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Measurements and Accuracy of IgM and IgG Anti Phenolic Glycolipid-1 Levels in Blood Serum for Early Detection *Mycobacterium leprae* by using Enzyme-Linked Immunosorbent Assay (ELISA): A Reality of a Laboratory

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ABSTRACT

Indonesia was the third most recent case of leprosy globally in 2020 with 11,173 people, after India and Brazil. Most of the leprosy manifestations are asymptomatic. This is possibly as subclinical leprosy which individuals without leprosy symptoms but have leprosy specific antibodies high levels, so it has the potential to become a transmission and disability. Therefore, an ELISA test need for early detection in preventing leprosy transmission. This study aims to measure IgM and IgG antibody levels in leprosy patients and assess the accuracy of the measurement results. This research is a cross-sectional study. Five patients' blood samples have analyzed for IgM and IgG anti-PGL-1 antibody levels by ELISA. Accuracy interpretation of this measurement based on the %CV. Antibody levels were classified based on the cut-off <605 u/ml as IgM seronegative or <630 u/ml as IgG seronegative, 605–1000 u/ml as low seropositive IgM or 630-1000 u/ml as low seropositive IgG, and >1000 u/ml as high seropositive IgM and IgG. Among five patients examined, 40% had high seropositive leprosy with anti-PGL-1 IgM and IgG antibody titers>1000 u/ml, and 60% of patients had seronegative leprosy. Accuracy in this ELISA test shows high accuracy with %CV <10% in the conversion of OD to antibody titer levels. IgM and IgG Anti PGL-1 antibody titers by ELISA as one of the parameters in identifying patients at higher risk of leprosy. A significant portion of patients with high seropositive leprosy with high accuracy.

Keywords: leprosy, early detection, ELISA, Anti-PGL-1, seropositive.

Highlights: The ELISA test can detect the presence of Anti Phenolic Glycolipid-1 (PGL-1), specific for *M. leprae.* Therefore, sensitive and specific early detection tool by ELISA anti-PGL-1 needs to be able to control the transmission of *M. leprae*

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INTRODUCTION

Leprosy or Morbus Hansen is one of the Neglected Tropical Diseases (NTD) with a chronic infection caused by *Mycobacterium leprae*. Leprosy has been found for thousands of years BC, it is still endemic in several countries, including Indonesia.[1] Based on data from the World Health Organization (WHO) for 2020, Indonesia ranks third with the most recent cases of leprosy globally with 11,173 people, after India and Brazil.[2] *M. leprae* first attacks the peripheral nerves where it can then attack the skin, upper respiratory tract mucosa, and tends to cause defects, especially in the organs of the hands and feet. Most of the manifestations of leprosy are asymptomatic, with clinical signs and symptoms in leprosy patients usually seen after five years incubation periods. Leprosy sufferers begin to experience white and red spots on the skin, a feeling of tingling, and organ dysfunction.[1]

Most of the leprosy control still focused on clinical leprosy following the recommendations given by WHO. Multi-Drug Therapy (MDT) in Indonesia has existed since 1980.[3,4] Healthy individuals have a natural immune system capable of fighting *M. leprae* infection, and only 15% of individuals with a weak immune system may become infected with *M. leprae*.[1] This is considered as subclinical leprosy in individuals who look healthy without any clinical signs and symptoms of leprosy but have high levels of specific antibodies against leprosy bacilli. Subclinical leprosy allows the development of clinical leprosy within 2-10 years later. Poor management of subclinical leprosy cases can potentially become a source of transmission and disability.[3–7] Therefore, sensitive and specific early detection tool needs to be able to control the transmission of *M. leprae*.

In addition to the presence of clinical symptoms, detections of antibody levels through the Enzyme-Linked Immuno-Sorbent Assay (ELISA) can show the activity and classification of the patient's current or previous infection. The ELISA test was introduced by Brett et al., in the 1980s.[8] ELISA is a well-based diagnostic test especially used in the study of immunology, that measures the concentration of antigens and antibodies in a sample through enzymes as markers. The ELISA test can detect the presence of Anti Phenolic Glycolipid-1 (PGL-1),specific for *M. leprae*.[4] Anti-PGL-1 antigen levels in blood serum can represent the level of antibody response given by the body.[4,6,7,9] Presence Anti PGL-1 is one of the biomarkers in identifying patients who are at higher risk of experiencing a reaction before the appearance of clinical manifestations of leprosy so that it can determine the best case management approach in preventing further disability and disease transmission as early as possible, especially in endemic areas.[6,9,10] Epidemiologically, 15% of leprosy cases in endemic areas are in the group experiencing a subclinical infection.[11] Research by Iswahyudi and Sujagat showed that 29.5% of subclinical leprosy cases in children were detected through ELISA antibody measurements.[12,13] Besides that, antibody measurements using for follow-up evaluation of treatment, which was accompanied by a decrease in circulating antibody titers.[14]

