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Original Article

The Prevalence of Anti-DFS70 Antibodies in SLE and Non-SLE Patients

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ABSTRACT

Background: The presence of anti-DFS70 antibodies was considered by some rheumatologists as a tool to help exclude the diagnosis of systemic autoimmune rheumatic diseases (SARDs) such as systemic lupus erythematosus (SLE). However, several studies reported the controversial result. The golden standard for anti-DFS70 antibody detection is indirect immunofluorescence (IIF) assay. Recently, ELISA method has been developed for the anti-DFS70 detection.
Objective: We aimed to investigate the presence of anti-DFS70 antibodies in SLE and non-SLE patients using IIF assay and ELISA.
Methods: We evaluated 45 SLE patients who fulfilled the Systemic Lupus International Collaborating Clinics (SLICC) classification criteria, SLEDAI score >5; 15 patients with atopic dermatitis (AD) with IgE >200 IU/ml, and 30 healthy subject individuals. Anti-DFS70 antibodies were measured by ELISA and IIF assay (ANA-DFS70 Cytobeads). Differences in the anti-DFS70 prevalence in SLE and non-SLE patients were analysed using a chi-square test. The sensitivity, and specificity of ELISA were calculated by McNemar's test.
Results: The presence of anti-DFS70 antibodies measured by IIF assay was found in 11.1% of SLE and 4.4% of non-SLE patients (p-value 0.434), while anti-DFS70 measured by ELISA was found in 17.7% of SLE and 6.6% of non-SLE patients (p-value 0.197). There was no significant difference in detecting anti-DFS70 of the two methods (p.0.05). The ELISA has a sensitivity of 85.7% and a specificity of 94% for anti-DFS70 detection.
Conclusion: Anti-DFS70 was detected more frequently in SLE rather than in non-SLE patients measured using both ELISA and IIF. There is good agreement between ELISA and IIF assay for the anti-DFS70 detection.

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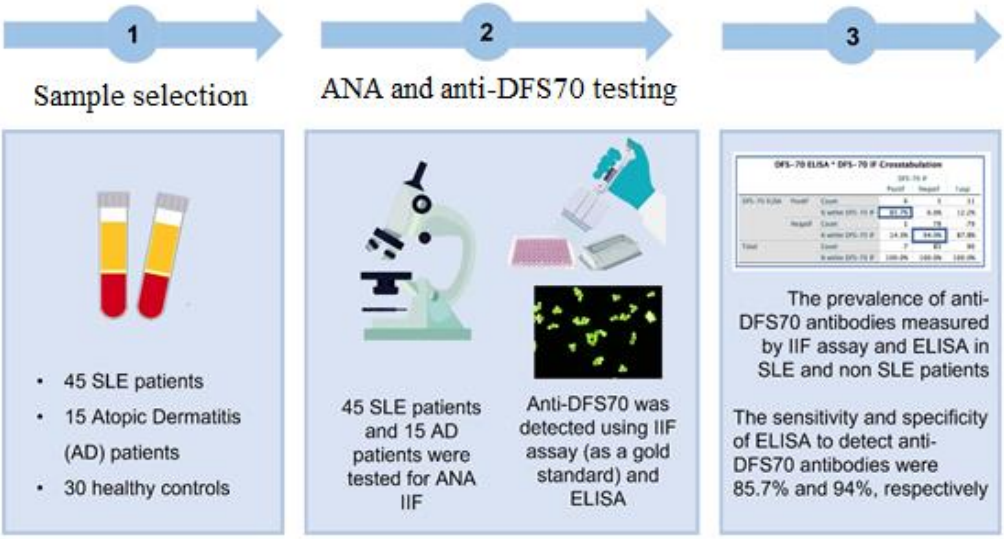
GRAPHICAL ABSTRACT



Introduction

Indirect immunofluorescence (IIF) antinuclear antibodies (ANAs) testing is a sensitive assay recommended in screening test for autoimmune systemic rheumatic disease (SARD) [1-4].

However, it was reported that ANA can be positive in healthy individuals up to 20%, and positivity in the majority of cases might be associated with anti-DFS70 antibodies [5-7] .

Although the ANA presence is a hallmark of SARD, the anti-DFS70 antibodies are considered by some rheumatologists as a tool to help exclude the SARD diagnosis such as systemic lupus erythematosus (SLE) [8, 9].

In healthy people with positive ANA test, 33% of them have positive anti-DFS70 [10, 11].

