Comparison of Immunogenicity of Antigen O *Salmonella typhi* Between Isolates from Makassar Strain and Surabaya Strain in Inducing Formation of Specific Antibodies

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ABSTRACT

The aim of this research was to know the difference of immunogenic ability of antigen O *Salmonella typhi* in inducing antibody formation (IgM) between isolates from Makassar strain and isolate from Surabaya strain, also mixture of both isolates (mixed isolate). The study sample was suspension of O antigen *Salmonella typhi* isolate from Makassar strain, isolate from Surabaya strain and mixture of both isolates. The type of research used is true experiment, so that both the sample and the treatment given more controllable, measured and the effect of more reliable treatment. The method used is isotyping ELISA from Bio-Rad using the post-test design only controle group design, with 5 types of treatment. Each treatment was repeated 12 times, so there were 60 experimental units. The experimental animals used were Balb / c, male, 6-8 weeks old, muscle mice, 40 gram, can move agile, fluffy and bushy. Animals try to be prepared by acclimatization for 4 weeks. Each mouse was immunized with *Salmonella typhi* O antigen containing 50 μg of 0.5 ml per head (0.25 ml of 0.25 ml suspension of adjuvant $Al(OH)_2$) subcutaneously. After 10 days, the blood of the mice was taken through orbital, each 1 ml, then serum separation was performed to detect the antibody (IgM) using ELISA isotyping test. To test the hypothesis used Anova test. Based on the results of the study, it can be concluded that the immunogenic ability of antigen suspension of O *Salmonella typhi* isolate from Makassar strain is higher than the immunogenic ability of antigen suspension of O *Salmonella typhi* isolates from the Surabaya strain and mixture of both, in inducing IgM antibody formation in experimental animals.

Keywords: Immunogenicity, Antigen O, *Salmonella typhi*

INTRODUCTION

Typhoid fever caused by *Salmonella typhi* is a systemic infectious disease, endemic, and still a health problem in many developing countries in the world[1],[2],[3] *Salmonella typhi* as an agent is also a cause of morbidity with an estimated 13 cases occurring each year in Asia[4].

In Indonesia, the disease tends to increase with incidence of 9.2 per 10,000 population in 1990 to 15.4 per 10,000 population in 2004. The mortality rate due to typhoid fever is estimated to be 2-5% of all deaths[5],[6]. On the course of typhoid fever, about 3-5% of patients become asymptomatic careers. This is a threat as a source of new disease to the surrounding community. South Sulawesi Provincial Health Office in 2010 reported that cases of typhoid fever were the fourth of 26 types of diseases. From 2,245 cases found death rate 0.49%[7].

Increased cases of typhoid fever in urban areas are affected by several factors, such as increased urbanization, rapid population density and spread, poor sanitation and undetectable asymptomatic care[3]. The tendency to increase the cases of typhoid fever is caused by several factors, among which are the clinical symptoms that are displayed vary widely, routine blood tests do not give specific results, while the process of laboratory examination, seed examination, long enough that there is delay in diagnosis enforcement. The use of bile transport medium that is "gall culture" increases the positive result of culture.

Several methods of laboratory diagnosis of typhoid fever have been developed including using monoclonal antibodies[8], PCR (polymerase chain reaction) (EIU Dot (enzyme immuno assay dot)[9] and ELISA (enzyme linked immunosorbent assay)[3],[10] known as non-conventional methods.

The diagnosis of typhoid fever can be established by isolation of *Salmonella typhi* from blood, fecal, urine, bone marrow and other body fluids, but the seed yields are less satisfactory because the seedling takes a long time. The positivity of the seed results is largely determined by the taking of the material for the examination as well as

the effect of previous antimicrobial administration[11]. In areas where there is inadequate laboratory facilities, the diagnosis of typhoid fever is based on clinical features and a significant increase in antibody titre[12].

The serological test for the diagnosis of typhoid fever that is widely used today is the Widal test, which is an agglutination reaction. The Widal test is a test that uses serum containing antibodies against the O antigen *Salmonella typhi*, where the antigen-bonding reaction with antibodies results in agglutination in the form of clumps such as white sand[13]. Antibodies that play a role against antigen O *Salmonella typhi* primarily are immunoglobulin M. In *Salmonella typhi*, its antigenic specificity is determined by lipid A, which is a series of nuclei and ends of repeated units of lipopolysaccharide in the cell wall of Gram-negative bacteria[13]. However, until now, no difference has been found between *Salmonella typhi* immunogenesis from isolates of a particular region and other regions in inducing antibody formation.

