella sps. The dot bloot test and Western Blotting showed no significant differences between S. dysenteriae and S. boydii, but there were significant differences with *S. fexneri* and *S.* sonnei [**9].**

The results of our latest study by administering the sub-unit *S. dysenteriae* protein with a molecular weight of 49.8 kDa conjugated with ISCOM showed an increase in s-IgA. This increase in s-IgA is followed by resistance to colonization of *S. dysenteriae* and damage to colon epithelial cells [**10]**. The results of this study provide an opportunity to get the Shigellosis vaccine candidate caused by *S.dysenteriae*. The results of the Khoirul study (2014) showed that the immune response between the sub-unit proteins of *S. dys*enteriae and S. flexneri with a molecular weight of 49.8 kDa was not identical [**11]**.

The Mice Illeal Ligated Loop (MILL) method is a new method that aims to measure the fluid in the intestines of mice due to diarrhea. We have developed this alternative method in the our central laboratory of biomedical in order to overcome ethical clearance [**11]**. The MILL method is expected to replace the Rabit Ileal Loop Test (RILT) method. RILT is still being used in diarrhea research. As animals, they are rabbits. The experimental animal model on RILT was more tormented compared to the MILL experimental model [**11,12]**.

One vaccine that has been successfully marketed is the pertussis vaccine. This vaccine contains three components, namely; pertussis toxin (PT) is an extra cell product, pertactin (PRN) autotransporter protein produced by Bordetella bronchiseptica cluster, such as *B. pertussis, B. parapertussis*, and *B. bronchiseptica* and filamentous hemagglutinin (FHA) which are adhesin molecules. FHA is the most potent component among these 3 components [**13].**

Peptide vaccines are an attractive alternative strategy that relies on usage of short peptide fragments to engineer the induction of highly targeted immune responses, consequently avoiding allergenic and/or reactogenic sequences [**14].**

The last our study, with bioinformatics studies it was found that there was have a 100% similarity from prospective of our vaccine candidates shigellosis with ATLGATLNRLDFNVNNLK (A-K) amino acid *Bacillus thuringiensis* pili sub unit protein.

The results our researh show that the anti-body A-K amino acid inhibited the pili hemagglutination of subunit 49.8 kDa *S. flexneri*. By using the dot blot method found that the effective concentration of antigen antibody reaction, western blotting test and the effectiveness of diarrhea in mice.

Material and Methods

Bacterial Strains

The bacteria obtained from Labkesda Jogjakarta Indonesia isolates were examined for morphologicolony characterization on Mac Conkey Agar, Salmonella Shigella Agar (SSA) and then incubated for 24 hours at 37^0-C followed by Gram staining. Bacterial identification is done with the 12A microbact system.

Anti-ATLGATLNRLDFNVNNLK amino acid anti-body induction.

Immunization was carried out intra-peritoneally by 5 mice. The dosage used was 50 µg AK amino acid sequences which were dissolved with a solution of normal saline and Freund's complete adjuvant. The interval between doses is 7 days for 4 weeks, the serum is harvested 10 days after being given the last dose.

Isolation of Antibodies Anti-ATLGATLNRLDFNVNNLK amino acid sequences anti-body.

Blood is taken from the heart of a mouse. Blood was collected in sterile tubes and stored in an incubator at 37^0-C with a sloping position for 30 minutes. Blood is then stored in the refrigerator at 4^0-C for 10 minutes, then centrifuged at 10,000 rpm for 5 minutes. The formed supernatant was taken and put into a sterile tube and then stored at -20 0C [[**15].**

Agglutination Resistance Test

Agglutination resistance tests were carried out and adjusted according to the instructions from **Jones and Freter**[**16].** Hemaglutin protein 49.8 kDa pili sub unit is isolated as Yulian did. Samples resulting from IgG isolation of mice that have been immunized with the A-K amino acid diluted half a series in a well contained on microplate V with a volume of 50 µl each in their wells, whereas as a dilution solution used is PBS pH 7 , 4. Furthermore, in each of the wells above, a hemagglutinin protein of 50 µl was added which contained 2 times the highest titers resulting from the hemagglutination reaction. Then the incubation was carried out on a water bath by shaking 60 times a minute at 37^0C for half an hour. After the incubation time was completed in each well, 50 ml of blood cells of mice were added to a concentration of 0.5%. The results of the agglutination inhibition reaction were read after incubation at room temperature for 1 hour. As a negative control, there is a hemagglutination inhibition reaction using pre-serum serum.