In SLE patients, the positive rates of anti-DFS70 ranged from 0% to 22.1% by different methodologies among several studies [12-16].

The majority of them regarded the anti-DFS70 antibodies as less prevalent in SLE patients than in healthy individuals, except for one research performed by Japanese scholars, indicating that there was no significant difference in the prevalence of anti-DFS70 antibodies between SLE patients (22.1%) and healthy individuals (16.4%) [17]. The diversity of prevalence of anti-DFS70 antibodies may be affected by genetic, ethnic, and environmental factors, as well as detection methods. To date, few studies have investigated the anti-DFS70 antibodies in SLE patients in Indonesia.

Anti-dense fine speckled-70 (DFS70) antibodies, known as lens epithelium-derived growth factor (LEDGF) antibodies, detected in the ANA IIF pattern, which was indicated by the fluorescence of dense fine speckled granular spots in the cell nucleus that are irregularly distributed in interphase and metaphase chromatin [18].

The DFS70/LEDGF antigen is known to be a transcriptional coactivator that can upregulate several stress and inflammation protective genes [19-21].

These functions may contribute to the cell viability under environmental stresses associated with both health and disease. Alterations in the function or structure of DFS70 can trigger disease pathogenesis through the autoantibodies formation [22, 23].

Anti-DFS70 autoantibodies are generally IgG class and target specific regions within the C-terminal domain of DFS70 [24]. The anti-DFS70's prevalence was reported between 0.8% to 16.6%. This variance is probably associated with the differences in patient's criteria and methodological issues such as different HEp-2 substrates, inter-observer variability in pattern assignments, and serum dilution variability.

In addition, the lack of agreement on anti-DFS70 IIF detection methods with other methods has led to many variations in reported results. These differences were related to the use of different LEDGF antigens, analytical sensitivity/specificity, and manufacturing limitations of various confirmatory tests [25, 26].

Although anti-DFS70 antibodies have been originally discovered in patients with interstitial cystitis and later in patients with chronic inflammation, atopic dermatitis (AD), tumor, and even apparently healthy individuals, their clinical impact is still unknown [27-30].

An anti-DFS70-positive subject reported that there were no symptoms of SARD after 4 years of follow-up [31]. A previous study reported that the presence of anti-DFS70 could be used as a biomarker to exclude SARDs from healthy individuals with positive ANA [32].

Knowing the serological and clinical profile of anti-DFS70-positive subjects may help to prevent unnecessary cases to specialists and requests for further testing in healthy individuals [32-34].

The gold standard for anti-DFS70 testing is the IIF assay. This is relatively complex and can only be done in certain laboratories [35, 36].

Recently, an easier method to test for anti-DFS70 have been developed, such as ELISA. The aim of this study was to assess the presence of anti-DFS70 antibodies in SLE, non-SLE and to establish the sensitivity, specificity, and suitability of the IIF assay, and ELISA methods for anti-DFS70 antibodies detection.

Materials and Methods

Samples collection

Disease group

Serum specimens were obtained from 45 adult SLE patients who were recruited from the outpatient and inpatient departments of Internal Medicine Dr. Saiful Anwar Hospital (Malang, Indonesia) from April to September 2022. All patients fulfilled the 2012 SLICC classification criteria, with SLE Disease Activity Index score >5 and recorded all drugs consumed. SLE patients who were complicated by other SARD or malignancies were excluded.

Control group

The healthy control (HC) cohort included 30 age- and gender-matched healthy individuals from Dr. Saiful Anwar Hospital, without any known history of SARD or chronic diseases. The disease control (DC) enrolled 15 atopic dermatitis (AD) who went to Dr Saiful Anwar Hospital during the same period as the SLE. All DC patients fulfilled the American Academy of Dermatology Diagnostic Criteria for Atopic Dermatitis, with IgE >200 U/ml. This study was approved by the Institutional Review Board of Dr Saiful Anwar Hospital (Approval no. 400/108/K.3/102.7/2022.) and informed consent were required from all patients involved in this study.

To provide sufficient power to detect correlation, at least 47 samples were needed according to the sample size formula:

$$N = \frac{Z\alpha + Z\beta\ 2}{0.5\ In\ \{(1+r)/(1-r)\}} + 3$$

$$N = \frac{1.96+1.645}{0.5\ In\ \{(1+0.5)/(1-0.5)\}} + 3 = 47 \text{ samples}$$

Based on this formula, the minimum sample size calculation result is 47 samples. We used a total of 90 samples for better analysis.