In general, the ELISA test consists of the stages of coating, blocking, carrying out several items of washing, incubation, carrying out several washing processes again, staining, stopping the staining process, and finally reading the optical density (OD) with a spectrophotometer. OD will be converted into antibody levels in units per milliliter by the Biolise software. Classifications of the antibody threshold value are determined based on the percentage of 80-90% of the lowest antibody level results.[14] The cut-off value of IgM Anti PGL-1 is 605 u/ml, and the cut-off value of IgG Anti PGL-1 is 615 u/ml what it has used as the classifications of a

patient's results are included in leprosy seropositive or seronegative. The ELISA serological test can detect antigen and antibody concentrations at the level of 0.01 µg/ml with high specificity that can correctly identify people who do not have the disease at an ability level above 80%.[15]

MATERIAL AND METHODS

Materials

The tools used to research the detection of *M. leprae* by the ELISA test using blood serum samples include Immunowash (BIORAD model 1575), microplates, micropipettes, and tips with a size of 50 µl – 1000 µl, vortex, spindown, Biolise/X-read, Eppendorf tube of 1.5 µl, and incubation contacts. The materials used include 0.5 ml blood serum in a capillary tube, NT-P-BSA, Coating Buffer pH 9.6 ($NaHCO_3$, Na_2CO_3, NaN_3, Distilled Water), Phosphate Buffered Saline (Na_2HPO_4, KH_2PO_4, NaCl, KCl, Distilled Water), PBST (PBS, Tween20), Blocking Buffer (1% Skimmed Milk, NaN_3, PBS), Washing Buffer (PBST, Distilled Water), Stopping Solution (H_2SO_4 pk, Distilled Water), and Substrate Solution (Citrate-phosphate buffer, Ortho Phenylene Diamine, 30% H_2O_2). NT-P-BSA is a synthetic form of the anti-PGL-1 *M. leprae* whose manufacture and distribution is regulated by T. Fujiwara from Institute for Natural Science, Nara University.

Methods

Sample. This research is a descriptive study with a cross-sectional design. Five patient blood samples have been analyzed for IgM and IgG anti-PGL-1 levels by ELISA at the Leprosy Laboratory, Institute of Tropical Disease, Airlangga University, Surabaya for identifying patients with seropositive or seronegative status of leprosy. The five blood samples of the patients have been coded in the sample name IF, DT, SR, AS, and SO. Blood samples had centrifuged to separate serum to use in the ELISA test. The blood serum separation has transferred into a new 0.5 ml tube. Sampling was carried out using non-probability purposive sampling. The inclusion criteria in this study were a sample of suspected leprosy patients. Meanwhile, exclusion criteria included patients with other chronic infectious diseases, patients with other acute infections, and or patients with resistance to anti-leprosy drugs.

Antigens. Blood serum was analyzed using Indirect ELISA quantitatively using synthetic anti-PGL-1 (NT-P-BSA).

Anti-PGL-1 antibody assay. This study used the indirect ELISA method quantitatively. The ELISA method had described in the work instructions for serological examination of leprosy (ELISA Anti-PGL-1).[16] Each serum dilution was tested in duplicate on an Anti-PGL-1-coated microplate and a control microplate without antigen. The microplate was coated by 50 µl NT-P-BSA and 50 µl Coating Buffer pH 9.6 according to the specified scheme for overnight incubation at 4^0C. The microplate has washed with PBST solution, then coated again with 200 µl of Blocking Buffer, and then incubated at 37°C for one hour. The blocking buffer was discarded, and 50 µl of serum was added to the microplate. Fifty µl of serum volume was diluted 1:300 in Dilution Buffer. Each standard and blank well was diluted in five different concentration ratios (0, 5, 10, 15, 20). Samples were tested in duplicate and incubated for one hour at 37°C. The microplate was washed, and 50 µl of secondary antibody IgM and IgG conjugated with the enzyme was added, which was diluted 1:2000 in Dilution Buffer. Microplate has incubated at 37°C for one hour. The microplates were washed and then stained with 100 µl of the substrate containing Ortho Phenylene Diamine (OPD) and 30% Hydrogen Peroxidase in Citrate-Phosphate Buffer. The microplate was incubated until a yellow or orange color developed. The staining reaction stopped after 10-30 minutes by adding 100 µl of Stop Solution containing

sulfuric acid. The result of the ELISA test has read by using ELISA Reader at the wavelength of 492 nm/620 nm.

