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# TECHNIQUES OF PURIFICATION of ANTIGEN O ISOLATES of Escherichia coli AND IN VIVO ANALYSIS OF ANTIGEN IMMUNOGENICITY IN POULTRY

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**ABSTRACT**
Lipopolysaccharide (LPS) is a major surface component of most Gram-negative bacteria and is recognized by immune cells as pathogen-associated molecules. Antibodies against O antigens can provide protection against infection and vaccines have been developed by conjugating O antigens to carrier proteins to enhance immunogenicity. This study aims to produce O antigen from Escherichia coli and analyse the in vivo test results in poultry using ELISA technique. The methods used in this study were *E. coli* Bacterial Culture, O Antigen Purification, SDS PAGE, Inoculation on Experimental Animals, Rapid Plate Agglutination Test, and ELISA. Based on the results of the research conducted, it can be concluded that the O antigen of Escherichia coli injected into chickens can induce the formation of specific antibodies against *E. coli* in the blood. Specific antibodies to *E. coli* formed can be detected using the rapid plate agglutination test and ELISA technique.



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## 1. INTRODUCTION

Lipopolysaccharide (LPS) is a major surface component of most Gram-negative bacteria and is recognized by immune cells as pathogen-associated molecules. LPS consists of a polysaccharide chain of repeating units, O antigens, bound to a core oligosaccharide containing 10–12 sugar units. Antibodies against O antigens can provide protection against infection and vaccines have been developed by conjugating O antigens to carrier proteins to enhance immunogenicity. An important precursor for the production of O antigen vaccine conjugates is the purification of the O-antigen which is separated from all contaminants including LPS, because LPS contamination of the vaccine will cause undesirable local and systemic reactogenicity (Micoli *et al*., 2014).

In most published protocols for producing O antigen-based vaccines, O antigen purification is performed after LPS extraction and hydrolysis. Various procedures have been used for the isolation of LPS, such as methods using trichloroacetic acid, ether, EDTA, liquid butanol, Triton X-100/Mg2+, cold ethanol, diethylene glycol or liquid pyridine, extraction in water at 100 °C, and methanol-chloroform. Another extraction method, using phenol, chloroform, petroleum-ether, and methanol, has been carried out specifically for crude LPS, while another method using sodium dodecyl sulphate has been developed to efficiently extract both types of LPS, fine and coarse. However, due to the higher yield, the most frequently used method for LPS extraction is the Westphal procedure, commonly known as phenol-heat extraction. Several modifications are already present in the basic protocol to reduce contamination and produce higher yields, and for the hot-phenol extraction method, although it is cancerous and toxic, it is commercially used to extract large quantities of LPS.

The traditional isolation and purification process for the overall O antigen is very complex and requires longer time and uses large volumes of harmful phenols and toxic LPS intermediates (Aulia *et al*., 2015). Therefore, there is a need for clarity to improve the process of safer O antigen extraction and purification from Gram negative bacteria, especially in relation to large-scale production. (Micoli *et al*., 2014) describes the development of a direct extraction method, which employs hydrolysis of acetic acid at 100 °C in direct bacterial cultures. This type of treatment can cleave the labile bond between KDO, at the proximal end of the core oligosaccharide, and lipid A,

the O antigen chain attached to the core sugar is released. So, it is hoped that through this purification method, pure O antigen can be produced and can be used to produce high antibodies in poultry through the Elisa technique.

## 2. RESEARCH METHOD

### A. Culture of E. coli

Bacterial isolates were cultured on TSA media by stretching using a sterile tube, then incubated at 37 °C for 24 hours (Wulandari *et al*., 2019). After growing the bacterial colonies, 1 colony of bacteria that grew on the agar medium was taken using a sterile tube and then the colonies were cultured on 100 ml TSB media and incubated in a shaker at 37 °C for 24 hours (Wulandari *et al*., 2019).

### B. Purification O Antigen

100 ml of bacterial culture on TSB media was then transferred to a 95 ml flacon bottle. Then it was centrifuged at 3000 g for 15 minutes, the supernatant was discarded. Then the pellet was added with PBS (0.15 mM CaCl2 and 0.5 mM MgCl2) until it reached the initial volume (95 ml). Centrifugation and washing was repeated 3 times, then the supernatant was discarded. The pellet was transferred to a microtube and then diluted with 500 l of 0.85% NaCl solution. After being diluted using 0.85% NaCl, the pellets were given 2 treatments. The first treatment is using the heating method. Pellets in microtubes that have been diluted with NaCl are heated using a water bath at a temperature of 65 °C for 30 minutes. The second treatment used the acid method, pellets that had been diluted with NaCl were then added with acetic acid and mixed using a vortex so that the final pH was 3.5–4.7. Both are then labelled and stored in a freezer at -20 °C.