ANA testing

All SLE and AD patients were tested for ANA IIF assay (EUROIMMUN AG, Lüebeck, Germany). All assays were performed according to the manufacturer's instructions. Patient's serum that has been diluted were incubated with Hep2 cells for 30 minutes. The subsequent washing was performed and further incubation with fluorescein isothiocyanate-labeled human anti-immunoglobulin G (IgG) for 30 minutes. Slides were re-incubated and washed. The specific antibodies that attached to the antigen was preserved and read using a Leica LED fluorescence microscope (DM 1000 LED, Leica Microsystems).

Positive results were reported include nuclear staining patterns and titers. An ANA test was reported as positive if there was a nuclear staining pattern and a titer of 1:80.

Anti-DFS70 antibodies IIF assay

Commercial anti-DFS70 Cytobeads ANA-DFS70 (Generic Assay, Germany) was used in this study. 60 µl diluted patient serum and 60 µl serum control were dropped into each well and incubated for 30 minutes at room temperature in a moist chamber.

After that, aspirate fluids from well were washed for 5 × 2 minutes with fresh PBS solution in a staining chamber. Next, 60 µl of conjugate was applied into each well and completely converted. The slides were incubated for 30 minutes at room temperature in a moist chamber, and protected from direct light.

Thereafter, aspirate fluid from wells were washed for 5 × 2 minutes with fresh PBS solution in a staining chamber.

Next, one drop of mounting medium was applied per well carefully, and placed a cover slip onto the slide and pressed hard on the cover slip or tapping the slide to remove any air bubbles occurring, and then the slide was read under a fluorescent microscope, starting from the centre circle which contain Hep-2 cells, and proceeded to the right and left compartments. The results were positive if there was a green fluorescence in the centre circle (Hep-2) and the left and right compartments (Figure 1). Results are reported with presence or absence. Validation of kit test was done using standard serum that already provided from manufacture [37].

Anti-DFS70 Elisa

Anti-DFS70 antibodies were detected using a commercial kit (Euroimmune). Prepared ELISA well-plates that have been coated with DFS70/LEDGF antigen. 100 µl of the calibrator was transferred; positive or negative control or diluted patient samples were also transferred into the individual microplate wells according to the pipetting protocol. Incubate for 30 minutes at room temperature (18-25 °C). The reagent wells were washed with 45 µl of working strength wash buffer. The buffer was washed in each well for 30-60 seconds per washing cycle, and then the wells were emptied.