Statistics. ELISA results in Optical Density (OD) are presented in a standard curve to determine the relationship between concentration and absorbance. The regression line and the correlation coefficient in a standard curve had depicted in a 4-parameter regression. OD results can be converted to units/ml using the Biolise software to determine the levels of detected antibodies. The results had expressed as the average value of the antibody titer for each sample obtained based on the concentration and dilution factor in each sample well. Antibody levels in u/ml were classified using an IgM cut-off value of <605 u/ml as seronegative, 605–1000 u/ml as low seropositive, or >1000 u/ml as high seropositive, and an IgG cut-off value of <630 u/ml as seronegative, 630-1000 u/ml as low seropositive, or >1000 u/ml as high seropositive leprosy. The accuracy of the ELISA test in converting the OD results into concentrations levels (units/ml) is shown in the results of the percentage coefficient of variation (%CV) by Biolise software.

RESULTS AND DISCUSSION

Results Optical Density

The microplate schematic consisting of IgM and IgG Anti-PGL-1 schemes are analyzed simultaneously.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.066	0.009	0.009	1.299	0.149	0.099	0.039	0.029	0.034	0.418	0.168	0.488
B	0.330	0.328	0.318	1.231	0.133	0.097	0.330	0.345	0.346	0.454	0.170	0.477
C	0.771	0.770	0.742	0.100	0.100	0.088	0.502	0.512	0.463	0.197	0.174	0.489
D	0.870	0.868	0.811	0.096	0.101	0.098	0.767	0.779	0.739	0.195	0.157	0.529
E	1.113	1.085	0.981	0.442	0.069		0.923	0.940	0.883	0.203	0.381	
F	0.064	0.134	0.145	0.455	0.067		0.171	0.204	0.231	0.206	0.370	
G	0.075	0.128	0.149	0.138	0.057		0.150	0.204	0.236	0.217	0.412	
H	0.043	0.204	0.205	0.135	0.067		0.037	0.305	0.305	0.217	0.415	

Figure 1. Optical Density Result

All rows in the first to sixth columns are IgM schemes, while the seventh to twelfth columns are IgG schemes. The scheme consists of the standard in an orange well, the blank in a blue well, and the sample in a white well. Each sample has been analyzed in duplicate on both antigen and antibody coatings. Each standard and blank well has five different concentration ratios (0, 5, 10, 15, 20). The OD results are different for each sample well, standard, and blank.

The OD results on standard wells and well blanks were lower than the OD results on sample wells. The lowest OD results for standards and blanks were the standard and the blanks wells with a concentration of zero, namely 0.009/0.029 in standard wells and 0.043/0.037 in well blanks. The lowest OD result in the SO sample well was 0.057, and the highest in the IF sample well was 1.299.

Standard Curve

The standard curve consists of the X-axis showing the concentration level and the Y-axis showing the absorbance value. The X-axis stretches horizontally, and the Y-axis stretches vertically on the standard curve. The regression equation on the standard curve is $y = (a-d) / (1+(x/c)^b) + b$.

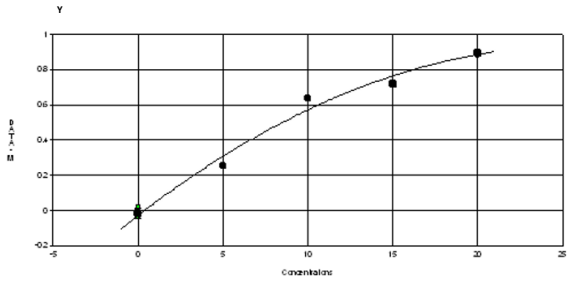

Figure 2. IgM PGL-1 Curve Standard

On the IgM standard curve with the equation $y = 0.001422x2+0.0745x–0.02879$ with an R^2 of 0.9830.