Dot Blot Method

This method is carried out by referring to Harlow & Lane, 1988 [**15]**. Nitrocellulose membranes are immersed in sterile H2O for 30 minutes. Then installed on the dot blot device. Through the tool hole, the membrane which has been moistened with FFB, dripped with 50 µl antigen, is incubated overnight at 4^0C. Next, blocking with blocking buffer FFB was carried out, incubated overnight at 4^0C, blocking solution removed. The membrane with primary antibodies was 50 µl, incubated for 2 hours at room temperature and placed on top of the shaker. The solution was removed, then washed 3 times with TBS-Tween-20 0.05%. Secondary antibody added with 1: 500 dilution in FFB solution, incubated at room temperature for 1 hour, above the shaker. Washed again 3 times with TBS tween -20 0.05%. Chromogen substrate was added and incubated at room temperature for 30 minutes. The reaction is stopped by adding H2O. Positive results when a dot-dot is formed on the nitrocellulose membrane. Quality results are seen based on color gradations

Western Blot Method

Western blot examination using the method from Towbin, 1979 [**17]**. The gel sheet from SDS-PAGE containing protein bands was transferred to nitrocellulose paper using a semi-dry blotter made by Biorad. The way to transfer protein bands to nitrocellulose paper is to use 100 mA of electricity for 120 minutes

Protectivelyan anti-A-K amino acid antibodies against discharge to the intestinal lumen.

This method refers to our previous research which we named the Ile Legated Loop (MILL) Mice with modification (Yulian). There were six treatment groups: the first positive control was injected with *S. flexneri* 10^5, the second negative controls without injected bacteria, the third was injected with *S. flexneri* 10^5 coated with a concentration of 1^{-1} anti AK amino acid sequences, the fourth was injected *S. flexneri* 10^5 which was coated with a concentration of 10^{-3} anti AK amino acid sequences, the fifth was injected S. flexneri 105 bacteria coated with a concentration of 10^{-4} anti AK amino acid sequence and the sixth was injected with *S. flexneri* 10^5 pre-immunization. The volume of every group is 200 ul.

Small intestine samples were obtained from six mice. Each mice was taken three intestines each with a size of ten cm, each end of the piece tied with colored thread that can be used as a marker. Eighteen small intestinal were divided into six groups randomly. Each group of small intestine samples was injected with each grouping of bacterial treatment.

Then each intestinal group (three) is included in a flash media containing RPMI. Each intestine before being inserted into the flash is weighed. This weighing is also done at the end of incubation. Incubation is placed in an incubator which is shaken at 37^0 C for 5 hours.

Result

The presence of an anti-A-K amino acid anti-antibodies response is indicated by:

Results of examination of haemagglutination tests and haemagglutination inhibition.