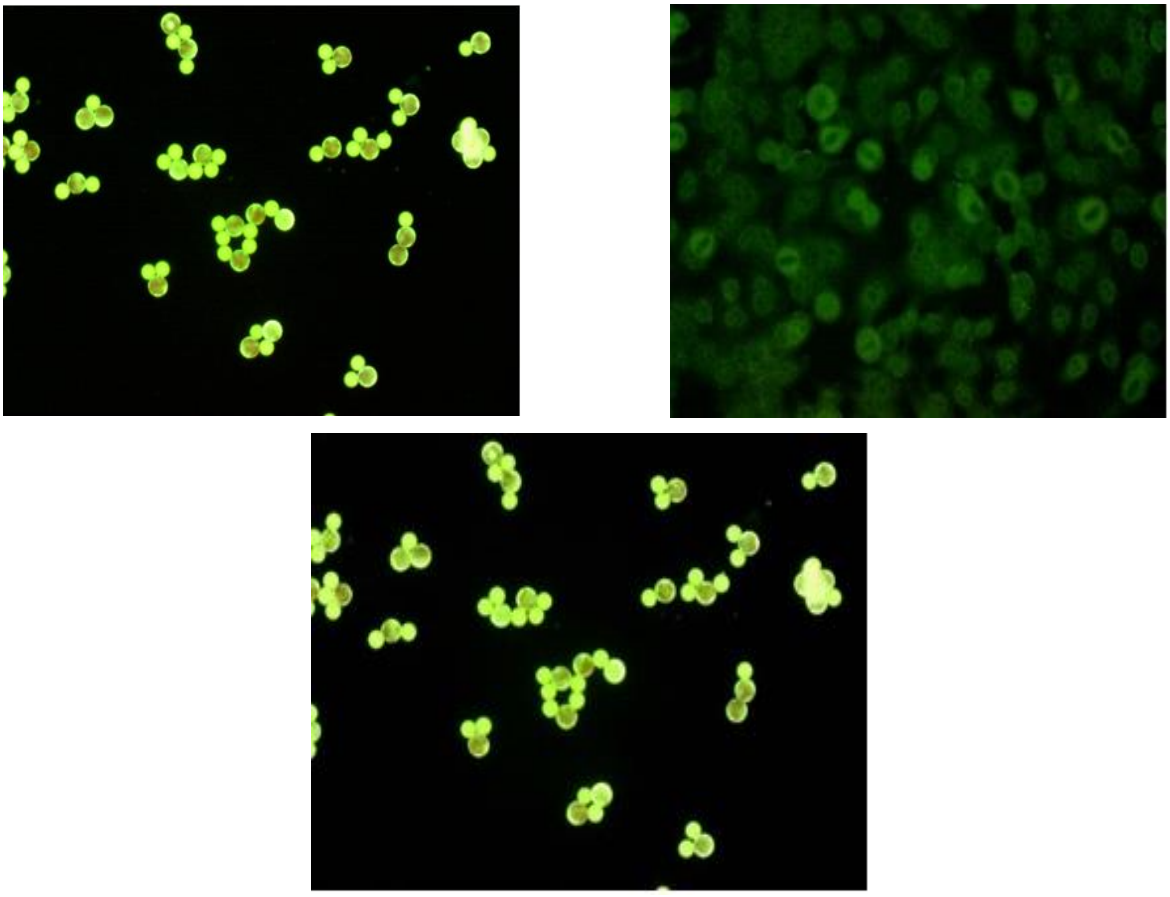


Figure 1: Results were positive if there was green fluorescence in the centre circle (Hep-2) (Left Figure) also the left and right compartments (Right figure) (taken by author)

After washing, all liquid was thoroughly disposed from the microplate by tapping it on absorbent paper with the openings facing downwards to remove the total residual wash buffer. Pipette 100 µl of enzyme was conjugated (peroxidase-labeled anti-human IgG) into each of the microplate wells and incubates for 30 minutes at room temperature (18-25 ºC). The wells were emptied, washed as described above.

Next, pipette 100 µl of chromogen/substrate solution into each of the microplate wells was incubated for 15 minutes at room temperature (18-25 ºC), protect from direct sunlight. Pipette 100 µl of stop solution was introduced into each of the microplate wells, in the same order, and at the same speed as the chromogen/substrate solution. Photometric measurement of the color intensity should be made at a wavelength of 450 nm and reference wavelength between 620-650 nm within 30 minutes of adding the stop solution. Prior to measure, the microplate was slightly shaken to ensure a homogenous distribution of the solution. The result of < 1.0 was considered as negative while ≥ 1.0 was considered as positive. Validation and standard of kit test were done using standard serum that already provided from manufacture [38]. All examination were conducted in double blind.

Testing of anti-dsDNA autoantibody

The quantitative determination of anti-dsDNA antibodies was performed by ELISA using the Alegria®-Orgentec Analyzer. Assay was performed according to the manufacturer's instructions.

Statistical analysis

Statistical tests performed included descriptive analysis and comparative tests. Normally and non-normally distributed continuous data were, respectively, represented by mean ± standard deviation (SD). Categorical data were expressed as counts and percentages. The comparative test of nominal data was performed with two-tailed Chi Square test. $p < 0.05$ was considered statistically significant using Fisher's exact test. Furthermore, diagnostic tests were carried out using 2×2 tables and the agreement test was performed with McNemar test. All statistical tests were performed with the SPSS 21.0 program.

Results and Discussion

The 6-months study included 45 SLE patients, 15 AD patients and 30 healthy subjects. Subjects' ages ranged from 16 to 70 years old (91.1% were 16 to 55 years old and 8.9% were 55 years and older). The subjects were 65 females and 25 males. There were no age or gender differences between SLE and non-SLE patients. ANA and anti-dsDNA were found in 100% and 73.3% of SLE patients respectively, and 20% of AD patients showed positive ANA (Table 1).

There were 8 of 45 SLE patients (17.7%) and 3 of 45 (6.6%) non-SLE patients showed positive anti-DFS70 antibodies examined by ELISA (p-value 0.197) (Table 2). Moreover, 5 of 45 (11.1%) SLE patients and 2 of 45 (4.4%) non-SLE patients were positive measured by IIF assay (p-value 0.434) (Table 3). Two of the three positive anti-DFS70 antibodies were AD patients.

