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The effectiveness of using ID broth in identifying the outer membrane protein of *Salmonella typhi*

Pestariati, Suhariyadi
Department of Medical Laboratory Technology, Polytechnic Ministry of Health Surabaya, Surabaya, Indonesia

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ABSTRACT

Typhoid fever is an infection that affects the digestive system. It spreads through contaminated food and drinks due to the *Salmonella* bacteria. One way to develop immunity against *Salmonella typhi* is by using outer membrane protein (OMP), which activates the cellular immune system. This research aimed to determine the effectiveness of using ID Broth to identify OMP *Salmonella typhi*. The study was conducted experimentally at the Institute of Tropical Disease from April 2023 to May 2023. For the study, we obtained three samples of *Salmonella typhi* isolated from East Java, and each sample was replicated three times. We isolated the bacteria and extracted the OMP to measure its levels and perform electrophoresis with SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a standard protein analysis method. To address sample loading challenges due to stacking gel transparency, an acidic dye was added to improve visibility without affecting gel performance. In this study nutrient agar from MacConkey medium and ID broth were used as variables. We cultured *Salmonella typhi* and extracted OMP using the sonication technique. We measured protein levels through the nanodrop method. *Salmonella typhi* from ID broth produced higher protein levels than *Salmonella typhi* cultured from MacConkey Medium. It affected the identification of OMP using SDS-PAGE. Lower protein levels lead to fewer protein molecules in the same band zone, causing reduced visibility and readability of the protein bands. The ID broth stabilizes the bacteria's condition before being grown on nutrient agar media.

Corresponding Author:

Suhariyadi
Department of Medical Laboratory Technology, Polytechnic Ministry of Health Surabaya
Surabaya, Indonesia
Email: suhariyadi@poltekkesdepkes-sby.ac.id

1. INTRODUCTION

Typhoid fever is a digestive infection caused by the Salmonella bacteria. The word "typhoid" is derived from the Greek word "typhos," which means fog. This is because people affected by this disease often experience impaired consciousness, ranging from mild to severe. In 2019, an estimated 9 million cases of typhoid fever resulted in approximately 110,000 deaths. This serious and potentially life-threatening infection is caused by the bacterium Salmonella Typhi, typically transmitted through consuming contaminated food or water. Once in the body, the bacteria multiply and spread into the bloodstream, leading to severe symptoms and complications [1], [2]. Salmonella bacteria produce endotoxins, which are complexes of lipopolysaccharides. Endotoxins induce fever and inflammation, causing typhoid fever. Additionally, they can stimulate macrophage and leukocyte cells in inflamed tissues to produce cytokines, which are mediators of the onset of fever and proinflammatory symptoms [3], [4].

Salmonella typhi is a gram-negative, non-spore, motile, capsulated, and flagellated rod bacterium. These bacteria can live at pH 6-8 at temperatures of 15-41 ºC (optimal temperature 37 ºC). Through boiling and pasteurization, as well as chlorination, Salmonella can die for one hour at 54.4 ºC and for 15-20 minutes at 60 ºC. Some antigens found *in Salmonella typhi* include O antigen (somatic), H antigen (flagellar), Vi antigen, and outer membrane protein (OMP) antigen. *Salmonella typhi* can cause hemolysis on blood agar plate (BAP) media. However, it is a non-lactose fermenter (NLF) and cannot ferment lactose. On the other hand, fermentation occurs in glucose, mannitol, and maltose media without the formation of acid and gas. The bacterial confirmation test relies on the IMViC Test, which is essential for effectively differentiating Gram-negative rods, especially within the Enterobacteriaceae family. When subjected to IMViC assays (indole, Methyl Red, Voges-Proskauer, and citrate), *Salmonella typhi* yields negative indole, positive MR, negative VP, and most likely positive citrate results. These bacteria do not hydrolyze urea and do not produce H_2S. Microscopic *Salmonella typhi* on media appears convex, slippery, and clear [5], [6].

OMP antigens are located on the surface of gram-negative bacteria. They are known to be good at inducing immunity against *Salmonella typhi* by activating the cellular immune system. Researchers are studying OMP as a potential vaccine candidate against typhoid fever. The pathogenicity of *Salmonella* in inducing diarrhea is a complex phenomenon that involves several mechanisms, including the production of enterotoxin [7]. Studies have shown that vaccines based on OMP are effective in inducing protective antibodies against typhoid, making OMP from *Salmonella* a potential candidate for protection. However, vaccines based on polysaccharides and O antigens may not be effective and can even increase susceptibility to extracellular *Salmonella* growth, which can be dangerous. In recent years, researchers have conducted extensive studies on the molecular structure and function of OMP from *Salmonella* as a potential vaccine candidate, virulence factor, and diagnostic antigen. Despite this, only a limited number of OMPs have been fully characterized so far [8].