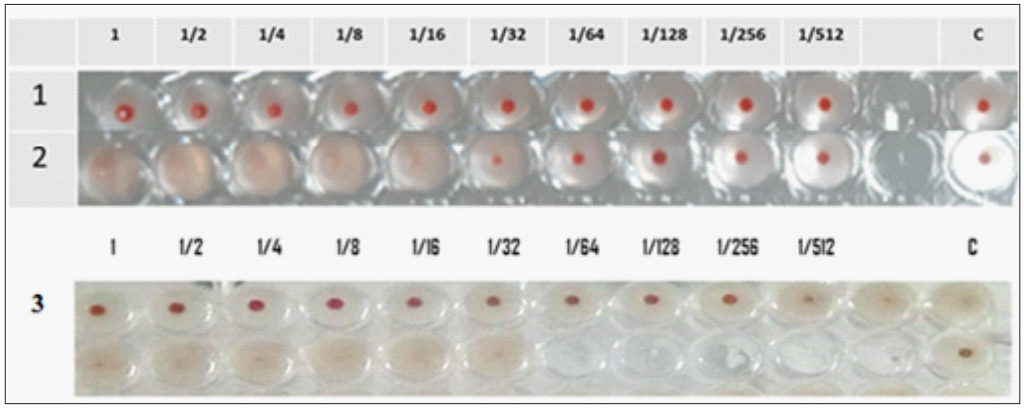

Fig 1. *Results of hemagglutination examination and hemagglutination inhibition*

Description

[1] The results of the haemagglutination examination of A-K amino acid

[2] The results of haemagglutination protein examination 49.8 kDa pili subunit S.flexneri

[3] The protein 49.8 kDa pili subunit S. flexneri have activityhaemagglutinationandithemagglutination inhibited by anti-A-K amino acid antibody have capacity inhibition against hemagglutinin protein 49.8 kDa sub-unit pili S.flexneri.

The A-K amino acid does not show a hemagglutination reaction. While the 49.8 kDa protein pili S.flexneri subunit showed hemagglutination with 1/16 titers, furthermore, anti-A-K amino acid antibodies can inhibit hemagglutination of the 49.8 kDa protein pili S.flexneri subunit with a high titer of 1/10242.

The result of examination anti-A-K amino acid anti bodies response to the A-K amino acid by using Dot blot method. The results of the test can saw in Fig 2

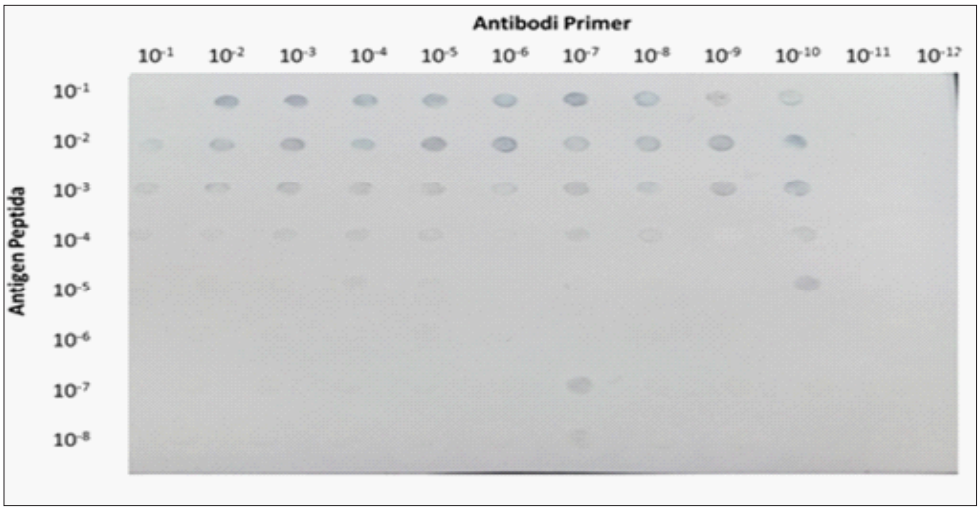

Fig2. *The results response anti-A-K amino acid sequences antibodies throughout A-K amino acid with the dot blot examination.*

Semi-quantitative calculations of antigen-antibody reactions are carried out with Corel Photo-Paint, the highest titer in the highest antibody dilution is 10^{-7}. This highest titer antigen-antibody reactions of A-K sequences is 10^{-1}.

Result of examination an anti-A-K amino acid sequences anti-antibodies throughout whole bacterial cells with the Dot blot method. The results can saw in Fig 3.