Table 1: Patient characteristics of the SLE, health subject, and disease control

Parameters	SLE Patients, N = 45	AD Patients, N = 15	Healthy Subjects, N=30
Sex Woman (%) Man (%)	 40 (88.9%) 5 (11,1)	 13 (86.7%) 2 (13.3%)	 27 (90%) 3 (10%)
Age (years)	41,6 ± 16,8	42,6 ± 17,1	43,45 ± 15,5
Positive ANA (%)	45 (100 %)	3 (20%)	N/A
Positive Anti-dsDNA (%, IU/ml)	32 (73, 3 %) (186.3 ± 68.8)	0	N/A
SLEDAI score	12.4 ± 6.1	N/A	N/A
Positive IgE (> 200 IU/ml)	N/A	15 (100%) 460.67 ± 212.5	N/A

Table 2: Prevalence of DFS70 antibodies in SLE and non-SLE patients with ELISA

Parameters	SLE patients, N= 45	non-SLE N=45	P-value
Anti-DFS70 ELISA positive (%)	8 (17.7%)	3 (6.6%)	0.197
Anti-DFS70 ELISA negative (%)	37 (82.2%)	42 (93.3%)	

Table 3: Prevalence of DFS70 antibodies in SLE and non-SLE patients with IIF assay

Parameters	SLE patients, N= 45	non-SLE N=45	P-value
Anti-DFS70 IIF positive (%)	5 (11.1%)	2 (4.4%)	0.434
Anti-DFS70 IIF Negative (%)	40 (88.9%)	43 (95.6%)	

Table 4: Sensitivity and specificity of anti DFS-70 ELISA method against IIF assay

	DFS-70 IIF positive	DFS-70 IIF negative
DFS-70 ELISA Positive (%)	6 (86%)	5 (6%)
DFS-70 ELISA Negative (%)	1 (14%)	78 (94%)

The sensitivity and specificity of the anti-DFS-70 ELISA against the IIF assay as the gold standard were 85.7% and 94%, respectively (Table 4). There was no significant difference (p-value 0.125) between the results of the IIFA assay and ELISA for detecting anti-DFS70 examinations, thus illustrating a good agreement.

Systemic autoimmune rheumatic diseases (SARDs) are a cluster of diseases characterized by the presence of autoantibodies to intracellular antigens, particularly antinuclear antibodies (ANA). Positive ANA is one classification criterion for systemic lupus erythematosus (SLE), Sjögren's syndrome (SjS), and systemic sclerosis (SSc) [38, 39]. These autoantibodies are traditionally detected by indirect immunofluorescence (IIF) assay on HEp-2 cells, and this method was suggested as the gold standard by the American College of Rheumatology (ACR) [24]. However, about 20% of healthy subjects are detected positive ANA, and in most cases, these positive results are related with anti-dense fine speckled 70 (anti-DFS70) antibodies [9]. Previous studies have shown that the existence of anti-DFS70 antibodies in the absence of other antibodies is extremely rare in SARDs patients and therefore the existence of these antibodies may rule out the SARDs diagnosis [12, 13].

The primary purpose from this research is to establish the existence of anti-DFS70 antibodies in autoimmune patients, especially SLE, allergic (atopy), and healthy subjects. The results of this research showed that anti-DFS70 antibodies are found in both SLE and non-SLE patients. Anti-DFS70 was detected more frequently in SLE patients than in non-SLE patients. Anti-DFS70 testing using the ELISA was found to identify the presence of this antibody higher than the IIF method in both SLE (17.7% vs 11.11%) and non-SLE patients (6.66% vs 4.44%). The results of this study are inconsistent with other studies in European countries [24].

A previous study, included 3175 samples, found an anti-DFS70 prevalence of 1.7% in the general population and 4.6% in ANA-positive samples. They reported that anti-DFS70 in male population could be useful as a biomarker to predict the absence of other autoantibodies developing in these subjects [24].

In a cohort study of anti-DFS70 autoantibody prevalence in Japan involving 250 healthy subjects and 276 SARD patient specimens, the predominance of anti-DFS70 antibodies in healthy subjects was 16.4%, of which 12.8% were males and 20.0% were female (sex difference; P = 0.12). In SARDs patients, the prevalence of anti-DFS70 antibodies found in SLE, mixed connective

tissue disease, systemic sclerosis, dermatomyositis/polymyositis (DM/PM), Sjögren's syndrome, and rheumatoid arthritis (RA) was 22.1%, 14.3%, 14.3%, 3.0%, 21.3%, and 18.1%, respectively. There was no reliable variation in anti-DFS70 between SARDs patients, except DM/PM, and healthy individuals [14].

