

Detection of Specific Autoantibodies in Sera with Negative Antinuclear Antibody by Indirect Immunofluorescence Assay but Positive by Enzyme Immunoassay

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ABSTRACT

Objective: The aim of this study was to investigate the predictive value of positive enzyme immunoassay (EIA) for detection of specific autoantibodies in sera negative for antinuclear antibody (ANA) by indirect immunofluorescence (IIF) assay, but positive for ANA by EIA.

Methods: Eighty sera that tested negative for ANA by IIF, but positive for ANA by EIA were included. All sera were tested for specific autoantibodies by line immunoassay (LIA). The positive predictive value (PPV) of EIA was calculated using LIA result as a reference standard. Medical records of patients were reviewed. Clinical findings at the time of blood sampling for ANA testing and at 5 years after sampling were obtained.

Results: Twenty-eight sera (35%) were found to be positive by LIA. The PPV of EIA for detection of specific autoantibodies at the manufacturer's recommended cut-off was 35.0% (95% CI: 24.5-45.5%). The most prevalent antibodies were anti-SSA/Ro60 (64.3%, 95% CI: 46.5-82.0%), anti-Ro52 (25.0%, 95% CI: 9.0-41.0%), and anti-SSB/La (10.7%, 95% CI: 0-22.2%). A diagnosis of systemic autoimmune rheumatic disease was established in 7 patients (25%) at the time of blood sampling, and 4 patients (14.3%) were diagnosed with non-rheumatic autoimmune disease.

Conclusion: EIA testing in IIF-ANA negative sera yielded a chance to detect antinuclear antibodies. However, the poor PPV of EIA may have low benefit in real-life clinical practice. Anti-SSA/Ro60 was the most prevalent antibody detected. A high proportion of LIA-ANA positive patients were not diagnosed as autoimmune disease at the time of antibody detection.

Keywords: Antinuclear antibody; extractable nuclear antigen; enzyme immunoassay; indirect immunofluorescence assay (Siriraj Med J 2019;71: 234-239)

INTRODUCTION

Antinuclear antibodies (ANA) are essential autoantibodies for screening and diagnosis of systemic autoimmune rheumatic diseases (SARD), such as systemic lupus erythematosus (SLE), as well as non-rheumatic

autoimmune diseases.¹⁻⁵ Certain specific antibodies are included in the diagnostic criteria for certain specific diseases.⁶⁻⁹ Indirect immunofluorescence (IIF) assay is the gold standard method that is most commonly used for detection of ANA.¹⁰ IIF uses Hep-2 cells as

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a substrate, which provides almost all of the antigens that are required for ANA testing. However, enzyme immunoassay (EIA) is more rapid and simple than IIF for detecting ANA. EIA contains disease-relevant native or recombinant antigens that are coated on a microtiter plate. IIF using Hep-2 cells could yield a false-negative result for anti-Ro and anti-Jo-1 antibodies, while EIA could detect them.^{11,12} However, EIA has been associated with both false-positive and false-negative results.¹³ During 2010, the Clinical Pathology Laboratory at Siriraj Hospital implemented a protocol that calls for IIF to be used for initial screening of ANA. In cases of negative result by IIF, secondary screening is performed using EIA. We observed that among sera positive for ANA by EIA, some sera samples were positive and some were negative for specific antibodies subsequently requested by the physician.

Accordingly, the aim of this study was to investigate the predictive value of positive EIA for detection of specific autoantibodies in sera negative for ANA by IIF, but positive for ANA by EIA. The secondary objective was to determine and report the prevalence and clinical findings of certain antibodies.

MATERIALS AND METHODS

Patient sera

Sera negative for ANA by IIF, but positive for ANA by EIA were randomly selected from stored sera sent to the Clinical Pathology Laboratory at Siriraj Hospital for ANA testing during 2010. Sera were stored at -20°C before analysis. Siriraj Hospital is Thailand's largest national tertiary referral center.

Medical records of patients whose sera were included in this study were reviewed. Clinical findings at the time of blood sampling for ANA testing and at 5 years after sampling were obtained and recorded.