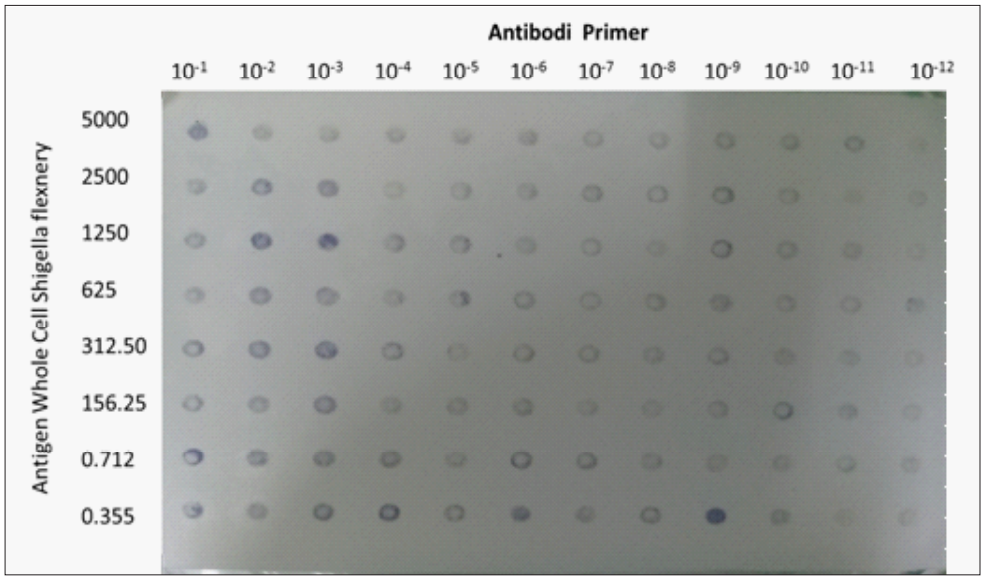

Fig 3. *The results response immune of anti-A-K amino acid sequences antibodies throughout to whole bacterial cells with the Dot blot method.*

Semi-quantitative calculations of antigen-antibody reactions are carried out with Corel Photo-Paint, the highest titers of antibody dilutions are 10-10. While the highest titer is the number of bacteria is 355 per ml. These findings used for western blotting tests and the protection of discharge into the intestinal lumen.

A result of examination an anti-A-K amino acid sequences anti-antibodies response throughout to against various kinds of antigen with Western Blotting method and the results are displayed Fig 4.

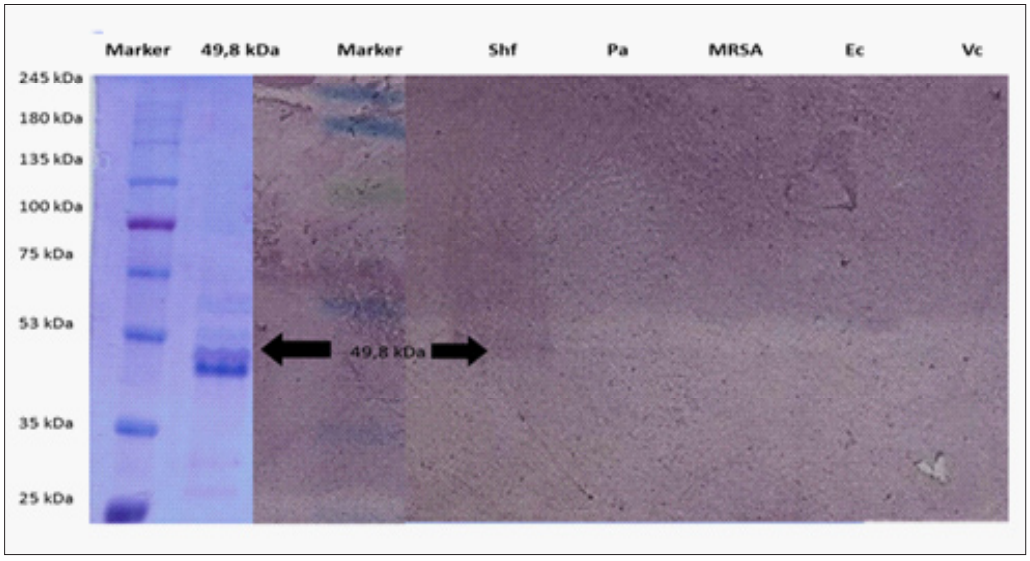

Fig 4. *Results of Western Blotting an anti-A-K amino acid sequences anti-antibodies with the 49.8 kDa protein pili S. flexneri subunit, V. cholerae, E. coli, S. aureus, and P. aeruginosa*