SDS-PAGE, which stands for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, is a technique used to separate proteins based on their molecular weight. This method utilizes the ability of proteins to migrate towards an electrode in the polyacrylamide gel based on their size under an electric current [9], [10]. Proteins are separated by passing through a gel matrix made of acrylamide during electrophoresis. This separation relies on the molecular mass of the proteins, with smaller molecules moving through the gel faster than larger molecules. Electrophoresis is a highly sensitive technique that does not alter the structure of proteins, even with slight variances in charge and molecular weight affecting their movement [11].

Growth media is a substance that contains nutrients essential for the growth of microorganisms. This medium can be used for isolating, identifying, and creating pure cultures of microorganisms, whose nutritional requirements vary depending on the type. Generally, they require macronutrients, micronutrients, vitamins, and minerals to thrive. Macronutrients such as carbon, hydrogen, and nitrogen are necessary in larger quantities, while micronutrients are required in smaller amounts and contribute to enzyme function and protein structure. Micronutrients are categorized into trace elements and growth factors, with trace elements serving as enzyme cofactors, including iron, manganese, and zinc. Culture media is a material consisting of a mixture of nutrients used for cultures of a microorganism. The creation of culture media aims to provide a balance of nutrient mixtures needed by microorganisms by providing an artificial environment as a simulation of natural conditions that are important for growth. Microorganisms arrange their cell components by assembling small molecules utilizing nutrients in the growth medium. Different microorganisms require different nutritional materials. Therefore, there are culture media in varying shapes and compositions adapted to the type of microorganisms being cultured [12], [13].

MacConkey medium is a selective and differential medium for the growth of gram-negative bacteria based on the bacteria's ability to break down lactose. The main components of MacConkey medium include crystal violet dyes, bile salts, lactose, and neutral red (pH indicator). Crystal violet dyes and bile salts stop the growth of gram-positive bacteria. MacConkey medium contains a pH indicator that turns pink under acidic conditions. Bacteria that can ferment lactose will produce organic acids, especially lactic acid, which can lower pH. The presence of neutral red as a pH indicator will change from faded white to bright red or pink when the pH is less than 6.8. Thus, pink colonies will form on lactose fermenter bacteria, while non-lactose fermenters will form opaque white colonies. Pathogenic bacteria, especially bacteria that cause gastroenteritis such as *Escherichia* and *Salmonella*, are widely distinguished using this medium [14].

Nutrient agar is one of the complex media. Per liter, agar nutrients contain beef extract (3 g), peptone (5 g), sodium chloride (5 g), and agar (15 g). The main components of nutrient agar media are carbohydrates and proteins found in meat extracts and peptones. These nutrients provide a source of nitrogen and carbon, as well as vitamins to aid in bacterial growth [15]. The nutrient composition of agar and nutrient broth can be replaced with alternative ingredients with similar nutritional content to support bacterial growth [16].

The *Salmonella typhi* isolate to be identified needs to be isolated and enriched to obtain appropriate results. The enrichment phase of *Salmonella typhi* uses non-selective media to help repair damaged bacterial cells, dissolve inhibitory substances, and provide adequate nutrition. To detect Salmonella, a specific culture

process is necessary that involves using optimal medium formulations to revive the bacteria while reducing the presence of competing bacteria. Nutrient agar is an example of a non-selective enrichment method used for isolating *Salmonella* [17].

ID broth is a broth used as a manufacturer of bacterial inoculum for automatic antibiotic susceptibility testing with the Phoenix system. Positive cultures of growing bacteria need to be tested for antimicrobial susceptibility testing (AST) resistance. AST is a critical process in clinical microbiology to evaluate antibiotic effectiveness. It identifies when bacteria become less susceptible or resistant to antibiotics, reducing their ability to eliminate bacteria or inhibit their growth [18]. Phoenix's automatic AST resistance test was performed by making an inoculum suspension on the ID broth in the amount of 0.5-0.6 McFarland. The bacterial suspension from ID Broth is transferred to AST broth and continued with automated testing [19]. The inoculum suspension on ID Broth can be used as an advanced testing material. In this study, testing will be carried out on the effectiveness of enrichment of *Salmonella typhi* bacteria from ID broth on nutrient agar media to identify OMP antigens.