The protocol for this study was approved by the Siriraj Institutional Review Board (SIRB), Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand (Si 211/2557).

IIF assay

IIF assay was performed using a Mosaic Hep-20-10/Liver (Monkey) Kit (Euroimmun AG, Lübeck, Germany). Briefly, sera were diluted to 1:100 and then incubated with Hep-20-10 cells. The Hep-20-10 cells contained an increased spectrum of Hep-2 cells in the mitotic phase. After staining with fluorescent conjugate, slides were visualized under a fluorescent microscope at 400x. Fluorescence intensity was compared with that of the end-point positive control. Fluorescence intensity

equal to or greater than the fluorescence intensity of the end-point positive control was considered a positive result.

EIA testing

EIA testing was performed using an IMTEC-ANA Screen Kit (Human Diagnostics, Wiesbaden, Germany). The microtiter strips came coated with HeLa cell nuclei. Sera were diluted to 1:101 before being added to the plates. The manufacturer defined any value above 55 U/ml as being a positive result.

Antigen-specific assays

Line immunoassay (LIA) was performed using an IMTEC-ANA-LIA Kit (Human Diagnostics). The strips contain the following antigens: nucleosomes, histones, SmD1, nRNP, SSA/Ro60, Ro52, SSB/La, Scl-70, CENP-B, Jo-1, and ribosomal P proteins (Rib-P). Sera showing positive reactivity on the Human Diagnostics LIA were tested again using a EUROLINE ANA Profile 3 Kit (Euroimmun AG) to confirm the result. The strips in that kit contain the following antigens: nRNP, Sm, SSA/Ro60, Ro-52, SSB/La, Scl-70, PM/Scl, Jo-1, CENP-B, PCNA, dsDNA, nucleosomes, histones, Rib-P, and AMA M2. Sera were diluted to 1:101 before incubation with the strips. Positive reactivity on both LIA kits for at least 1 identical antibody (Ab) type was considered a positive result.

Statistical analysis

All data were collected and analyzed using Microsoft Excel 2016 spreadsheet software (Microsoft Corporation, Redmond, WA, USA). Rate of positive or negative LIA, predictive value of positive EIA, prevalence of each specific antibody, and 95% confidence interval (CI) were calculated.

RESULTS

Eighty sera that tested negative for ANA by IIF, but that tested positive for ANA by EIA were included. An overview of LIA results from both LIA test kits is shown in [Fig 1](#). Seven sera with positive LIA by only the Human Diagnostics LIA (samples 31-37), and 2 sera positive for different antibody types using either the Human Diagnostics LIA or the Euroimmun LIA (samples 29-30) were considered negative LIA ([Table 1](#)). Therefore, 28 sera (35%) were considered positive LIA (samples 1-28).

The manufacturer's recommended cut-off value of 55 U/mL could not discriminate positive from negative LIA ([Fig. 2](#)). Using the LIA result as a reference standard, the