Widal's serology test is still an important tool in the diagnosis of typhoid fever[3] because the inspection process is easy and simple, it also requires a low cost. Convenience is also supported by the use of antigen factors that can be produced by laboratories in the region[14]. Nevertheless, some researchers have reported that the Widal test gives less sensitive and specific results. This is due to the cross-reaction with antibodies produced by other *Salmonella*, where the possibility of contamination with other organisms around it is considerable.

Considering the incidence of typhoid fever data, which is closely related to disease diagnosis, it is necessary to pay attention to the use of Widal Test reagents, so that the sensitivity of the examination results can be improved. If these shortcomings persist, the data on the incidence of typhoid fever remains unreliable, or incident data tends to increase. Mortality rates also increase due to improper treatment[3].

Widal agitamination reaction qualitatively states the presence of aglutinin in serum of patients, whereas quantitatively it is to know the level or titer aglutinin in serum patient. In Indonesia as an endemic area, individuals are constantly exposed to *Salmonella typhi*. In unhealthy individuals, antibody titers are higher than in non-endemic areas. Thus, the assessment of the results of these reactions should be considered with regard to clinical manifestations[15]. In addition, the antigen used should have high sensitivity, which is supported by proper manufacturing and storage processes[14].

In terms of the immune response, the antigen exhibits two different reactivities. First, it is immunogenicity, ie the ability of the antigen to induce immunity indicated in antibody production (humoral response), as well as an increase in the number of lymphocytes (cellular responses). Second, is the specificity of antigenicity, the ability of antigens that interact specifically with antibodies. The antigenicity of a molecule is not always the same as its immunogenicity, which means that the molecule has an antigenic determinant and is capable of interacting with appropriate antibodies, not necessarily immunogenicity. Various requirements for a molecule to be immunogenic, among others; molecular size, antigenic determinants on the surface of antigen molecules, immunogenicity and immunity, and the role of specific amino acids in immunogenicity[16].

Enzyme Linked Immunosorbent Assay (ELISA) is an immunoassay test that uses enzyme-labeled antibodies in IgM having high sensitivity and specificity[17]. Given the importance of early and precise diagnosis of typhoid fever, it is necessary to find the right laboratory diagnostic, at a low cost and easy to work on.

The aim of this research was to know the difference of immunogenic ability of antigen O *Salmonella typhi* in inducing antibody formation (IgM) between isolates from Makassar strain and isolate from Surabaya strain, also mixture of both isolates (mixed isolate).

METHODS

The study sample was suspension of O antigen *Salmonella typhi* isolate from Makassar strain, isolate from Surabaya strain and mixture of both isolates. The type of research used is true experiment, so that both the sample and the treatment given more controllable, measured and the effect of more reliable treatment[18]. The method used is isotyping ELISA from Bio-Rad using the post-test design only controle group design, with 5 types of treatment. Each treatment was repeated 12 times, so there were 60 experimental units. To know the internal validity, the skill is divided into 5 groups of treatment at random.

The experimental animals used were Balb / c, male, 6-8 weeks old, muscle mice, 40 gram, can move agile, fluffy and bushy[19]. Animals try to be prepared by acclimatization for 4 weeks. Each mouse was immunized with *Salmonella typhi* O antigen containing 50 µg of 0.5 ml per head (0.25 ml of 0.25 ml suspension of adjuvant $Al(OH)_2$)[20] subcutaneously. After 10 days, the blood of the mice was taken through orbital[19], each 1 ml, then serum separation was performed to detect the antibody (IgM) using ELISA isotyping test.

The reagents used were wash buffer (NaCl 0.9 gram, Triton X-100 0.05 NaN_3 ml 20 ml, sufficient with aquades up to 100 ml, pH 8.6), Buffer coating (Na_2CO_3 1.59 gram, $NaHCO_3$ 2.93 grams, treated with Aquades up to 1000 ml pH 9.6), substrate buffer ($MgCl_2$ 10 mg, Diethanolamine 9.7 ml, NaN_3, 80 ml Aquades, concentrated HCl to pH 9.8 were sufficient with Aquades up to 100 ml pH 9.8), blocking buffer (BSA 0.5 grams is sufficient with PBS Tween to 100 ml, pH 7.4), PBS (NaCl 0.8 gram, KCl 0.02 gram, KH_2PO_4 0.02 gram, Na_2HPO_4 12 H_2O 0.289 gram is sufficient with aquades up to 100 ml, pH 7.4).

