

# A simpler diagnostic method using blood collection on filter paper to determine anti-natural octyl disaccharide-leprosy infectious disease research institute diagnostic in household contacts of leprosy patients

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## Abstract

The high prevalence among children shows continued transmission from adult and lack of disease control by the health system. Detection of anti-NDO-LID-1 has been known to be more effective to predict the development of leprosy in household contacts than just detecting PGL-1 or LID-1 alone. However, serodiagnosis is not available in poorer-settings area and the procedure (venepuncture) is still inconvenient to some people. These problems can be solved by using finger-prick blood sample on filter paper. This study aims to prove the effectiveness of using capillary blood samples on filter paper to detect the positivity of Ig G antibody against NDO-LID-1 antigen in asymptomatic household contacts. Seventeen samples of capillary blood on filter paper and sera were tested for IgG anti-NDO-LID-1 using ELISA. There was no significant difference between IgG level from filter paper and serum ( $p=0.754$ ) and there was also a strong positive correlation ( $R=0.906$ ) between the two procedures. These findings show that the use of filter paper and NDO-LID-1 is worthy of further investigations, especially for those with lower bacillary load or contacts of leprosy patients.

## Introduction

The strong commitment of national governments, availability of multidrug treatment, and other efforts have resulted in a significant milestone in the elimination of leprosy as a public health problem when analysed year to year. Nonetheless, new cases continue to occur and new important problems should be noticed. One of the main targets at a global level to be achieved by 2020 as stated by WHO is a reduction to zero cases of new grade 2 disabilities (G2D) child cases. Indonesia, however, remains one of the three countries (along with India and Brazil) that reported >10 000 new cases in 2016 and ranks second in the number of children among the new cases.<sup>1</sup> Leprosy is a chronic infectious disease; its prevalence among children shows continued transmission from adult and lack of disease control by the health system.<sup>2</sup> This circumstance reveals the need to continue develop a method that is accurate, applicable to be performed without significant struggle to patients of any age, can be done even in a modest infrastructure and more desirably can predict the development of leprosy in asymptomatic prone individuals (household contacts/HHC).

According to the national guideline, diagnosis of leprosy in Indonesia is still referring to clinical sign and symptoms findings. This method is not efficient and can cause a delay of diagnosis due to the requirement of obvious signs or symptoms that usually are only present in the late/advanced spectrum of the disease. Serological test for diagnosis of leprosy is one of the tools that can diagnose or even predict the presence of *Mycobacterium leprae* (*M. leprae*) in either patient with clinical leprosy or HHC that are prone and likely to be infected but didn't show any signs or symptoms.<sup>3-6</sup> Detection of Immunoglobulin M (IgM) antibodies against phenolic glycolipid (PGL-1), a cell wall component of *M. leprae*, through enzyme-linked immunoabsorbent assay (ELISA) has been widely used and proven can support the diagnosis of leprosy.<sup>7</sup> However, the positivity of anti-PGL-1 is limited to patients with high bacillary load (multibacillary/MB),<sup>6</sup> therefore this method lacks the capability of early detection. More recently, there has been finding of novel antigenic proteins from *M. leprae* nucleic acid (ML0405 and ML2331). These antigens are shown to be highly specific to *M. leprae* and significantly reacting to Immunoglobulin G (IgG) antibodies of patients with lower bacillary load (paucibacillary/PB).<sup>8</sup> The fusion of these proteins, Leprosy Infectious Disease

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Research Institute Diagnostic-1 (LID-1) has been studied and proven can improve the sensitivity of serological test throughout the clinical spectrum.<sup>3-5</sup> Moreover, subsequent studies also found that the conjugation of LID-1 and PGL-1 epitopes in ELISA could further improve sensitivity and specificity in a diagnostic test. This conjugate is well known as Natural Octyl Disaccharide-Leprosy IDRI Diagnostic-1 (NDO-LID-1). Their positivity in both MB patients and non-patients support a role to diagnose the entire spectrum of leprosy, including people at greater risk of developing clinical disease that needs regular monitoring.<sup>3,4</sup>

The need to regularly monitor the progression of the disease also raises another problem to poorer-settings area since it lacks diagnostic tools like ELISA to perform a serological test. To improve this condition, a good referral system and as stated before, a patient-friendly procedure is important. To date, the commonly used method in diagnosing leprosy is quite invasive. The collecting of sera from the patients for serodiagnosis are mostly done with venipuncture. This kind of sample has some challenges since it needs special treatment to be appropriately stable for further testing in the referred laboratory; such as temperature maintenance, preservatives, and special storage for a quite lengthy period of time (until it is centrifuged for the collection of serum). It is also less convenient to be performed on children and elderly.<sup>9</sup> It has been widely acknowledged that we