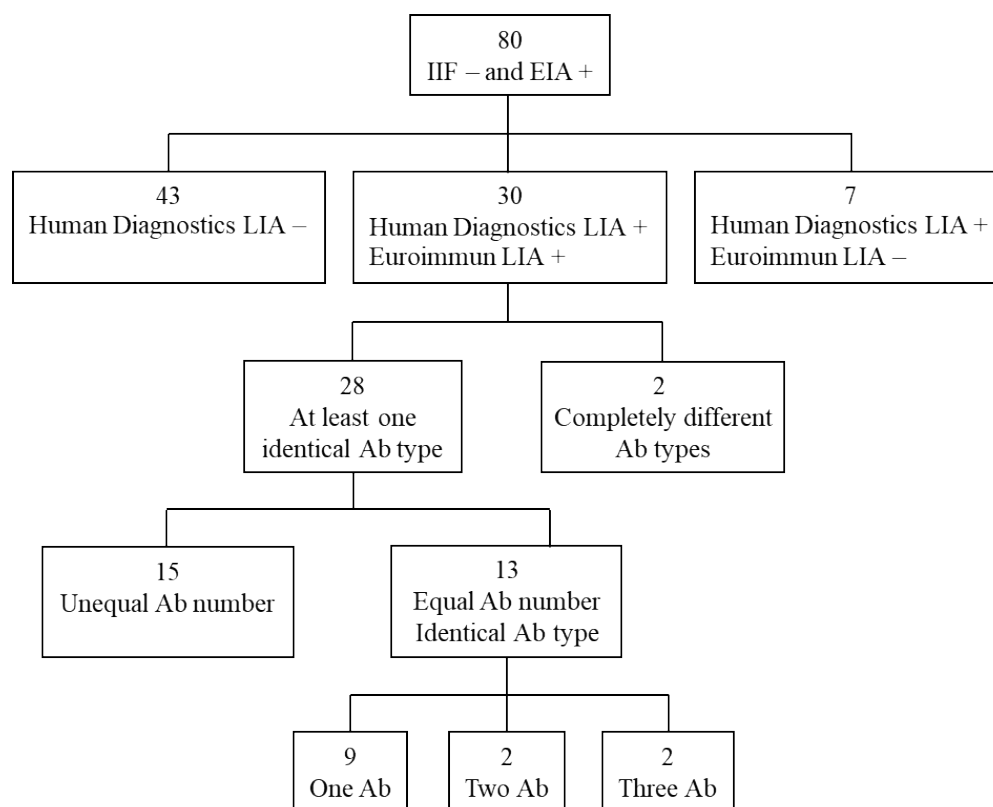


Fig 1. Overview of LIA results of 80 sera negative for ANA by IIF, but positive for ANA by EIA.

Abbreviations: Ab = antibody; EIA = enzyme immunoassay; IIF = indirect immunofluorescence assay; LIA = line immunoassay, - = negative; + = positive

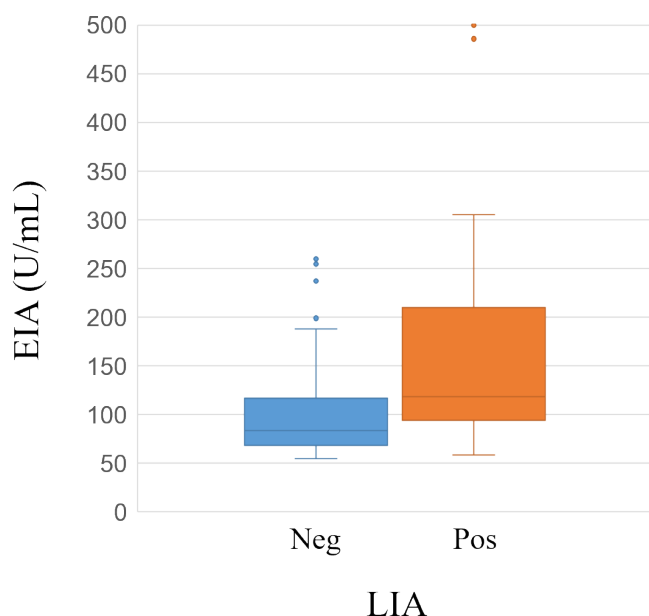


Fig 2. Comparison of EIA values between positive and negative LIA.

Abbreviations: EIA= enzyme immunoassay; LIA= line immunoassay; Neg= negative; Pos= positive

positive predictive value (PPV) of EIA for the detection of specific autoantibodies at this value was 35.0% (95% CI: 24.5-45.5%). The PPV of different EIA values are shown in Table 2.

The most prevalent antibodies were anti-SSA/Ro60 (64.3%, 95% CI: 46.5-82.0%), anti-Ro52 (25%, 95% CI:

9.0-41.0%), and anti-SSB/La (10.7%, 95% CI: 0-22.2%) (Table 1). A diagnosis of SARD was established in 7 of 28 patients (25%) at the time of blood sampling. Four patients (14.3%) were diagnosed with non-rheumatic autoimmune disease. Seventeen patients had insufficient evidence for diagnosing autoimmune disease (60.7%).