2. METHOD

In this study, we used *Salmonella typhi* which was isolated and identified from suspected typhoid fever patients in East Java in 2022 during previous research. The experimental research was conducted at the Department of Medical Laboratory Technology, Polytechnic Ministry of Health Surabaya, and the Institute of Tropical Disease from April 2023 to May 2023, using three samples of *Salmonella typhi* isolates from East Java with three replications. This study was approved by the Health Research Ethics Committee at Polytechnic Ministry of Health Surabaya with a certification number No.EA/1600/KEPK-Poltekkes_Sby/V/2023 dated 11/04/2023.

2.1. Making culture media

Media production occurs in the Bacteriology Laboratory of the Department of Medical Laboratory Technology at the Polytechnic Ministry of Health in Surabaya. In this research, we utilized MacConkey medium CM0115 (OXOID) and nutrient agar CM0003 (OXOID). The media is prepared following the OXOID procedures.

To create MacConkey medium CM0115 (OXOID) media, 49.53 grams of media powder, consisting of peptone (17 g), proteose peptone (3 g), lactose monohydrate (10 g), bile salts (1.5 g), sodium chloride (5 g), neutral red (30 mg), crystal violet (1 mg), and agar (13.5 g), is dissolved in one liter of aquadest. The media should be dissolved while stirring at 80 ºC. After dissolving, check the pH and adjust it to the final pH of NA media, which is 7.1±0.2. Finally, wrap the media and sterilize using a 121 ºC autoclave for 15 minutes.

To create nutrient agar CM0003 (OXOID) media, 28 grams of media powder, consisting of peptone (5 g), yeast extract (2 g), sodium chloride (5 g), beef extract (1 g), and agar (15 g), is dissolved in one liter of aquadest. The media should be dissolved while stirring at 80 ºC. After dissolving, check the pH and adjust it to the final pH of NA media, which is 7.4±0.2. Finally, wrap the media and sterilize using a 121 ºC autoclave for 15 minutes.

2.2. *Salmonella typhi* isolation

For this study, two types of media - MacConkey medium and ID Broth - were used to inoculate *Salmonella typhi* bacteria. The bacteria were grown on MacConkey medium using the streak technique and incubated at 37 ºC for 24 hours. In ID Broth, the bacteria were inoculated to a level of 0.5-0.6 McFarland. After growth, *Salmonella typhi* from each variable was also grown on Nutrient Agar as a supplementary medium. To obtain a pure culture of the bacteria for further testing, the samples were streaked on Nutrient Agar and then incubated at 37ºC for 24 hours [20].

2.3. OMP *Salmonella typhi* extraction

Salmonella typhi isolate from nutrient agar media was suspended at 10 mL 10 mmol Tris HCl pH 8.8 on a 15 cc valcon tube. OMP extraction of *Salmonella typhi* used sonication technique with a frequency of 35 kHz for 30 seconds and repeated for 15 cycles with a gap of 30 seconds. The sonicated isolates were then centrifuged at 4 ºC to 15,000 rpm for 30 min. The supernatants were separated, and the pellets were resuspended with 10 mL PBS and 2% triton-X and incubated at 37 ºC for 20 min. Centrifuges return isolates at 4 ºC to 15,000 rpm for 30 minutes. The pellets were resuspended with 500 µL PBS. Store at-20 ºC until ready to use.

2.4. Protein content calculation

Protein levels were measured using thermo scientific nanodrop at a wavelength of 280 nm [21]. SDS-PAGE To extract the *Salmonella typhi* OMP antigen, we used SDS-PAGE with a 12% separating gel concentration and a 4% stacking gel. We mixed the crude samples and loading buffers in microcentrifuge tubes at a ratio of 2:1 and homogenized them using spin down. Next, we heated the sample for 5 minutes between 90 ºC-95 ºC. We prepared the gel in a chamber with a running buffer and filled the available wells with 20 µL of prepared samples. We also filled one well with 10 µL of protein markers. We ran electrophoresis at 100 V 40 mA for 90 minutes, until the sample almost reached the gel bottom. Finally, we stained the gel using coomassie brilliant blue R-250 (Bio-Rad).