don't need a big amount of serum for serological test and many studies diluted the serum from 1:100 until 1:500 of dilution.<sup>6,7,10</sup> In fact, 50  $\mu$ l of serum is ample for diagnosis and it can be obtained from a couple drops of blood.<sup>9</sup> Dried blood spot on filter paper is an agreeable candidate in this condition. The storage could be as simple as a zipper bag (gas impermeable, containing desiccant sachets) and it can be stored in room temperature for up to 14-30 days. There also have been studies that prove it to be equally effective (compared to using serum samples) when situated in various kinds of temperature and length of storage.<sup>11,12</sup> The use of capillary blood samples on filter paper in measuring quantitative IgM for *M. leprae* PGL-1 has also shown significant correlation with serum samples and therefore useful for diagnostic tests.<sup>9</sup> This study aims to prove the effectiveness of using capillary blood samples on filter paper to detect the positivity of IgG antibody against NDO-LID-1 antigen in asymptomatic HHC. Hopefully it can benefit health workers of rural area in referring blood samples of those who need to be diagnosed to central laboratory and therefore widen the screening of leprosy in the area.

## Materials and Methods

### Study site and participants

This study population was comprised of 17 sera and capillary blood samples of HHC of leprosy patients in Dermatology Venereology outpatient clinic of Dr. Soetomo General Hospital. HHC were those individuals who reside or have resided with the patients for at least 5 years. Blood samples were collected following local ethics committee approval from Dr. Soetomo General Hospital and after participants signed their informed consent forms.

### Preparation of serum

Blood (5 mL) was collected with venipuncture (antecubital vein) with proper disinfection prior to the procedure. The serum was obtained by centrifuging 2 ml of the samples. It was then added with preservative and stored at 2-10°C until the ELISA was performed.

### Preparation of filter paper

The skin of the palmar side of the tip distal phalanx of the third or fourth finger of the non-writing hand is cleaned first with a 70% isopropyl alcohol. Capillary blood samples were obtained by using single-use safety lancet and then transferred to a filter paper without touching the surface directly with the fingertip. The filter paper was then

allowed to dry and then inserted to a zipper bag. Drying process was complete when the blood spots had a uniformly dark brownish color and no visible red areas. To elute the dried blood spot, the filter paper was first cut into tiny pieces and then put into a 1.5 ml tube. The tube is then filled with PBS solution and then vortexed for 5 minutes. The samples were then either tested or stored in freezer.

### Quantitative Indirect ELISA Measurement

The 96 well microplates were coated with 50 mL coating buffer or NDO-LID-1 antigen working solution and then incubated for 1 hour at 37°C. After three times washing with PBST solution, blocking was performed with 200 ml blocking buffer (Skimmed milk, Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, and PBS). 50 mL of the samples that was previously eluted from the filter paper were then added to the plates to be incubated for 1 hour at 37°C. Subsequently, plates were washed and incubated for 1 hour with diluted IgG antibody. After washing, reactions were developed by adding 100 mL of substrate solution (citrate-phosphate buffer, OPD stock solution, and 30% peroxidase) and then stopped by the addition of 100 mL H<sub>2</sub>SO<sub>4</sub> after 10-30 minutes (the solutions turned into yellow/orange). The optical density (OD) was determined at 492 nm. The anti-NDO-LID-1 serology threshold for positive responses was considered OD > 0.031.

## Results

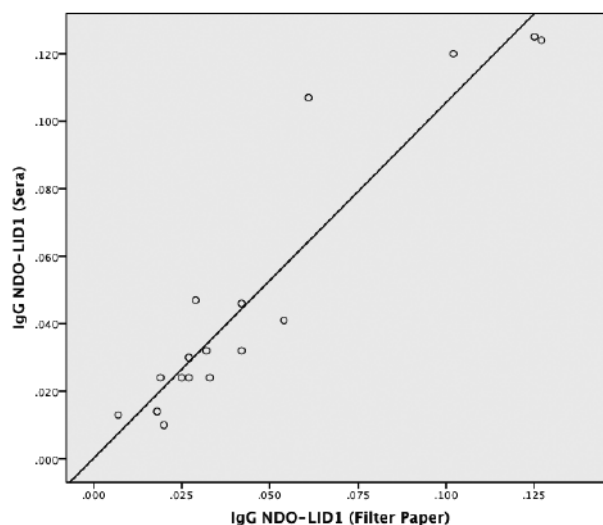
Seventeen sera and capillary blood samples on filter paper from HHC were tested

using ELISA to determine antibody IgG level against NDO-LID-1. Using a cut-off of 0.031, both groups interestingly showed similar percentage on positive ELISA result (9/17, 52.9%). The mean level of IgG antibody from capillary blood samples (filter paper), however, was lower than the sera ( $\bar{x}$ =0.046 and 0.049, respectively). Nonetheless, there was no significant difference between them ( $p=0.754$ ). We also observed a strong positive Pearson's correlation ( $R=0.906$ ) between the two procedures.

