Immunoglobulin AMG Anti Natural Disaccharide Octyl - Leprosy IDRI Diagnostic (NDO-LID) Serologic Test for Leprosy Diagnosis: A Pilot Study

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Abstract

Leprosy remains a public health problem in Indonesia. Diagnostic tools have been developed in order to aid early diagnosis and prompt treatment. Phenolic glycolipid (PGL)-1 has been considered as a good candidate for leprosy diagnosis but has been found to have several limitations. More recently, the conjugation between natural disaccharide octyl (NDO) and leprosy IDRI diagnostic (LID)-1, known as NDO-LID, shows great promise because of its high specificity and sensitivity and its ability to detect leprosy before any clinical signs are present. The test incorporates the detection of IgM antibodies towards NDO and IgG antibodies towards LID-1. This study aimed to show the profile of IgM, IgG, and IgAMG antibody titers against NDO-LID to further discover its diagnostic potential. Sera from eight new leprosy patients from Surabaya, Indonesia were evaluated using ELISA detecting levels of IgG, IgM and IgAMG antibodies against NDO-LID antigen. Skin slit biopsy was also taken for smear and histopathology test. This study shows that the titer levels IgAMG anti NDO-LID were consistent with the results from smear and were consistently higher compared to IgM or IgG titer alone. IgAMG might have the potential to improve the sensitivity of NDO-LID serologic tests but further investigation is needed.

Introduction

Despite the World Health Organization's efforts to eradicate it, leprosy remains as a serious health problem in some countries in the world such as India, Brazil and Indonesia.[1] Leprosy is a chronic disease caused by *Mycobacterium leprae (M. leprae)*. It primarily affects the skin and peripheral nerves. Previously, the diagnosis of leprosy was based on its clinical manifestation, bacilloscopy and histopathology; diagnosis relied on the observation of the wide range of manifestation it presents. Tuberculoid leprosy is characterized by few skin lesions, low or absent bacteriologic index, strong *M. leprae*-specific Th-1 type cell mediated immunity and low or absent specific antibodies., Lepromatous leprosy, on the other hand, is characterized by multiple skin lessions, high BI, Th2-type immunity with abundant antibody production and low *M. leprae*-specific CMI.[2] Recently, researchers have been developing diagnostic tools which aim to be able to detect *M. leprae* infection before any clinical symptoms are visible (subclinical leprosy).[3] These tools include immunohistochemistry, serologic and PCR tests. Currently, research regarding leprosy is prioritizing the identification of molecular markers specific to *M. leprae* for early diagnosis and treatment. Serologic tests are inexpensive, among the easiest to perform and easily applicable in the field. One of the most commonly used antigens is phenolic glycolipid I (PGL-1) which was discovered in the early 1980s. The level of immunoglobulin M (IgM) antibody towards PGL-1 reflects bacterial load which makes it predominant in multibacillary type leprosy - with seropositivity ranging from 80-100% - while scarce in paucibacillary type leprosy, with seropositivity ranging from 30-60%).[4] Some studies also suggest that one of the drawbacks of the tests involving PGL-1 is its high false-positive results in endemic areas. Another problem of PGL-1 antibodies is that they remain in tissues for a long time, which stimulates the production of IgM antibodies in the absence of viable bacilli. Therefore, their presence does not always mean active disease.[5] More recently, the Infectious Disease Research Institute developed a fusion that incorporated ML0405 and ML2331, (*M. leprae* protein antigens found in the nuclei) which is now known as Leprosy IDRI Diagnostic 1 (LID-1); this is a chimeric protein with better sensitivity than the original proteins alone.[6] A study by Duthie and friends demonstrated that LID-1 can be used to diagnose MB leprosy patients regardless of geographic location, can be

used for the diagnosis of some PB patients and can provide a clear leprosy diagnosis before the onset of clinical symptoms; some studies have also shown that anti-LID-1 antibody levels begin to increase markedly as early as one year prior to clinical diagnosis. When conjugated with the natural disaccharide octyl (NDO), it forms a NDO-LID 1 conjugate which is able to identify patients early in the spectrum of leprosy and is more sensitive towards paucibacillary type leprosy.[7] NDO-LID also provides great differentiation of positive and negative results. The NDO-LID rapid diagnostic test generally involves the detection of IgM antibodies against NDO and IgG antibodies against the LID-1. NDO-LID test is considered bet-

ter than the PGL-1 from other researches, which might be due to the presence of one glycolipid and two protein antigens which are capable of detecting both IgM and IgG antibodies. This pilot study aimed to evaluate the profile of levels IgG, IgM and a combination of IgG, IgM and IgA[8] antibodies towards NDO-LID test.

Materials and methods

Study Site and Participants

New leprosy patients who had yet to undergo any treatment from the Dermatovenereology Outpatient Clinic of Dr. Soetomo Teaching Hospital, Surabaya were enlisted for this pilot study during October to November 2017. They were then selected based on the inclusion and exclusion criteria. Inclusion criteria were: new leprosy patients who were willing to participate in the research and who had not yet received multidrug therapy for leprosy (MDTL) and patients who had been receiving MDTL for less than three months. Exclusion criteria were: leprosy patients who had been receiving MDTL for more than three months or who were currently pregnant or breastfeeding. Blood samples were collected following local ethics committee approval from Dr. Soetomo General Hospital and after participants signed their informed consent forms. Patients were fully characterized on the Ridley-Jopling scale by slit skin smear and biopsy.