Thus, our results are consistent with those reported in Japan. Anti-DFS70 cannot be used to differentiate between SLE and non-SLE patients and therefore cannot be used to rule out the SLE diagnosis.

Furthermore, a previous study found that positive anti-DFS70 autoantibody cannot exclude SARD [40].

A Chinese study found a significantly higher prevalence of anti-DFS70 in SLE patients (20.7%) than in healthy subjects (10.8%, p=0.002). In multivariate analysis, anti-DFS70-positive SLE patients were related to younger age (OR = 0.982; 95% CI = 0.969, 0.995) and greater frequency of anti-dsDNA (OR 1.598; 95% CI 1.107, 2.306). It became clear that anti-DFS70 antibodies appear to predominate in Chinese SLE patients. The positive correlation between anti-DFS70 and anti-dsDNA, and the persistent dynamic fluctuations between anti-DFS70 and anti-dsDNA during the evaluation, suggest a potential link between anti-DFS70 and anti-dsDNA in SLE patients [41].

A study reported 30% of AD patients were found to have anti-DFS70 antibodies. 16% of asthma patients and 9% of interstitial cystitis patients had also antibodies with the same specificity [42]. Another important finding was that IgE antibodies reactive to DFS70 were further present in the serum of AD patients [43]. This suggests that the presence of autoantibodies reactive to DFS70 may be associated with AD in asthma, interstitial cystitis, and other conditions. Although the role of these antibodies in AD pathogenesis has not yet been established, they are likely markers for a subset of AD patients. AD is widely associated with allergen-specific IgE and the formation of allergic disease, leading to the development of auto-reactive IgE-associated 'auto-allergic' disease [42].

Our next research objective was to assess the sensitivity, specificity, and suitability of the ELISA method compared to the IIF method in anti-DFS70 detection. This research showed that the ELISA method has a sensitivity of 85.7% and a specificity of 94%. There was a good agreement between the ELISA and IIF Cytobeads. The basic principle of the ANA-DFS70 Cytobeads method is the use of beads attached to the LEDGF antigen (placed in the left and right compartments) as a comparison (reference) pattern on the Hep2 substrate placed in the centre compartment. The use of beads is very helpful in interpreting the Hep2 pattern, especially for novice observers.

Not many studies have assessed the performance of ANA-DFS70 Cytobeads against the conventional IIF [25, 26].

Furthermore, the frequency of anti-DFS70 antibody positivity is greater in healthy individuals than in SARD patients, and anti-DFS70 antibodies cause IIF to be positive for ANA in healthy individuals. Consequently, SARD overdiagnosis, based on her positive ANA results, may increase patient anxiety and lead to increased requests for other unnecessary tests. The high prevalence of anti-DFS70 antibodies found in Japan has caused confusion among physicians when treating patients with probable SARD and non-SARD patients, complicating the differential diagnosis of SARD in ANA screening [43].

Limitations of this study are the small size of the samples included, and the SARDs patients included in this study were only SLE. Other antibodies were not examined in our patients such as anti-ENA which is reported to be related to the existence of anti-DFS70.

Conclusion

Anti-DFS70 was assayed in 90 sera using two different methods: IIF Cytobeads and ELISA. It is found in both SLE and non-SLE patients. This study revealed that anti-DFS70 antibodies were more prevalent in Indonesian SLE patients than in non-SLE patients. Both methods used for anti-DFS70 detection showed good agreement. Further research is needed involving a larger number of samples, involving autoimmune diseases other than SLE.

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Disclosure Statement

No potential conflict of interest was reported by the authors.

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All authors have done work in the field of human and animal also have observed the principles of professional ethics and human and animal rights. The Ethics Commission of General Hospital Dr. Saiful Anwar Malang, Indonesia, with regards of the protection of human rights and welfare in medical research, has carefully reviewed this research and approved with the ethical approval no. 400/108/K 3/102.7/2022 on May 25, 2022.

Authors' Contribution

Kusworini Handono, Hani Susianti, and Syahrul Chilmi made the design study, Rahmatul Yasiro and Natalia Sukarta collected serum samples, took clinical data from patients, and did the experiments. The statistical analysis and data interpretation were done by Andrea Aprilia, Kusworini Handono, and Syahrul Chilmi. All authors did the manuscript preparation and literature search.

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