### C. SDS Page

The prepared O antigen was added with 2x SB (sample buffer) in a volume of 30 l with a ratio of 1:1. The mixture was boiled at 65 °C for 5 minutes. The gel was put into the SDS PAGE electrophoresis bath which had been filled with running buffer. The SDS PAGE agar mold made consisted of 10 wells, 9 wells filled with 10 l of O antigen samples and 1 well filled with 10 l of marker as a positive control. Furthermore, electrophoresis at a voltage of 100 V with a current of 50 mA for ± 3 hours.

### D. Antigen Inoculation

O antigen that has been tested with SDS PAGE which shows a positive result is inoculated into experimental animals. The experimental animals used were male and female chickens, 1 each.

### E. Rapid Test Agglutination Test.

The rapid plate agglutination test is very simple. Antibody serum that has been harvested from experimental animals is dripped (2-3 drops) on an object glass. Then the O antigen is added to the serum and stirred. The result is positive if there is precipitation such as greyish white sand grains in the tested serum.

### F. Elisa

The ELISA used in this study is Indirect ELISA. Its purpose is to detect antibodies.

## 3. RESULT AND DISCUSSION

*E. coli* bacteria that have been grown on trypticase soy agar (TSA) media on April 6 2018, growth observations were carried out on April 7 2018. The growth of *E. coli* on TSA media is shown in Figure 1, the result is positive *E. coli* bacteria growing on TSA media, the colonies are light cream in color and the surface of the colonies is raised. *E. coli* bacteria were seen growing to form several single colonies which were then taken to be grown in trypticase soy broth (TSB) media. TSA media is an agar medium for the isolation of microorganisms because it is fertile and rich in nutrients so that it can be used to grow various kinds of aerobic microorganisms. TSA media contains natural ingredients derived from soybeans as a source of nutrition for bacteria, in order to compact the TSA media, casein for amino acid sources, sodium chloride plays a role in maintaining osmotic balance and phosphate as a buffer to maintain the pH of the media(Becton Dickinson, 2019).

*E. coli* bacteria that have grown on TSA media and grow to form single colonies will then be taken and grown in trypticase soy broth (TSB) media on April 12, 2021 On April 13, 2021, *E. coli* bacteria that had been grown in TSB media were extracted to obtain O antigen. The purpose of growing *E. coli* bacteria in trypticase soy broth media is so that bacteria grow more optimally because it uses a liquid medium and during incubation, agitation is carried out in an incubator at 37 °C for 18 hours. The content of TSB media is almost the same as TSA media, the difference is that TSB media does not contain agar so that the media is in liquid form, TSA media can last more than 2 weeks while broth media can only last for 3 days. . Soy broth trypticase media is widely used to isolate bacteria from laboratory specimens and supports the growth of the majority of bacteria that are pathogenic media (Becton Dickinson, 2019).

Figure 1. Escherichia coli bacteria culture results on TSA media

*E. coli* bacteria have three types of antigens, namely O antigens, K antigens and H antigens. O-antigens are the core of lipopolysaccharides and polysaccharide units, usually O-antigens are associated with special diseases in humans, for example the O-specific type of *E. coli* is found in diarrhea. The K-antigen is a capsule of polysaccharide, while the H-antigen is a flagellar antigen (Setiawan *et al*., 2014). In this practical activity, the O antigen was extracted by centrifuging several times and one of the micro tubes was added with glacial acetic acid and in the final stage heating. The results of O antigen extraction were then tested using the SDS PAGE method to see the level of purity of the sample extraction results and also used to determine the molecular weight of a protein (Rapley, 2005). TA3 code is extraction without the addition of glacial acetic acid. While the absorbance value of O antigen extraction measured using an ELISA reader the results were not significantly different between the extraction with the addition of acid and without the addition of acid, namely 281 (A3/added acid) and 242 (TA3/without the addition of acid). The effect of adding acid on the extraction of O antigen in several studies showed a higher value of O antigen concentration although not significantly.