TABLE 1. EIA values, specific antibodies, and clinical findings of 37 sera showing reactivity on LIA.

Sample no.	EIA (U/ml)	Human Diagnostics LIA	Euroimmun LIA	Clinical findings
1	95	Ro60	Ro60	Urticarial vasculitis
2	98	Ro60	Ro60	Chronic urticaria, allergic rhinitis
3	94	Ro60	Ro60	Tubulointerstitial disease of unknown cause
4	305	Ro60, Ro52	Ro60, Ro52	Dead fetus in utero at 13 weeks
5	252.57	Ro60, Ro52, La	Ro60, Ro52, La	SLE
6	113.63	Ro60, SmD1, Rib-P	Ro60, Sm, Rib-P	Discoid LE, lupus profundus
7	>500	Ro60	Ro60, Ro52	SLE
8	139	Ro60	Ro60, Ro52	Amaurosis fugax
9	114	Ro60	Ro60, Ro52	RA, secondary Sjogren syndrome
10	193.41	Ro60	Ro60, AMA-M2	Primary biliary cirrhosis
11	>500	Ro60	Ro60, histone	Nonalcoholic steatohepatitis
12	486	Ro60	Ro60, Rib-P	AIH
13	206	Ro60	Ro60, Ro52, AMA-M2	Nephrotic syndrome (FSGS)
14	198.6	Ro60	Ro60, Ro52, La, CENP-B	RA, leukopenia
15	120.3	Ro60, nRNP	Ro60	DM, AKI on top of CKD
16	211	Ro60, Ro52	Ro60, Ro52, PM/Scl	Chronic ITP
17	90	Ro60, SmD1, nucleosome	Ro60, Ro52	Alopecia areata
18	288.3	Ro60, histone, nucleosome	Ro60, dsDNA	Lupus nephritis type IV
19	58.41	Ro52	Ro52	Myofascial pain syndrome
20	106	Ro52	Ro52, Ro60	Common variable immune deficiency
21	63	Ro52	Ro52, AMA-M2	Chronic hepatitis C
22	117	Ro52, Jo-1	Ro52, Jo-1	RA
23	148.54	CENP-B	CENP-B	Suspected AIH
24	72	Jo-1	Jo-1	Progressive obstructive jaundice of unknown cause
25	88.9	La	La	Beta-thalassemia/Hb E disease, AIHA
26	83	La	La, Jo-1	Erythema nodosum
27	124.29	Scl-70	Scl-70	Chronic urticaria
28	96.87	SmD1	Sm	Unilateral posterior uveitis
29	78.9	SmD1	Ro60	N/A
30	146	nRNP	Sm	N/A
31	68	Ro60	-	N/A
32	72	SmD1	-	N/A
33	77.8	SmD1	-	N/A
34	135	Histone	-	N/A
35	158	nRNP	-	N/A
36	107.4	SmD1, Ro60	-	N/A
37	81	Nucleosome, histone, nRNP	-	N/A

Abbreviations: Ab= antibody; AIH= autoimmune hepatitis; AKI= acute kidney injury; AIHA= autoimmune hemolytic anemia; CKD= chronic kidney disease; DM= diabetes mellitus; FSGS= focal segmental glomerulosclerosis; ITP= immune thrombocytopenia; LE= lupus erythematosus; N/A= not applicable; RA= rheumatoid arthritis; SLE= systemic lupus erythematosus

TABLE 2. Positive predictive values of different EIA values.

EIA value (U/mL)	Positive LIA n	Negative LIA n	PPV (95% CI)
>260	5	0	100% (100-100)
>250	6	2	75.0% (45.0-100)
>200	8	3	72.7% (46.4-99.0)
>150	10	9	52.6% (30.2-75.1)
>100	18	19	48.6% (32.5-64.8)
>55	28	52	35.0% (24.5-45.5)

Abbreviations: EIA= enzyme immunoassay; LIA= line immunoassay; PPV= positive predictive value; CI= confidence interval

Five years later, 13 of those (76.5%) had not developed autoimmune disease, and 4 patients (samples 19, 21, 23, and 28) had missing data.