Figure 1 shows the linear regression for the paired data and indicates that the results obtained from elution of dried capillary blood spot on filter paper were closely related compared with the results obtained from the sera. The regression was  $y=1.055x$ , with  $y$  being IgG anti-NDO-LID-1 level from serum and  $x$  being IgG anti-NDO-LID-1 level from filter paper.

## Discussion

Collecting good samples is the first step allowing us to further control leprosy. Without good and adequate amount of samples, screening of leprosy won't run effectively and therefore keeping us from reaching the target of reducing leprosy or its complications incidence by 2020.<sup>1</sup> Despite the availability of modern diagnostic tools such as serologic tests, leprosy screening still has some limitations for there are difficulties and disobediences of either the health workers or the patients/contacts to refer samples to central laboratory. These difficulties are usually due to the inconvenience of taking or sending the samples. To overcome these limita-



**Figure 1. Comparison of ELISA IgG anti-NDO-LID-1 paired data from capillary blood samples collected on filter paper versus sera.  $R^2=0.906$ ,  $p=0.000$ .**

tions, it is important to establish a new tool to improve the referral activities from the peripheral areas.

We evaluated the effectiveness of using capillary blood and filter paper as a sample for diagnostic test or early detection. This procedure is convenient for the patient and easy to mail therefore can improve the referral activities. Our study revealed that detection of anti-NDO-LID-1 level using capillary blood samples on filter paper is equally effective as using the serum from venipuncture ( $p=0.754$ ). We also formulated a regression that is useful to convert the level of IgG anti-NDO-LID-1 obtained from filter paper to determine the titer of the antibody in the blood. A similar study by Prakoeswa *et al.* in 2007 also show the effectiveness of utilizing filter paper to detect IgM anti-PGL-1 in a total of 30 leprosy patients. There was a significant difference between anti-PGL-1 antibodies from filter paper and serum ( $p<0.01$ ). Nonetheless, this study also found a strong correlation ( $R=0.977$ ) between the two procedures and constructed a linear regression to further convert anti-PGL-1 concentration into the titers in the serum.<sup>9</sup> This study differs from ours since our participants include only HHC without any signs and symptoms. Despite that, our study interestingly showed no significant difference between filter paper and serum. This was possibly derived from the use of different antigen, which was NDO-LID-1 instead of PGL-1. It was determined that relative to PGL-1, NDO-LID-1 demonstrates a greater potential to identify infected HHC and individuals from general populations who were infected with *M. leprae*.<sup>13</sup> This statement is in accordance to a subsequent study stating that some people lacking anti-PGL-1 antibodies have antibodies that recognize LID-1, and vice versa.<sup>6</sup> PGL-1 and LID-1 has been proved to be specific for *M. leprae*.<sup>8</sup> Supposedly, a reagent conjugating the two antigens will improve the sensitivity of detecting antibodies against *M. leprae*. There also have been studies that utilize finger blood prick on filter paper to detect IgM anti-PGL-1 for mass screening of leprosy contacts in Bangladesh. One study successfully revealed a prevalence of 7.3/1000 among contacts.<sup>14</sup> However, a 6 year follow up study of another mass screening with a similar method showed that there was no significant difference of IgM anti-PGL-1 level at first intake between contacts who developed leprosy and contacts who did not.<sup>15</sup> A consequent study using LID-1 showed that IgG antibody response to LID-1 was detected a year earlier than the appearance of the symptoms in 64% of HHC, which was way earlier than the increase of anti-PGL-1 response in the same individual. However, a study using NDO-LID-1 showed

a more promising result. A former longitudinal study also showed that 3.6% of the HHC have come out with positive responses to NDO-LID-1 (through ELISA) years before they reveal any signs and symptoms. This study also figures up a probability of 10.4% in developing leprosy for contacts who were seropositive for IgG anti-NDO-LID-1, which was higher than those who were tested for only IgG anti-LID-1.<sup>3</sup> Our study however, did not focus on bacillary load of the patients that shared contacts with our respondents, which will be useful in constructing a probability equation to calculate a chance of contact for developing leprosy based on IgG anti-NDO-LID-1 on first intake. We also did not log the time between first intake of capillary blood samples and the ELISA test to determine whether storage time affects the detection of IgG anti-NDO-LID-1. Moreover, our samples were too small in size while most of the prior studies used a large-scale population. This prevents us from extrapolating the statistical analysis results to the overall population. We suggest a subsequent study with improvements to the limitations of this study.

## Conclusions

In conclusion, the use of filter paper and NDO-LID-1 is worthy of further investigations, especially for those with lower bacillary load or contacts of leprosy patients. Having the expectation of a better-designed research, we hope that, in the future, a more reliable referral system and diagnostic method can be established to further succeed the eradication of leprosy.

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