Whereas the response to other bacteria (whole cells) does not appear *V. cholerae, E. coli, S. aureus,* and *P. aeruginosa.*

The results of an anti-A-K amino acid antibodies against discharge to the intestinal lumen shown in the table below and Fig. 5.

NO	SAMPLE	Mean ± SE
1	control (+)	19.56 ±3.123a
2	control (-)	5.44±.556b
3	Ab 1/1000	12.00±1.080c
4	Ab 1/1	10.56±1.684c
5	Ab 1/10.000	16.78±1.199a
6	pre-immun	16.33±2.082a

The same or different subscriber numbers that statistically indicate a significant difference or not.

Evaluation of these results clarified with Fig 5 shown below.

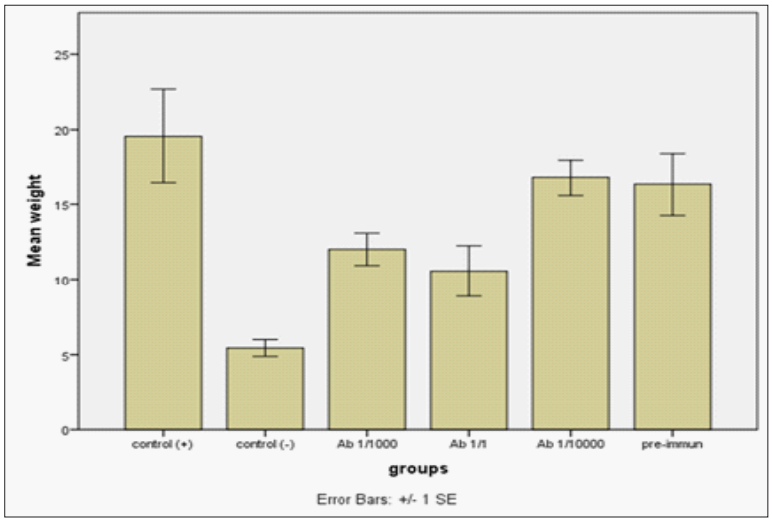

Fig 5. *The response of the various concentration of anti-peptide A-K examination of the anti-antibody sequence against S.flexneri of the protective test for the discharge of fluid into the intestinal lumen.*

Discussion

The peptide vaccine consists of 20-30 amino acids which can cause stimulation of the immune response. Synthesis of vaccine peptides refers to peptides found in microbes that are pathogenic or are part of a specific cancer protein. This synthetic peptide vaccine is an antigen-specific epitope that plays a role in microbial virulence or is part of those associated with chronic diseases including cancer [18].

Because peptides can cause stimulation of the immune response, the formation of antibodies can use for diagnostic tools. There are reports of peptides found in *M. leprae* (ML1419c p113-121) which can be useful for diagnostic tools and vaccine candidates. Data show that cohort observations in Brazil show that ML1419c p113-121 induces CD8 + T cells that provide protective immunity against *M. leprae* and induce B-cells to produce specific IgG [19].

The results of our latest study show that the sub-unit of the pili protein Shigella sp. 7.9 kDa and 49.8 kD are likely candidates for the shigellosis vaccine [7]. Furthermore, with bioinformatics studies, it was found that there was a 100% similarity to the two subunits of the protein with ATLGATLNRLDFNVNNLK (A-K) amino acid sequence B. thuringiensis pili subunit protein.