Lipopolysaccharide (LPS) is a major component of the surface of most Gram-negative bacteria and is recognized by immune cells as a molecule associated with pathogenic properties. LPS consists of a polysaccharide chain of repeating units, the O antigen associated with a core oligosaccharide containing 10–12 sugar units. The covalent nucleus is bound via 2-keto-3-deoxyoctonate (KDO) to lipid A. Lipid A is highly conserved and plays a role in endotoxic activity, while antigen O is a distinct chain between serovars and plays a major contributor to serology.

The release of O antigen from LPS utilizes the acid-labile glycosidic bond between lipid A and KDO at the end of the LPS core region. Hydrolysis of LPS with the addition of acetic acid in 1% AcOH at 100 °C, sodium acetate buffer at pH 4.5 has been shown to efficiently cleave the lipid A-polysaccharide bonds. AcOH will hydrolyze O antigen from lipid A while still attached to the bacterial membrane so that the O antigen chain plus the nucleus will be released in solution and can be taken by centrifugation to obtain O antigen pellets (Rosner & Markowitz, 2013). Therefore, the extraction process with the addition of acid resulted in a slightly higher concentration of O antigen than the extraction without acid.

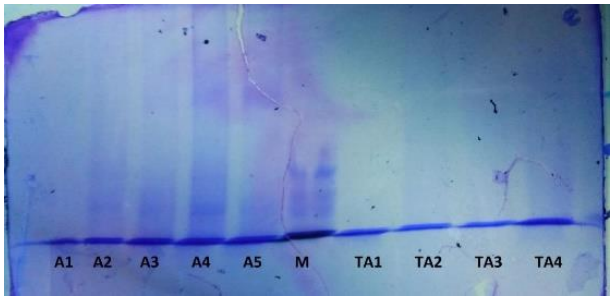


Figure 2 SDS PAGE test results for the O antigen of *E. coli* bacteria

The results of SDS-PAGE extraction of O antigens show that the protein band size is between 28–36 kD and the protein bands formed look single and sharp. The results of SDS-PAGE extraction of O antigens show that the protein band size is between 28–36 kD and the protein bands formed look single and sharp. In SDS PAGE, proteins can separate due to heating. Protein molecules will migrate from the negative pole to the positive pole in the presence of electricity. Separation of protein molecules based on migration rate and molecular weight in an electric field.

Rapid Plate Agglutination (RPA) is a serological test method that is suitable and easy to detect the presence of antibodies produced when animals are infected or after animals are vaccinated, this serological test method only shows the presence or absence of antibodies so it cannot be determined the high and low value (titer) of the antibody produced. found in animal bodies. This test method is relatively flexible because it can be carried out in the laboratory as well as directly in the cage (WHO, 2004).

The rapid agglutination reaction has the same principle as the antigen-antibody relationship. The important difference is that a soluble complex is not formed in this agglutination. The result of a rapid agglutination reaction between homologous antigens and antibodies will cause agglutination or clumping in the form of visible aggregates so that they can be seen directly by the eye without the need for a microscope. The factors that influence the agglutination reaction are particle size, surface electrostatic charge density, or the immunochemical properties of the antibody as well as the specific physicochemical state.

The agglutination process in the first phase of antigen-antibody union occurs as in precipitin and depends on ionic strength, pH, and temperature. The second phase, lattice formation, depends on overcoming the electrostatic repulsion of the particles. Red blood cell agglutination, for example in antigenic receptor sites, may lie in deep depressions. On the cell surface, the antibody binds tightly to the receptor site on one cell. Lattice formation cannot occur until a valence free receptor is attached to the antigen between adjacent cells. If the cells are separated by repulsion, the free end of the antibody molecule will not approach the antigen tightly enough to form a strong bond so that agglutination cannot occur (Zmijewski, 1993).

Rapid plate agglutination was carried out on July 19, 2018 and positive results were obtained with the formation of particles of agglutination between the O *E. coli* antigen and chicken serum containing homologous antibodies to the O antigen. This positive result indicates that the O antigen injected into chickens on 24 May 2018 was able to induce the formation of specific antibodies so that agglutination was formed as shown in Figure 4.