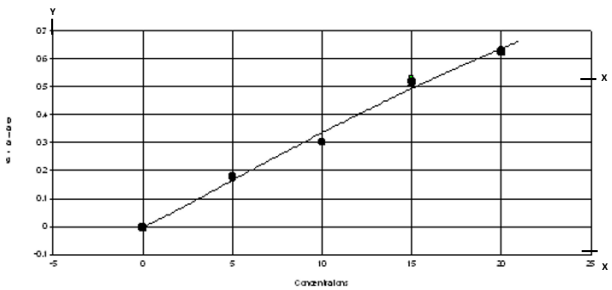

Figure 3. IgG PGL-1 Curve Standard

On the IgG standard curve with the equation y = -2.6753693 / (3.675+(x/56.44)$^{1.123}$) with an R^2 of 0.9920.

IgM PGL-1 and IgG PGL-1 Antibody Titer Levels

IgM and IgG anti-PGL-1 in ELISA results are displayed in the form of a distribution which is classified based on level seropositive or seronegative.

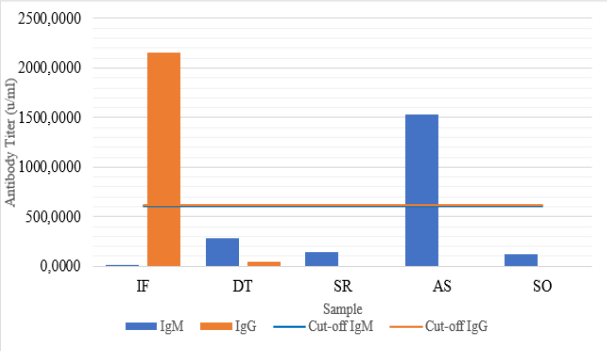

Figure 4. Distributions of Antibody Titer Levels

The results of the ELISA serology showed that out of all five samples tested, three blood samples showed seronegative leprosy, while the other two showed high seropositive leprosy in IgM anti-PGL-1 and IgG anti-PGL-1 antibody titers.

ELISA Test Method Accuracy

The accuracy of the ELISA test in converting the OD results into concentrations levels (units/ml) is shown in the results of the percentage coefficient of variation (%CV).

Table 1. Percentage CV of ELISA Test

Stand.	Conc.	%CV	
		IgM	IgG
1	0	0	0
2	5	2.0176	3.8769
3	10	0.1045	1.6557
4	15	0.2077	2.9767
5	20	0	1.5772

Based on the percentage coefficient of variation of IgM and IgG standard solutions (<10%) shows that the ELISA test method used has high accuracy.[16]

Discussion

Early detection of *M. leprae* is important in preventing further disability and breaking the transmission chain, especially in cases of subclinical leprosy which often go undetected.[3,5–7] Phenolic Glycolipid (PGL-1) is the dominant component of carbohydrates and lipids in *M. leprae*. This antigen is found in all tissues infected with *M. leprae* and can survive until the tissue dies.[17] Although it can stimulate the body's immunity, PGL-1 is not enough against *M. leprae*. Therefore, the ELISA test had carried out by measuring antibody titers in blood serum against anti-PGL-1 as a specific antigen for *M. leprae* and was used to detect leprosy early.[18]

The reading of the ELISA test results is in the form of Optical Density in Figure 1. Each well has an optical density (OD) with different values and concentrations. These differences have been influenced by the number of antigen and antibody bonds formed using enzyme-labeled secondary antibodies as markers. Antigens with non-specific antibodies or vice versa will not form specific bonds. In the microplate schematic, the positions of rows A and the first until third column or the seventh until ninth column do not show any antigen and antibody reactions where the wells only contain antigens without antibody titers. The position of rows F, G, and H, and the first until third column or the seventh until ninth column only contains antibodies without antigens so that reactions with specific antigen-antibody bonds do not occur. In line with the results, the lowest OD results are well standard and well blank. The position of rows B, C, D, and E, and the first until third column or the seventh until ninth column show a reaction in which antigen, antibody, and enzyme-labeled secondary antibodies have formed in the wells as markers. In line with the results of Optical Density in Figure 1., the well samples had

higher OD results than well standard wells and well blanks without a specific binding reaction between antigen and antibody.