3. RESULTS AND DISCUSSION

MacConkey medium is a type of bacterial growth medium that is both selective and differential, meaning it is used to identify and isolate gram-negative bacteria. This media has a composition of bile salts as inhibitors of gram-positive bacteria, neutral red dye as an indicator of pH, lactose, and peptone. *Salmonella typhi* appears as white colonies that do not cause any changes to the pH in the absence of lactose fermentation. MacConkey is a commonly used growth medium that selectively promotes the growth of gram-negative bacteria and distinguishes them based on their fermentation profile. This media is widely used to differentiate pathogenic bacteria, especially in species that cause gastroenteritis [14], [22].

ID Broth (BD Phoenix ID Broth) is a solution used to create bacterial suspensions for use in Phoenix's automated susceptibility identification and testing system. It contains potassium chloride, calcium chloride, tricine glycine, and polysorbate 80 in a one-liter solution. The amount of bacterial suspension is determined using McFarland standards. Glycine is one of the essential amino acids and is a source of condensed nitrogen [23]. Tris-tricine has been found to be a superior buffer for separating serum proteins and is commonly used in conjunction with glycine to stabilize pH. ID Broth contains not only tricine and glycine, but also polysorbate 80. Tricine is a recommended method for controlling pH, as it closely resembles physiological solutions [24], [25]. Therapeutic protein formulations use Polysorbate 80 as a surfactant to prevent adsorption and promote protein aggregation [26]. Polysorbate impacts *Salmonella typhi* by stabilizing bacterial cells and reducing their clumping, which can interfere with measuring protein levels. Polysorbate can also influence the integrity of the bacterial cell membrane and alter protein structure within the cells [27].

Nutrient Agar is a commonly used non-selective media for rapidly growing various bacterial species found in water, waste, and feces. It consists of peptone, beef extract, and agar, providing essential nutrients for bacterial growth. This makes it an excellent choice for bacterial identification, especially for cultivating large quantities of Salmonella bacteria [28]. In a study conducted by Khosravi (2022), immunomagnetic beads were developed to isolate Salmonella bacteria from contaminated milk samples. The researchers successfully detected at least 3×104 CFU/mL of bacteria in the polluted samples using nutrient agar media [29].

Salmonella typhi isolate is extracted using sonication techniques by utilizing ultrasonic energy to move the constituent particles of the cell membrane so that the protein components can be released. OMP extraction of *Salmonella typhi* in this study used a sonication technique with a frequency of 35 kHz for 30 seconds and repeated for 15 cycles with a gap of 30 seconds. After the sonication process, phosphate-buffered saline (PBS) surfactant and Triton-X are added to assist in lysing the bacterial cell surface so that OMP extract is obtained to be analyzed. PBS is a water-based salt solution used to maintain the pH and osmolarity for cells, tissues, and biological samples [30].

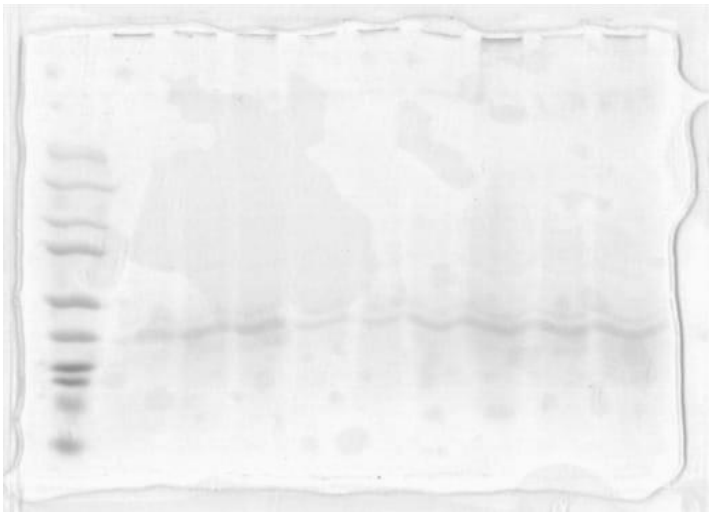
The protein content of OMP *Salmonella typhi* extract was measured using a Thermo Scientific Nanodrop at a wavelength of 280 nm. Thermo Scientific nanodrop utilizes a UV-Vis spectrophotometer to measure absorbance. Purified proteins absorb light at a peak of 280 nm [21]. Table 1 shows that the protein levels in *Salmonella typhi* OMP extract obtained from ID Broth culture samples ranged from 8.310 mg/mL to 8.985 mg/mL, while samples from MacConkey cultures ranged from 1.105 mg/mL to 5.471 mg/mL. The difference in bacterial growth between solid and liquid culture media may be due to differences in nutrient availability.