DISCUSSION

The present study set forth to investigate for specific antibodies in sera negative for ANA by IIF, but positive for ANA by EIA. We found a higher rate of positive specific antibodies than previous study (35% vs. 16.9%).¹⁴ Compared to previous studies that performed antigen-specific assay in all IIF-ANA negative sera and who found a rate of positivity of 0.7-14%¹⁵⁻¹⁷, we demonstrated that screening with EIA-ANA before performing antigen-specific assay in IIF-ANA negative sera may increase the probability of identifying specific antibodies.

The manufacturer's recommended cut-off value yielded a low PPV. Even though the EIA value was as high as 250 U/mL, the PPV of 75% was not sufficiently high. Therefore, a positive result from this EIA kit may not be applicable to real-life clinical practice. However, reporting the EIA result along with its specific PPV may provide benefit relative to a decision to request or not request antigen-specific assay.

Similar to previous studies^{14,16}, the most prevalent antibodies identified in this study were anti-SSA/Ro60, anti-Ro52, and anti-SSB/La, and these antibodies have been recognized as being associated with Sjogren syndrome and SLE.¹⁸ Anti-SSA/Ro60 was shown to have association with both subacute cutaneous lupus erythematosus¹⁹ and neonatal lupus erythematosus.^{20,21} In the present study, a large proportion of patients with positive LIA were not diagnosed as autoimmune disease by the 5-year follow-up. However, the presence of autoantibodies often portends the development of disease that does not fully clinically manifest until years into the future. Anti-SSA/

Ro60 could be detected before the onset of symptoms and diagnosis of SLE at intervals as long as 9.1 and 10.4 years, respectively.^{22,23}

This study has some mentionable limitations. First and consistent with the retrospective nature of this study, some data was missing or incomplete. Second, the number of included samples was relatively small. Third, random samples were selected from routine ANA testing; therefore, our findings may not be generalizable to patients tested in specialized clinic. Fourth and last, we investigated only one EIA kit that uses HeLa cell nuclear extract, which may be different from other EIA kits that use different combinations of defined native or recombinant antigens.

CONCLUSION

EIA testing in IIF-ANA negative sera yielded a chance to detect antinuclear antibodies. However, the poor PPV of EIA may have low benefit in real-life clinical practice. Anti-SSA/Ro60 was the most prevalent antibody detected. A high proportion of LIA-ANA positive patients were not diagnosed as autoimmune disease at the time of antibody detection.

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Conflict of interest: The LIA kits used in this study were provided free of charge by their distributors. The authors declare no other conflicts of interest.