Quantitative analysis of ELISA test results also compares the concentrations of antigens or antibodies in the sample by using the standard curve. OD results have been converted into a standard curve to determine whether there was an effect between the OD or absorbance value and the concentration. The concentration level has been obtained by the dilution factor of the standard solution, and the absorbance value has been obtained by the standard solution concentration. Regression standard curves for the corresponding absorbance values and concentrations of reference standard concentrations have shown at the R^2.[19] An R^2 curve has considered having the goodness of fit when the R^2 value is over 0.99.[20,21] R^2 value in the Figure 2. IgM Anti-PGL-1 standard is 0.9830, meaning the concentration value of the solution determined had influenced by the absorbance value of 98%. This value is considered insignificant to the relationship between the absorbance value and the concentration of the reference standard determined. R^2 value in the Figure 3. IgG Anti-PGL-1 standard is 0.9920, meaning the concentration value of the solution determined had influenced by the absorbance value of 99%. This value is considered significant to the relationship between the absorbance value and the concentration of the reference standard determined. The value of R^2 >0.99 shows a significant relationship between the absorbance value and concentration.[20,21] Thus, the interpretation of optical density results can be predicted accurately.

Each sample was analyzed in duplicate so that the sample antibody titer value was the average result of the sum of each multiplication between the concentration and the diluent factor in each sample well. Antibody titer value (unit/ml) is the result of conversion from OD using Biolise software to detect antibody titer values related to the presence or absence of *M. leprae* in the human body. When *M. leprae* manages to enter the body, Anti-PGL-1 will stimulate the immune system to produce antibodies against *M. leprae* has been recognized as foreign cells.[6] The IgM and IgG antibodies have produced by T lymphocyte cells that stimulate interferon and interleukin in activating the cellular immune response. T cells control the proliferation of B cells to produce IgM and IgG antibodies.[22]

When first infected, Anti-PGL-1 can stimulate the body's immune system to produce IgM antibodies. The IgM antibodies can appear after 1-2 weeks to 2-3 months after exposure to *M. leprae*.[23] The IgM antibodies to anti-PGL-1 indicate the patient has an acute immune response or is suffering from leprosy.[22,23] IgM Anti-PGL-1 antibody titer level can represent the bacterial load of *M. leprae* in the human body.[24,25] Patients whose seropositive IgM anti-PGL-1 titers do not correlate with clinical symptoms of leprosy indicate the possibility of subclinical infection.[24,25] This is due to the absence of likely cross-reactions between Anti-PGL-1 and other mycobacteria. In Figure 4. IgM and IgG PGL-1 Antibody Titer Levels, AS patients showed high seropositive results of IgM anti-PGL-1 titers above 1000 units/ml. The results of the ELISA serological examination in Figure 4. showed that the IgM anti-PGL-1 antibody titer in AS patients were 1528.1 units/ml, and the IgG anti-PGL-1 was 0 units/ml. The high titers of IgM antibody to anti-PGL-1 correlate with ongoing or recent infection in AS individuals. The IgM anti-PGL-1 antibody titers that exceed the threshold may indicate that leprosy infection in acute AS patients has a higher risk of developing leprosy manifestations in the next few years.[6,7,26] The association between anti-PGL-1 IgM positivity and the development of leprosy cannot imply that the anti-PGL-1

results reflect a recent infection with *M. leprae*. Laboratory tests on experimental animals showed that the response of anti-PGL-1 antibodies in leprosy patients has positively correlated with the bacillary burden of *M. leprae* in the body.[27] This correlation may indicate that anti-PGL-1 positive, healthy contacts have been exposed to *M. leprae* and have a high bacillary burden. This hypothesis is consistent with the fact that IgM antibodies exhibit an early response to infection. Nevertheless, animal models have shown that IgM antibodies can persist and participate in long-term protection against obligate intracellular bacteria.[28] In the research with animal models, IgM anti-PGL-I is still present at higher levels many years after infection.[29] These data corroborate the finding that IgM antibodies in human leprosy not only indicate recent infections. Interpretation of anti-PGL-1 IgM titer levels also requires the results of an anti-PGL-1 IgG examination. The tendency for low IgM anti-PGL-1 antibody levels to remain positive may be related to bacillary persistence. Persistence is possible due to the PGL-1 antigen can still stimulate the low antibody response in the absence of living *M. leprae* bacilli.[30,31]