Before protein electrophoresis, OMP extract was prepared by adding loading buffer to the microcentrifuge tube with a ratio of 2:1 and homogenizing using spin down. The sample was heated at a temperature of 90ºC-95ºC for 5 minutes. SDS-PAGE with 4% stacking gel and 12% separating gel was used in this study. The wells were filled with 20 µL of prepared samples, and one of the wells was filled with protein markers. The Bio-Rad Protein Standard with a size of 10 kDa to 250 kDa was used as a protein marker. Electrophoresis was run at 100V and 40 mA for 90 minutes until the sample almost reached the gel bottom. Finally, the gel was stained using coomassie brilliant blue R-250 (Bio-Rad). The OMP antigen of *Salmonella typhi* was identified by separating proteins by their molecular weight using SDS-PAGE. The protein bands were then visualized using coomassie brilliant blue as shown in Figure 1. In Figure 1(a), a

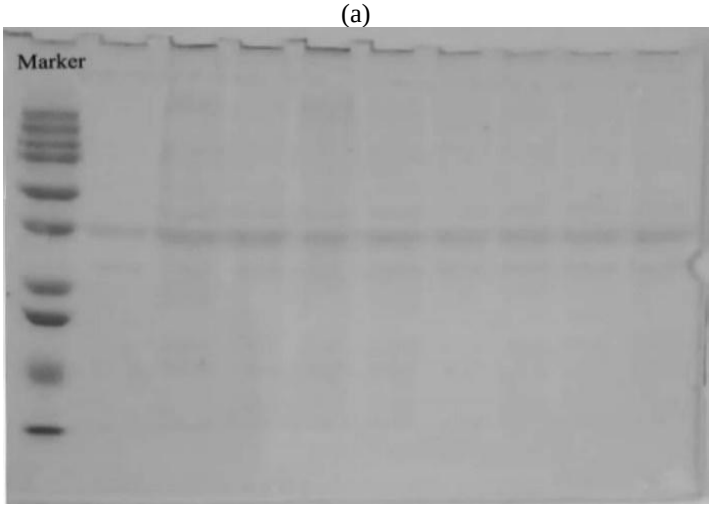
close-up image showcases the isolation of the OMP Salmonella typhi antigen from the MacConkey medium. Meanwhile, Figure 1(b) provides a detailed view of the ID broth culture.

Table 1. Protein content of the samples

Sample	*S. typhi* from Mac Conkey Agar (mg/mL)	*S. typhi* from ID Broth (mg/mL)
1a	1.105	8.985
1b	1.122	8.839
1c	5.054	8.484
2a	5.471	8.722
2b	2.406	8.310
2c	2.840	8.934
3a	3.324	8.838
3b	3.474	8.398
3c	4.076	8.887



(a)



(b)

Figure 1. Results of separation *Salmonella typhi* OMP antigen that cultured from (a) MacConkey medium and (b) cultured from ID broth

The molecular weight of protein bands is automatically calculated using the Gel Analyzer 19.1 application. The results of the SDS-PAGE analysis showed that the samples cultured on solid media resulted in the formation of 6 protein bands, with molecular weights ranging from 14 kDa to 97 kDa. On the other hand, samples cultured on liquid media resulted in the formation of 1 to 3 protein bands, with molecular

weights ranging from 30 kDa to 36 kDa. More details are available in Table 2. The variation in molecular weight of the protein formed came from the debris of the extracellular component of the sample because this study used crude samples without purification. The molecular weight of a protein can estimates the amino acid composition that a sample contains [31].