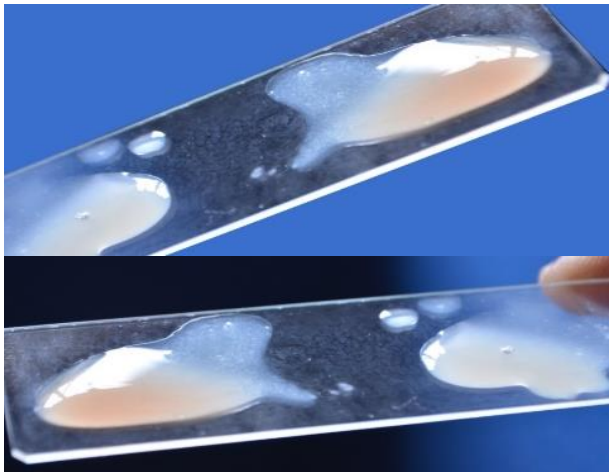

Figure 4. Results of the O *E. coli* . antigen rapid plate agglutination (RPA) test.

Measurement of protein concentration can be done by many methods, one of which is the Bradford method. This method is a simple but sensitive measurement method (Furstenburg & van Hoven, 1994). The protein concentration calculated in this practicum is derived from the suspension of O Escherichia coli antigen. The standard solution used to measure protein concentration is Bovine Serum Albumin (BSA). The measurement of the absorbance of the standard BSA solution at a wavelength of 595 nm (Table 1) showed a linear absorbance result with graded concentrations.

Table 1. BSA standard values

| Tube | Concentration (µg/ml) | Absorbance |
|---|---|---|
| 1 | 0 | 0.307 |
| 2 | 100 | 0.359 |
| 3 | 200 | 0.367 |
| 4 | 400 | 0.395 |
| 5 | 500 | 0.463 |
| 6 | 600 | 0.506 |
| 7 | 700 | 0.524 |
| 8 | 1000 | 0.531 |

The BSA absorbance values obtained in Table 1 were then calculated by linear regression. The line equation obtained from the BSA standard linear regression calculation (Figure 5) is = $0.0343x + 0.2271$ (y=concentration, x=absorbance). The value of the coefficient of determination ($R^2$) of the line equation is 0.96, which means that 96% of the protein concentration value can be explained by the line equation. The correlation coefficient value ($r$ = 0.98) is close to 1 indicating that the absorbance and concentration values have a very strong correlation or closeness(Sudjana, 2020). The higher the absorbance value, the higher the protein concentration.

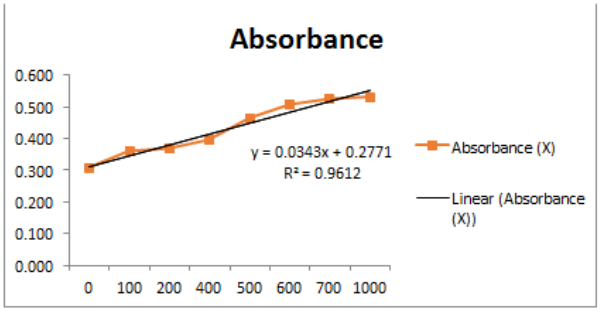


Figure 5. BSA standard curve

In general, to produce specific antibodies from eggs or chicken blood serum, it can be done by injecting certain required antigens (vaccines, bacteria, toxoids, or other biological materials). The method of injection can be done intravenously, intramuscularly, or subcutaneously depending on the required antigen preparation. Antigens derived from bacteria usually use bacterial cell standards in suspension form with physiological NaCl solvent (Mahardika *et al*., 2016).

The method used for purification in this practicum is easy and inexpensive, but it can denature protein quickly but is not precise (not specific), therefore it is better to use other methods such as gamma ray radiation or chromatography (Simaremare *et al*., 2015).

Table 2. Measurement of absorbance and protein concentration of O . antigen

| Kelompok | Metode Pemanasan | | Metode Asam | |
|---|---|---|---|---|
| | Absorbansi | Konsentrasi (mg/ml) | Absorbansi | Konsentrasi (mg/ml) |
| 1 | 0.266 | 0.2862 | 0.309 | 0.2877 |
| 2 | 0.285 | 0.2869 | 0.289 | 0.2870 |
| 3 | 0.281 | 0.2867 | 0.242 | 0.2854 |
| 4 | 0.272 | 0.2864 | 0.333 | 0.2885 |
| 5 | 0.264 | 0.2862 | 0.279 | 0.2867 |

The measurement of the absorbance value of the O antigen protein was carried out using a spectrophotometer. The absorbance value of O antigen obtained in Table 2 is entered into the regression line equation that has been obtained previously, then the protein concentration value is calculated. Average protein antigen concentration. The O obtained was 0.28 mg/ml for both the heating method and the acid method (Table 2). These results indicate that the protein concentration value of O antigen from the two methods was not significantly different because the two methods had similar concepts. The standard protein concentration of O antigen is also close to the value of *Eschericia coli* O157:H7 protein content after purification, which is 0.2 mg/ml(Sari *et al*., 2020).