The equipment used in this research are: Micro pipette / pipette Eppendrorf, Tips pipette, Micro tube, Microplate with 96 wells, Measuring pump, Pipette measure, Incubator, Pasteur paste, Rubber sucker, Magnetic stirrer, Magnetic Bar, Analytical Scales, pH meter, Aluminum foil and ELISA brand reader Immuno Mini NJ-2300. Before the measurement of IgM antibody with ELISA isotyping is done, first chessboard titration is done to obtain an optimal antigen, antibody and conjugate concentration comparison for use on ELISA isotyping. Optimal results are based on the antigen, antibody, and conjugate concentrations used in the ELISA isotyping assay examination based on the smallest antigen and conjugate dilution that can give great results.

The ELISA isotyping test process is as follows: antigens and antibodies prepared by certain dilutions based on chessboard results. 100 µl Oigenous *Salmonella typhi* antigen with 5 µg / ml content is fed into the ELISA microplant wells in 100 µl carbonate buffer and incubated in the refrigerator for 24 hours. Next washed with PBS Tween 3X, then blocked with 1% BSA in PBS as much as 150 µl per well for 2 hours at room temperature then washed with PBS Tween 3X. Added 100 µl of serum examined (primary antibody) each well at 1/50 dilution, allowed to stand for 1 hour, then washed with PBS Tween 3X. The next process is to add 100 µl anti-IgM (rabbit anti-mouse) (secondary antibody) to each well and incubated for 1 hour at 37°C, then washed with PBS Tween @ 15 minutes. The wells are filled with 100 µl tertiary goat anti-rabbit labeled alkaline phosphatase (tertiary antibody) at 1/100 dilution with PBS Tween, and incubated at 37^{0}C for 1 hour and again washed with PBS Tween 3X. It is then filled with 100 µl p-NPP in substrate buffer and incubated at 37°C for 1 hour. Then 50 µl stopping solution with 0.1 N NaOH was added and allowed to stand for 90 minutes. Read more with an ELISA reader with a wavelength of 405 nm. Interpretation of antibody concentrations based on optical density (OD). To test the hypothesis used Anova test.

RESULTS

The isolate group of Surabaya showed antibody titers (IgM) with OD (optical density) ranged from 0.148 to 0.248, with mean of 0.193. Makassar isolate group ranged from 0.209-0.294 with mean of 0.250. Group of OD mixed isolates ranged from 0.189 to 0.260 with a mean of 0.220. In control (Escherichia coli) OD ranged from 0.10 to 0.202 with mean of 0.132 and negative control (PBS) OD ranged from 0.099 to 0.157 with mean of 0.124.

Data from the OD readings on the ELISA isotyping test against IgM antibodies induced by O *Salmonella typhi* antigen suspension read in the 405 nm wavelength reader ELISA are as follows: The isolate strain group of Surabaya, the isolate of Makassar strain and the OD mixed isolate are read over the OD the mean of negative control as well as the average OD control of E. coli. This shows a significant difference (p <0.01) between immunogenicity of Surabaya isolate and Makassar isolate as well as mixed isolate.

The results of OD readings on ELISA isotyping test on IgM antibody formation after immunogenous suspension of antigen O *Salmonella typhi* in each treatment group showed mean and standard deviation (SD) as follows:

Table 1. Average of OD of antibody formation in mice after immunization with antigen suspension O
Salmonella typhi

Treatment group	*Optical Density* (OD)		
	Mean ±		Standard Deviation
Isolate of Surabaya	0.193 ^{d}	±	0.030
Isolate of Makassar	0.250 ^{a}	±	0.029
Mixed Isolate	0.220 ^{b}	±	0.027
Control (*E.coli* & PBS)	0.198 ^{c}	±	0.035

Subcrips showed significant differences in immunogenic ability (p <0.01)

DISCUSSION

This study used a "post-test only control group design" design with the assumption that within one population the rate of cell B reactivity before being given a treatment was the same. It is based on the clonal selection theory which states that; B cells have many specificities before contact with foreign immunogens. Only B cells with imunoglobulin receptors that match the immunogens in question will be able to differentiate into plasma cells[21],[22]. Based on these assumptions, the initial measurements are not made as they are the same for all groups, and come from the same population[18]. B cell populations can not form specific antibodies in the absence of a particular immunogen. The results of the detection obtained by ELISA isotyping method showed an overview of B cell responsiveness due to stimulation of the O antigen *Salmonella typhi* given to the treatment group in inducing antibodies. The OD (optical density) of the detected antibody is 1.5 times greater than the negative control COV[22], and since the mean OD value of negative control is 0.124, the OD is positive if $\geq$ 0.186

(1.5 X 0.124). The immunogenic ability of the O antigen suspension of *Salmonella typhi* in inducing antibodies from each treatment group showed significant differences.