REFERENCES

1. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982;25:1271-7.
2. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997;40:1725.
3. Solomon DH, Kavanaugh AJ, Schur PH, American College of Rheumatology Ad Hoc Committee on Immunologic Testing G. Evidence-based guidelines for the use of immunologic tests: antinuclear antibody testing. *Arthritis Rheum* 2002;47:434-44.
4. Alvarez F, Berg PA, Bianchi FB, Bianchi L, Burroughs AK, Cancado EL, et al. International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol* 1999;31:929-38.
5. Hennes EM, Zeniya M, Czaja AJ, Pares A, Dalekos GN, Krawitt EL, et al. Simplified criteria for the diagnosis of autoimmune hepatitis. *Hepatology* 2008;48:169-76.
6. Alarcon-Segovia D, Cardiel MH. Comparison between 3 diagnostic criteria for mixed connective tissue disease. Study of 593 patients. *J Rheumatol* 1989;16:328-34.
7. Tanimoto K, Nakano K, Kano S, Mori S, Ueki H, Nishitani H, et al. Classification criteria for polymyositis and dermatomyositis. *J Rheumatol* 1995;22:668-74.
8. Shiboski SC, Shiboski CH, Criswell L, Baer A, Challacombe S, Lanfranchi H, et al. American College of Rheumatology classification criteria for Sjogren's syndrome: a data-driven, expert consensus approach in the Sjogren's International Collaborative Clinical Alliance cohort. *Arthritis Care Res (Hoboken)* 2012;64:475-87.
9. van den Hoogen F, Khanna D, Fransen J, Johnson SR, Baron M, Tyndall A, et al. 2013 classification criteria for systemic sclerosis: an American college of rheumatology/European league against rheumatism collaborative initiative. *Ann Rheum Dis* 2013;72:1747-55.
10. Agmon-Levin N, Damoiseaux J, Kallenberg C, Sack U, Witte T, Herold M, et al. International recommendations for the assessment of autoantibodies to cellular antigens referred to as anti-nuclear antibodies. *Ann Rheum Dis* 2014;73:17-23.
11. Blomberg S, Ronnblom L, Wallgren AC, Nilsson B, Karlsson-Parra A. Anti-SSA/Ro antibody determination by enzyme-linked immunosorbent assay as a supplement to standard immunofluorescence in antinuclear antibody screening. *Scand J Immunol* 2000;51:612-7.
12. Bahrt KM, McCarty GA. A negative fluorescent antinuclear antibody test in a patient with Jo-1 antibody. *J Rheumatol* 1985;12:624-5.
13. Bayer PM, Fabian B, Hubl W. Immunofluorescence assays (IFA) and enzyme-linked immunosorbent assays (ELISA) in autoimmune disease diagnostics--technique, benefits, limitations and applications. *Scand J Clin Lab Invest Suppl* 2001;235:68-76.
14. Dahle C, Skogh T, Aberg AK, Jalal A, Olcen P. Methods of choice for diagnostic antinuclear antibody (ANA) screening: benefit of adding antigen-specific assays to immunofluorescence microscopy. *J Autoimmun* 2004;22:241-8.
15. Hoffman IE, Peene I, Veys EM, De Keyser F. Detection of specific antinuclear reactivities in patients with negative anti-nuclear antibody immunofluorescence screening tests. *Clin Chem* 2002;48:2171-6.
16. Lee SA, Kahng J, Kim Y, Park YJ, Han K, Kwok SK, et al. Comparative study of immunofluorescent antinuclear antibody test and line immunoassay detecting 15 specific autoantibodies in patients with systemic rheumatic disease. *J Clin Lab Anal* 2012;26:307-14.
17. Sener AG, Afsar I, Demirci M. Evaluation of antinuclear antibodies by indirect immunofluorescence and line immunoassay methods: four years' data from Turkey. *APMIS* 2014;122:1167-70.
18. Provost TT. Anti-Ro(SSA) and anti-La(SSB) antibodies in lupus erythematosus and Sjogren's syndrome. *Keio J Med* 1991;40:72-7.
19. McCauliffe DP. Cutaneous diseases in adults associated with anti-Ro/SS-A autoantibody production. *Lupus* 1997;6:158-66.
20. Cimaz R, Spence DL, Hornberger L, Silverman ED. Incidence and spectrum of neonatal lupus erythematosus: a prospective study of infants born to mothers with anti-Ro autoantibodies. *J Pediatr* 2003;142:678-83.
21. Jaeggi E, Laskin C, Hamilton R, Kingdom J, Silverman E. The importance of the level of maternal anti-Ro/SSA antibodies as a prognostic marker of the development of cardiac neonatal lupus erythematosus a prospective study of 186 antibody-exposed fetuses and infants. *J Am Coll Cardiol* 2010;55:2778-84.
22. Arbuckle MR, McClain MT, Rubertone MV, Scofield RH, Dennis GJ, James JA, et al. Development of autoantibodies before the clinical onset of systemic lupus erythematosus. *N Engl J Med* 2003;349:1526-33.
23. Eriksson C, Kokkonen H, Johansson M, Hallmans G, Wadell G, Rantapaa-Dahlqvist S. Autoantibodies predate the onset of systemic lupus erythematosus in northern Sweden. *Arthritis Res Ther* 2011;13:R30.