Anti-PGL-1 also stimulates IgG antibodies which indicate an immune response against chronic disease. The IgG anti-PGL-1 antibody titer using as a biomarker that can detect and predict *M. leprae* infection retrospectively.[32,33] Exposure history of patients who don't suffer from leprosy, but whose been exposed to *M. leprae* before is indicated by the level of IgG antibody titer.[22] In Figure 4. IgM and IgG PGL-1 Antibody Titer Levels, IF patients showed high seropositive results of IgG anti-PGL-1 titers above 1000 units/ml. The results of the ELISA serological examination in Figure 4. showed that the titer of IgM anti-PGL-1 antibodies in IF patients were 0.7081 units/ml and IgG anti-PGL-1 was 2153.3 units/ml. The high IgG titer indicates that the IF patients had been exposed to *M. leprae* or have been in contact with leprosy patients for months or years. The bacteria load of *M. leprae* in the body can slowly decrease with efficacy patient treatment.[27] Although IgG antibody titer levels are still high, IgM anti-PGL-1 antibody titers that have been low during the first year of treatment can be used for treatment efficacy evaluation. Research by Touw, Bach, and Khadge showed a significant decrease in IgM anti-PGL-1 antibody titers compared to IgG anti-PGL-1 was observed within one year after treatment.[34]

The study by Douglas et al., (2004) showed that individuals who were anti-PGL-1 seronegative had 75 times smaller risk than seropositive contacts to infected leprosy.[35] In Figure 4. IgM and IgG PGL-1 Antibody Titer Levels, the results of anti-PGL-1 seronegative antibodies have shown in DT, SR, and SO patients. The result of seronegative leprosy had shown by the cut-off IgM anti-PGL-1 titer below 605 units/ml and the IgG anti-PGL-1 titer below 630 units/ml. The results of the ELISA serological examination in Figure 4. showed that the titer of IgM anti-PGL-1 antibodies in DT patients was 285.62 units/ml, and IgG anti-PGL-1 was 49,955 units/ml. The results of the ELISA serological examination in Figure 4. showed that the titer of IgM anti-PGL-1 antibodies in SR patients was 137.92 units/ml, and IgG anti-PGL-1 was 0 units/ml. The results of the ELISA serological examination in Figure 4. showed that the titer of IgM anti-PGL-1 antibodies in SO patients was 121.46 units/ml, and IgG anti-PGL-1 was 0 units/ml.

Quantitative results of the ELISA test can assess seroprevalence and monitor changes in transmission time and place of transmission of *M. leprae*, especially in endemic areas at higher accuracy levels.[36] The accuracy of this ELISA test method has been assessed by the percentage coefficient of variation (%CV) of the solution standard with a known concentration (5 different concentrations – 0, 5, 10, 15, 20).[16] The conversion of Optical Density (OD) by Biolise software into a standard solution whose concentration is known can show the percentage coefficient of variation.[37] In Table 1. showed that the first

standard solution has a %CV IgM Anti-PGL-1 titer and IgG Anti-PGL-1 titer of 0%. In Table 1. showed that the second standard solution has a %CV Anti-PGL-1 titer of 2.0176% and an Anti-PGL-1 IgG titer of 3.8769%. In Table 1. showed that the third standard solution has a %CV Anti-PGL-1 titer of 0.1045% and an Anti-PGL-1 IgG titer of 1.6557%. In Table 1. showed that the fourth standard solution has a %CV Anti-PGL-1 titer of 0.2077% and an Anti-PGL-1 IgG titer of 2.9767%. In Table 1. showed that the fifth standard solution has a % CV IgM Anti-PGL-1 titer of 0% and an Anti-PGL-1 IgG titer of 1.5772%. There is no consensus for this accuracy determination. Most of the studies conducted use conventional ELISAs that are self-made, and due to other factors such as sampling, sample delivery, or sample preservation can cause variations in the accuracy of the assay.[38] Based on the percentage coefficient of variation, it shows that the conversion of absorption rates into units/ml has high accuracy at the %CV at the five concentration values being very small (below 10%). Research by Faizo et al. shows that the ELISA antibody detection protocol for SARS-CoV-2 uses the same consensus where %CV <10% indicates a high level of test accuracy.[39]

CONCLUSIONS

IgM and IgG Anti PGL-1 antibody titers by ELISA as one of the parameters can identify patients at higher risk of leprosy. The study results showed that a significant portion of patients has high seropositive leprosy with high accuracy. In the future, this test has been expected for early diagnosis of leprosy, especially in endemic areas so that leprosy does not develop into a transmission and further disability.

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CONFLICT OF INTEREST

All authors have no conflict of interest.

AUTHOR CONTRIBUTION

Designed the study, collected and analyzed the data, and also prepared the manuscript: SPKA and LNY. A scientific adviser in the field of leprosy and laboratory: DA, II, PAW, MD, RW, and CRSP. All authors read and approved the final manuscript.

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