To find out this synthetic A-K peptide is an epitope which means it can form antibodies then it is injected into mice intraperitoneally. The adjuvants used are adjuvant freunds. This adjuvant for future use needs deep attention because it can cause autoimmune [20,21].

To test the formation of A-K antibodies, we can make four types of examinations carried out, namely the first resistance of the hemagglutination reaction, the second dot blots, the three western blotting and the last four the protective test MILL method.

The first examination is the inhibition of the hemagglutination reaction can show in Fig 1.1. We found an interesting result, namely the decrease of the erythrocyte to the bottom of the well in columns one, two and three which preceded the deposition of erythrocyte compared to the control, i.e. well number 1.12. Erythrocyte in this well number 1.12 without added A-K. This phenomenon is similar to our study, which found a sub-unit of the protein pili S. dysentriae 7.9 kDa which is an adhesion molecule that inhibits hemagglutination of the sub-unit protein pili S. dysentriae 49.8 kDa [7].

Hemagglutination inhibition can do if hemagglutination is known as shown in Fig 1.2. The titers are 1/16.

Furthermore, the results of hemagglutination inhibition can show in Fig. 1.3. This titer of inhibition of hemagglutination was found to be quite high at 1/1024. Therefore from the results of the examination of A-K synthetic peptide immune response showing anti-A-K antibodies.

The second examination is the dot blot, the results of which shown in Fig 2 and 3. The usefulness of the results of this examination is first to find out whether the protein or peptide is an immunogen. The result is the same if the protein or peptide given to humans or experimental animals can respond, namely antibody production while the second use is to determine the titers of antigen-antibody dilution concentrations that still show antibody-antigen bond reactions.

The result of Fig. 2 and 3 due to the antigen same antibody reaction. The antibody, namely synthetic anti-A-K peptide antibodies. The antigen is different, for Fig 2 the antigen is A-K peptide. As for Fig. 3, the antigen is a whole S. flexneri bacterial cell.

From the results of the analysis of Fig. 2., it found that the color reduction of the dot when the A-K peptide antigen concentration lowered, while the level of the anti-A-K peptide antibody was the same. The same condition can show if anti-A-K peptide antibodies decrease while A-K peptide concentration is the same. Results that are not different can also found in Fig. 3 where the antigen is a different concentration, namely the whole *S. flexneri* bacterial cell.

The results of the anti peptides antibody test A-K sequences with the western blotting method can observe in Fig. 4. The A-K peptide concentration used was referring to Fig 3, which is 10^{-2} which showed a reaction with all levels of the whole *S. flexneri* bacteria used. The antigen used was the protein pili subunit 49.8 kDa *S. flexneri* as a result of our previous study (Yulia). As antigens used are those in *V. cholerae, E. coli, S. aureus,* dan *P. aeruginosa* bacteria. It turned out that the results showed a positive reaction to Shf lane (pili protein subunit 49.8 kDa *S. flexneri*). In other lanes, there is no positive reaction like lane Shf. This western blotting examination results along with the results of the dot blot examination.

Furthermore, the latest results obtained regarding the protective test of immunized mice used anti-A-K amino acid sequences antibodies. Bacterial adhesion anti-protein antibodies when binding to bacteria (opsonization) will cause attachment cannot perform in the host cell and failure of the proliferation process. This research method refers to our latest research with modifications as written in the research method. Although we do not show data the results MILL, the final results of the study can show in the table and Fig. 5.

Conclusions obtained from the analysis of research results are using tables and Fig. 5. The administration of anti-A-K antibodies concentrations 10^{1} and 10^{-3} can inhibit discharge of the intestinal lumen while anti-A-K antibodies concentration 105 cannot hinder. However, the release in the administration of anti-A-K antibodies concentrations 101 and 10-3 still had differences with the negative controls. The hope is that there is no difference when compared to negative controls.

Conclusions regarding the protective test of immunized mice used anti-A-K antibodies amino acid sequences were successful although less optimum. Therefore it still requires further research, namely the delivery of synthetic A-K peptides to be given by oral which refers to our previous study [22].

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