Serum and skin slit samples was taken from a total of eight patients diagnosed with multibacillary type leprosy. Sera were prepared by centrifugation, which was then tested within two hours of collection. Three different NDO-LID tests were performed in the Institute of Tropical Diseases, Surabaya, each detecting IgM, IgG and a combination between IgAMG antibodies against NDO-LID respectively.

Skin Slit Smear and Histopathological Examination

Skin slit smear samples were collected after taking four horizontal scrapes of tissue from an incision (5mm long and 2mm deep) made with a sterile surgical scalpel No.15 on the left and right earlobes and skin lesions without any contamination of blood along the skin-slit part. The tissue material was placed in a 700ml of 70% ethanol and mixed well in a micro-centrifuge tube. These samples were then sent to the Institute of Tropical Diseases, Surabaya for histopathological staining and examination according to procedure.

Quantitative Indirect ELISA Measurement

Blood samples were earlier withdrawn from patients by antecubital venipuncture and collected in an EDTA vial. Samples were then sent to the Institute of Tropical Diseases, Surabaya. Microplates were coated with 50 mL of coating buffer and NDO-LID working solution and incubated for one hour at 37°C. Then said microplate was washed three times with washing buffer (PBST). 200 mL of blocking buffer (PBS-T 1% BSA) was put inside the microplate and then incubated for another hour at 37°C. The blocking buffer was removed, 50ml of

Table 1. Subject characters according to histopathological examination.

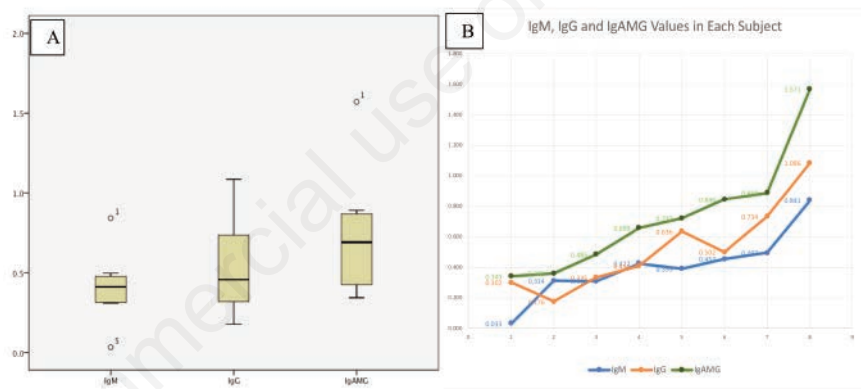| Subject	1	2	3	4	5	6	7	8
MH Clinical	BL	BL	BL	BL	BL	LL	LL	LL
HistoPA MH	BL	BL	BL	BL	BL	LL	LL	LL
BI	3	3	3	3	3	3	3	3
MI	3	3	0	7	3	3	3	7
PGL-1	345	3193	2805	6124	3128	5081	25091	62594

Figure 1. A) Haematoxylin and Eosin staining seen under 100x magnification, showing the infiltration of lymphocytes (shown with black arrow) and grenz zone (a narrow area of the papillary dermis uninvolved by underlying pathology) (shown with blue arrow) and B) Fite Faraco staining seen under 450x magnification, showing abundant acid-fast bacilli inside foamy macrophages (shown with blue arrow).
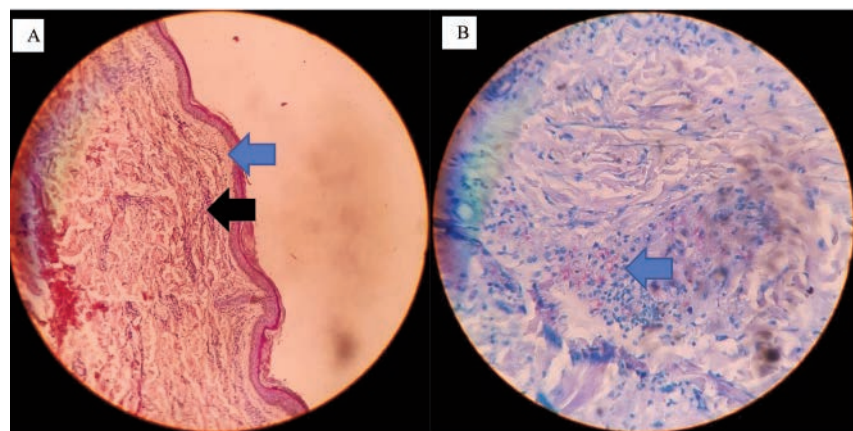

Figure 2. (A) Illustration of the descriptive data of the study showing minimum, maximum and mean values of IgM anti NDO-LID, IgG anti NDO-LID and IgAMG anti NDO-LID respectively. (B) Illustration shows the comparison between titer levels of IgM, IgG and IgAMG anti NDO-LID in each subject respectively.

serum was put in the microplate, incubated for onehour in 37°C, then washed three times. Plates were incubated for one hour at room temperature with IgG/IgM/IgAMG for NDO-LID serology. 100 ml of Substrate solution (peroxide color substrate) was then added to the microplate until it generated a yellow / orange color which was then stopped by the addition of 100 ml H_2SO_4 after 10-30 minutes. The optical density was then calculated using ELISA reader (Biolyse/Xread). Measurement was done according to procedure by experts in the Institute of Tropical Diseases, Surabaya.