Differences in the immune response of each treatment group were due to the influence of immunogens (antigen O *Salmonella typhi*) from different isolates. The difference in immune responses can also occur because of the structure of each protein that is immunogenic different, so it will be different immunogenic ability to induce antibodies and the immune response it causes. Various possible antigenic determinants can be found in protein molecules and polypeptides that determine the specificity of an antigen molecule in question. At times the whole molecule is not immunogenic but can react with antibodies produced by the presence of other similar molecules[23], as in this study.

Some researchers suggest that the sensitivity and specificity of the O antigen *Salmonella typhi* as one of the reagents in the Widal test used so far is less helpful in establishing early diagnosis of typhoid fever patients[24] and it is advisable to look for diagnostic tools for more sensitive typhoid fever and the results can be trusted[5]. This is also because in Indonesia, typhoid fever is an endemic disease which means there has been a constant exposure to the organism and produced a higher antibody titer[15],[25].

But the various statements are different from the results of ainnya researchers. The results of Kusumawati (1997) study using ELISA method to determine M and G immunoglobulin levels against OMP antigen *Salmonella typhi* in patients with typhoid fever showed that sensitivity and specificity were very high[26]. For the Widal test on a one-time blood sampling to help diagnose the patient with typhoid fever, it is better to examine the agglutinin titer O *Salmonella typhi* alone, because it has a high positive predictive value compared to H aglutinin titer which gives high sensitivity value, but this test has no diagnostic value for typhoid fever. To increase the sensitivity of Widal's test in diagnosing typhoid fever, blood sampling should be performed during the second and third weeks of the illness[3]. The argument is that *Salmonella typhi* H antigen is also owned by some other *Salmonella* germs, but not with germs that have the same O antigen[27]. In the immune response, antigens show two different reactions. First, immunogenicity, a capability of antigen compounds to induce an immune response (immune responsiveness) demonstrated both in humoral antibody production and the lymphocyte propagation of cell-mediated immune responses. Second, the specificity of antigenic or antigenicity, that is to interact, with the active side of antibodies or receptors (epitopes) of cells called antigenic[28].

Characteristics of the antigen to determine immunogenicity of the immune response that must be possessed is; (non-self) meaning to generate a molecular immune response must be known as "non-self". The most potent immunogenic molecular size is usually a large protein, having a molecular weight exceeding 10,000 daltons. It is known that small molecules independently can not boost the immune system, but when joined by a large and complex transporting molecule, it can generate an immune response. In this case, the size of a molecule is used as a requirement, whether a molecule will be immunogenic or not[16],[29]. Bryant (1982) from the results of his research, wrote that molecules with molecular weight less than 5000 are not able to act as antigens[30]. Molecular molecules of 14,000, when not joined by enhancer molecules, are weak antigens. Molecules with a molecular weight of 40,000 or more are good antigens. Molecules with a molecular weight of 50,000 or more, with a complex protein or polypeptide-carbohydrate structure are the best antigens. Referring to the description, the size of a molecule is one of the factors worth considering in the interaction between antigens. From a wide range of observations, tiny proteins and peptides of 4,000 to 5,000 molecules are sometimes capable of generating humoral and cellular immune responses, except that all small molecules are weak immunogens compared to large molecules. Thus it can be concluded that immunogenicity is not solely determined by molecular size[23].

The complexity of chemistry and structure in the sense of a certain amount of chemical complexity is indispensable. For example, amino acid homopolymers are less immunogenic than heteropolymers containing two or three different amino acids. The antigenic determinant or the smallest unit of a complex that can be bound by antibodies is called the antigenic determinant. Antigen may have one or more deteminants. Generally, a determinant has a size of five amino acids or sugars. A molecule must have a certain degree of complexity from its structure. The more complex or complex a structure, the higher the immunogenic level it has. The host genetic order is an ability to initiate an immune response to an antigen, depending on the genetic makeup of the animal or human being concerned. A pure polysaccharide will be immunogenic when injected to mice or humans, but its immunogenicity will be lost when injected into a guinea pig. Two animal strains of the same species may respond differently to the same antigen because of differences in gene composition of the immune response or differences in the host genetic order. Because the degree of immune response depends on the amount of antigen administered, the immune response can be optimized by carefully determining the dosage of the antigen. If the minimum dose of an antigen has been exceeded, the higher the dose the higher the immune response is comparable; but at certain doses will occur otherwise, ie decreased immune response or even the immune response is lost. This is called immunologic tolerance[16],[29].