The presence of antibodies in the blood can be detected by ELISA testing. In this experiment, the ELISA technique used is indirect ELISA. ELISA is an antigen or antibody detection technique using a labeled enzyme, such as the HRP enzyme (Mufidah *et al*., 2015). The ELISA technique has the advantage that it is more sensitive and faster (Syndrome, 1995). ELISA results obtained are displayed in absorbance values (optical density or optical density). The absorbance value obtained is a quantity value and is proportional to the antibody concentration. The higher the absorbance value, the higher the antibody concentration in the serum(Esfandiari *et al*., 2014). The results of the ELISA examination of chicken serum can be seen in Table 3. The ELISA reader readings showed that the chickens vaccinated with O *E. coli* antigen, both purified by acid and heating methods, all showed positive results. Determination of positive and negative serum from the value of Optical Density (OD) ELISA results were determined by calculating the threshold value(cut off). The cut off value is calculated by the following formula: $= OD + (3 \times )$ (Mufidah *et al*., 2015). The cut off value of the chicken serum sample in this experiment was 0.51. Based on the cut off value, the OD value of the chicken serum sample that is above the cut off line is declared positive and the OD value below the cut off line is negative. In Figure 6 it is known that the OD value of all chicken serum is above the cut off line so that it is declared positive. This indicates that all chicken serum samples taken after vaccination were significantly seropositive or contained specific antibodies against O *E. coli* antigen.

Table 3 Antibody titers of chicken serum vaccinated with O *E.coli* antigen

| Treatment | Group | Absorbance | Interpretation |
|---|---|---|---|
| Heating Method | 1 | 161 ± 0.143 | + |
| | 2 | 154 ± 0.119 | + |
| | 3 | 067 ± 0.098 | + |
| | 4 | 058 ± 0.120 | + |
| | 5 | 010 ± 0.078 | + |
| Acid Method | 1 | 0.926 ± 0.100 | + |
| | 2 | 0.941 ± 0.122 | + |
| | 3 | 0.937 ± 0.115 | + |
| | 4 | 0.986 ± 0.152 | + |
| | 5 | 1.056 ± 0.108 | + |

Information:
Information:
Control absorbance mean: 0.269 ± 0.080
Positive result if absorbance value 0.51
Negative result if absorbance value < 0.51

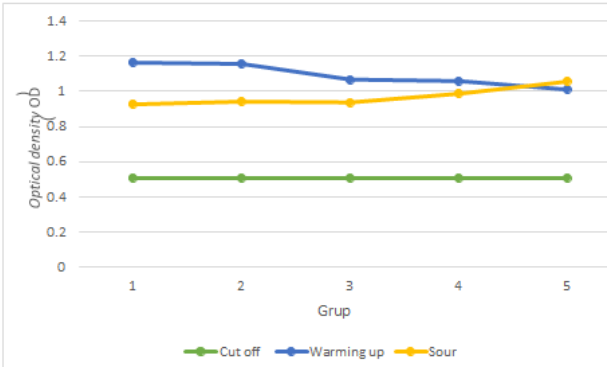


Figure 6. Antibody titer against O E.coli antigen by heating and acid method, ELISA cut off value = 0.51

Factors that influence the formation of antibody responses include the age of the animal, the size of the antigen molecule, the complexity of the chemical structure of the antigen, genetics, immunization route, antigen dose, time, and the number of repetitions of immunization/vaccination (Hendricks *et al.*, 1936). complexity of the chemical structure of the antigen, genetics, route of immunization, dose of antigen, time, and number of repetitions of immunization/vaccination. The concentration of antibodies formed (humoral response) is also influenced by the nature and duration of the antigen in the body. The condition of the animal's body greatly affects the response to antibody formation. The condition of the animal's body is influenced by the quantity and quality of the feed consumed.

## 4. CONCLUSION

Based on the results of the research conducted, it can be concluded that the Escherichia coli O antigen vaccine injected into chickens can induce the formation of specific antibodies against *E. coli* in the blood. Specific antibodies to *E. coli* formed can be detected using the rapid plate agglutination test and ELISA technique.

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