Results

A total of eight patients agreed to take part in this study. All of them were classified as MB type leprosy. Clinically, 62.5% (five patients) of these were classified as BL type and 37.5% (3 patients) were LL type. Histopathological examinations confirmed this result and showed that the clinical diagnosis of these patients was accurate. All the samples showed 3+ BI (1-10 bacilli were found on each microscope field) while morphological index varied: one subject had 0% MI, five had 3% and 2 subjects had 7%. A microscopical field view of the histopathological specimen stained with Haematoxylin and Eosin could be seen in Figure 1.

Figure 2 below represents a boxplot of the results of IgM, IgG and IgAMG titer results of each sample. From the IgM plot we could see two outlier data which represents the minimum (0.033 OD) and maximum of (0.841 OD), with a mean of 0.409 ± 0.225 SD. In the IgG plot, we have a minimum value of 0.176 OD, maximum value of 1.086 OD with a mean of 0.535 OD ± 0.298 SD. The IgAMG plot shows a minimum value of 0.343 OD, a maximum value of 1.571 (presented as outlier data), with a mean value of 0.396 OD ± 0.395 (Table 1).

Discussion

Early diagnosis and prompt treatment remain paramount in terms of eradicating leprosy. The ideal test should be able to detect all individuals with leprosy and those without; in other words, having no false positive or positive results and having 100% sensitivity and 100% specificity respectively.[9] Several approaches have been made: earlier methods considered as the "gold standard" of leprosy testing were the leprosy skin slit smear and the histopathological test. But these had some flaws i.e.

they were unable able to detect leprosy before a certain number of bacilli were present (10^4) and had their own technical difficulties, which sometimes produced unreliable results. In a study by Kumar et al., PGL-1 antibody assay was found to have 77% sensitivity and 93% specificity in MB patients;[10] it was also found that it could be used in detecting leprosy in household contacts.[6] NDO-LID antibody assay has shown better results and with a better predictive value than PGL-1. It could be seen that subjects on the higher spectrum of leprosy (LL) showed higher titers than the ones in the lower spectrum of leprosy (BL). This supports previous studies that state IgG and IgM titers correspond to the subject's bacterial load where subjects with a lower spectrum are suggested to have lower bacterial load than in the latter.

Kumar et al., in their research, found that antigens in the cytosol and cell walls raised IgG antibody titers while cell membranes had major numbers of antigens to raise IgA and IgM antibodies, suggesting that IgA should also play a part in the sensitivity of the test itself.[7] Hence, in this study, a combination of IgA, IgM and IgG level were measured. It is apparent that the IgAMG anti NDO-LID titer level is constantly higher than that of the IgG and the IgM anti NDO-LID alone. In the results section, it could also be seen that Ig AMG titers have higher mean values of titers compared to IgM and IgG. The trends of these titers correspond well with the clinical and histopathologic features of each subject, which shows that the combination is on track with IgM and IgG anti NDO-LID. Although not yet conclusive, this could suggest that IgAMG has a higher sensitivity than the other due to the interaction towards more antigens compared to IgM or IgG alone. This pilot study could indicate that leprosy serologic tests detecting IgAMG are more sensitive, more suitable and a better serologic test for leprosy.

Duthie et al., in 2007 wrote in their research that LID-1 is proven to be a good tool for the early diagnosis of leprosy.[11] It is said that it could give a six-to-eight months' time benefit when compared to clinical diagnosis. It might be possible that the utilization of the detection of IgAMG towards anti-NDO-LID, as was done in this study, with its suggested higher sensitivity compared to IgG and IgM alone, might improve the results of said study. This would give a further advantage towards the prompt treatment of leprosy, possibly affecting transmission rates by reducing the number of individuals who develop large bacterial burdens, as explained by Duthie et al.[12] This study focused mainly on MB patients. However, with the suggest-

ed increased sensitivity of this test, it might also be possible to expand this study towards assessing the sensitivity in PB patients, since it is known that LID-1 and NDO-LID 1's advantages are their ability to detect leprosy in PB type patients. With the limitations of this study being that the sample size was too small, we suggest that a similar research be done using a bigger and more representative sample which aims to assess the viability of the detection of IgAMG anti NDO-LID for leprosy diagnosis.

Conclusions

Although further investigation is needed, this pilot study shows that the IgAMG anti NDO-LID levels are consistently higher than IgM and IgG levels alone. It suggests that the detection of IgAMG might improve the sensitivity of NDO-LID for leprosy diagnostic tests.

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