Table 2. Calculation of molecular weight OMP

S. typhi from Mac Conkey agar		*S. typhi* from ID broth	
Lane	Molecular Weight (kDa)	Lane	Molecular Weight (kDa)
1 and 2	33	1, 2, 3, and 4	97 kDa
			42 kDa
3, 4, 7, 8, and 9	36		36 kDa
			27 kDa
	34 kDa		23 kDa
			14 kDa
	30 kDa	5, 6, 7, 8, and 9	54 kDa
			42 kDa
5 and 6	36 kDa		36 kDa
			27 kDa
	30 kDa		16 kDa
			14 kDa

The molecular weight of protein bands formed after OMP separation using SDS-PAGE was measured using Gel Analyzer 19.1 software, following staining with coomassie brilliant blue. From the measurement results, one to three protein bands were obtained in samples from Mac Conkey Agar and six protein bands from ID Broth with molecular weight details in Table 2. The difference in the number of bands formed due to the low OMP protein content of the sample extract allows the amount of protein to be separated slightly so that the visualization of protein bands is less optimal, protein bands cannot be read, and molecular weight cannot be identified [32]. The protein band formed in SDS-PAGE can be determined from the results of sample extraction carried out previously, considering that in this study sample extraction with the same method, the difference in results can be caused by the protein levels contained. The disadvantage of this SDS-PAGE method is that protein band visualization is done manually even though molecular weight calculations are done automatically using Gel Analyzer 19.1.

It is common to observe differences between the actual molecular weight of proteins on SDS-PAGE gels and their expected size. This difference can arise due to chemical modifications such as glycosylation and ubiquitination, which can significantly affect their mobility on the gel. It's worth noting that phosphorylation can sometimes cause slight variations in SDS-PAGE gels. However, when hyper-phosphorylation occurs, it shows up as slower stains. Therefore, it is important to examine whether the difference in size is due to chemical modifications or specific features such as the protein's amino acid composition [31].

4. CONCLUSION

Research has shown that OMP *Salmonella typhi* extract grown on Nutrient Agar from ID Broth contains higher protein levels than *Salmonella typhi* cultured from MacConkey medium. This variation is due to environmental conditions and nutrient absorption within the media. Using ID Broth before growing on Nutrient Agar stabilizes the bacteria's condition and decreases the likelihood of errors in growth media production. The protein levels in the extracted OMP samples have an impact on the identification of OMPs based on protein band formation. When protein levels are low, fewer protein molecules separate in the same band zone, which reduces the visibility and readability of protein bands, making it difficult to identify the accurate molecular weight of the protein. Therefore, using samples with higher protein levels yields better results in the SDS-PAGE method. It can be concluded that using *Salmonella typhi* from ID Broth is more effective in identifying OMP antigens than using MacConkey medium.

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BIOGRAPHIES OF AUTHORS

Pestariati Pestariati has received education from various institutions, including SDN and SMPN in Surabaya, a 4-year Analysis Management School (SPA) in Bandung, Health Analyst Academy in Bandung, a bachelor's degree in chemistry education, and a Magister program in Basic Medical Science with a particular interest in Medical Microbiology from Surabaya. Upon completing SPA, she became a class II/civil servant and later studied at the Bandung Health Analyst Academy to qualify for the II/c position. Currently, she holds the position of class IV/c. She is a lecturer for the Bacteriology course at the Department of Medical Laboratory Technology, Polytechnic Ministry of Health Surabaya, Surabaya, Indonesia. She can be contacted by email: pestariati@poltekkesdepkes-sby.ac.id.

Suhariyadi Suhariyadi's educational background is quite extensive. He attended SDN Cemandi for primary school, SMPN Juanda for middle school, the Health Analyst High School of the Ministry of Health Surabaya, IKIP PGRI Surabaya for his bachelor's degree in chemistry, and the Master Program of Faculty of Medicine Airlangga University for his master's degree in Immunology. Additionally, he has taken courses in Instrumentation, Immunology, and Molecular Biology. Suhariyadi has worked at PT. Sandai Farma, the Merpati Clinical Laboratory, and as an instructor and teacher at SMAK Ministry of Health Surabaya. Suhariyadi has an impressive work history, having held various positions in the health industry. He was the Head of the D3 Health Analyst Study Program, Secretary of the Health Analyst Department, and Head of the Clinical Chemistry Laboratory at Polytechnic Ministry of Health Surabaya Integrated Lab. He is also a laboratory consultant at the Food Security Agency of East Java Province and a resource person at the Food Security and Agriculture Office of East Java Province on food safety in all City Districts in East Java. Suhariyadi is currently a lecturer at the Polytechnic Ministry of Health Surabaya, where he teaches Immunology, Biomolecular, and Immunohematology. He serves as the Head of the Diploma 3 Study Program Department of Medical Laboratory Technology, Polytechnic Ministry of Health Surabaya, Surabaya, Indonesia (2022 – present). He can be contacted by email: suhariyadi@poltekkesdepkes-sby.ac.id.