The immune response of an immunogen is also affected by the mode of administration (including intervals between doses given). An immunogen which, when injected intravenously, is not capable of causing an immune response, may result in an immune response when subcutaneously injected. In general, immunogenous

immunization can be directly through the skin, respiration, alimentary tract, or injected subcutaneously, intraperitoneally, intravenously or intramuscularly, each of which may lead to different immune responses[16].

The antigeni ability of the *Salmonella* cell wall lies in a similar polysaccharide or polymer compound. These compounds act as antigens that are easily recognized by the immune response as they lie on the cell surface. This compound can withstand enzymatic exposures so that it will remain on the surface of the cell microorganisms after experiencing phagocytosis. In addition to polysaccharides that make up the cell wall, in microorganisms also found polysaccharides outside the cell called eksopolisakarida. Polysaccharide antigens are very diverse. In gram-negative bacteria, the major polysaccharide antigen is lipopolysaccharide. This highly complex macromolecule is called somatic antigen or O antigen. The antigenic specificity of the antigen O *Salmonella typhi* which is a surface antigen lies in repeating units at the ends of its polysaccharides[13].

The differences in immunogenicity of this study, allegedly also influenced by the process of making antigen suspension O *Salmonella typhi*, among others preparation of isolates, including the way of collection and handling, the quality of various media used, isolation and identification procedures, quality control procedures, standardize antigen suspension turbidity, gold standard, and equally important is the producing power (laboratory technician / analyst)[17].

From the results of this study, it was concluded that there were differences in immunogenicity capability of antigen O suspension *Salmonella typhi* between isolates of Surabaya, Makassar isolate and Mixed isolates in inducing antibodies. Similarly, there is a difference in the level of sensitivity in detecting IgM antibody levels. In this study, differences in immunogenity in inducing antibody formation as well as differences in sensitivity levels in detecting antibodies from the isolate treatment group of Surabaya, Makassar isolates and Mixed isolates may be due to differences in molecular weights of each O antigen. The O oxygen with a small molecular weight is not sufficient to optimize B cells. Another possibility is the difference in the antigenic determinant (epitope). The antigen of the isolate of Surabaya is likely to have a small molecular complexity so that its immunogenic capability is also low, while the high-sensitivity Makassar Isolate is estimated to have a greater degree of molecular complexity that has a high immunogenic ability. This suggests that the antigen receives an optimal response from the plasma cells that induce cell differentiation B to produce antibodies. In addition, different immunogenic and sensitivity capabilities in inducing and detecting IgM antibodies formed due to differences in the antigen content of the two isolates, ie, the suspension of Oigen O *Salmonella typhi* isolate strains from Makassar have antigenic compositions consisting of serotypes of O9 and O12 antigens , whereas isolate strains from Surabaya have antigenic compositions consisting only of O9 antigen serotypes, so there are differences in immunogenic ability and degree of sensitivity in inducing IgM antibody formation.

In this study, there are limitations, among others, not measuring the molecular weight of each antigen used. Another thing is the way of giving immunization with antigen suspension O *Salmonella typhi* in experimental animals that do not pass through the digestive tract (mouth / oral) as the entry of *Salmonella typhi* antigen in the patient / human body.

CONCLUSION

Based on the results of the study, it can be concluded that the immunogenic ability of antigen suspension of O *Salmonella typhi* isolate from Makassar strain is higher than the immunogenic ability of antigen suspension of O *Salmonella typhi* isolates from the Surabaya strain and mixture of both, in inducing IgM antibody formation in experimental animals.

Based on the results of the study with all the limitations available, it is suggested in the next study to compare how far the specificity of antigen suspension O *Salmonella typhi* isolate strain from Makassar, isolate strain from Surabaya and mixture of both in detecting IgM antibody, starting from antigen preparation process. Also, in the next research, the first measurement of molecular weight of the antigen and the provision of immunization to the animals is done by mouth to avoid bias, so the result can further increase the intensity and generalization of the research results.

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