Fluorescence intensity of anti–nuclear antibodies patterns on anti–extractable nuclear antigens detection

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ABSTRACT

OBJECTIVE
To provide assistive information for diagnosis of patients with autoantibodies.

METHODS
This study reviewed laboratory records of patients who were suspected of autoimmune diseases by clinicians and underwent ANA and anti–ENA tests at Khon Kaen Hospital, Thailand, from January 2012 to January 2016. 543 anti–ENA results with positive ANA were analyzed. Anti–ENA test was used to identify a group of specific autoantibodies, including anti–RNP, anti–Sm, anti–SSA, anti–Ro52, anti–SSB and anti–Scl70 antibodies.

RESULTS
The detection rate of anti–ENA antibodies in ANA positive serum samples was 66.1% (95% confidence interval (CI); 62 to 70). ANA patterns of homogeneous and fine speckled types with high fluorescence intensities from 3+ to 4+ showed 23.4%–35.9% of anti–SSA and anti–Ro52 antibodies detection. Meanwhile, the coarse speckle 3+ to 4+ could detect anti–SSA and anti–Ro52 antibodies by up to 50.0%–63.6% and started to detect anti–Sm antibody by 18.8% and 33.8%. Moreover, the coarse speckle 4+ showed 66.2% of anti–RNP and 20.8% of anti–SSB antibodies detection. Nucleolar 4+ showed the highest detection rate of anti–Scl70 antibody at 77.8%. Similar tendency of anti–Scl70 antibody detection could be found from low to high intensities of fine speckled and homogeneous patterns, which usually show up in mix patterns. Centromere pattern 4+ showed 10.5%–26.3% of anti–SSA, anti–Ro52, anti–SSB and anti–Scl70 antibodies detection. At low fluorescence intensities, fine speckled 1+ and homogeneous 1+ (titer 1:100) could detect anti–SSA antibody with highest probability of 17.5% and 13.5%, respectively. The coarse speckle 1+ also showed 5.6% of anti–SSA, anti–Ro52 and anti–SSB antibodies detection, while speckle 2+ had the increasing rate of anti–SSA, anti–Ro52 and anti–RNP antibodies detection from 15.1% to 33.3%.

CONCLUSION
The tendency of detection of some specific anti–ENA antibodies can be roughly estimated as the fluorescence intensity is 3+ or above. However, low intensities 2+ or below do not rule out the expected anti–ENA antibodies related to clinical diagnosis.

INTRODUCTION

Detection of anti–nuclear antibodies (ANAs) using indirect immunofluorescence assay (IFA) is a subjective screening test for autoimmune rheumatic diseases (ARDs) or connective tissue diseases. This method is recommended by the American College of Rheumatology (ACR) as the gold standard for ANA testing.[1] The staining pattern and fluorescence intensity or ANA titer will be reported by manual reading. The test plays an important role in supporting a diagnosis of many connective tissue diseases such as systemic lupus erythematosus (SLE), Sjögren's syndrome (SS), Systemic sclerosis (SSc) or scleroderma, polymyositis (PM) or dermatomyositis, Raynaud phenomenon and rheumatoid arthritis (RA).[2,3]

ANA by IFA showed high sensitivity and high specificity in SLE patients. However, it also gave a positive result in patients with multiple medical problems who had no symptoms of connective tissue diseases or in patients with non–autoimmune diseases.[4,5] It has also been reported that ANA positive results could be found at low titers of 1:80 and 1:160 even in normal healthy persons.[4,5,6] Moreover, many studies presented that healthy people had a prevalence range of ANA varying from 0.5% to 20.0% at titers no more than 1:80, and 0.1% to 3.7% at higher titers.[7] From guidelines for the laboratory use of autoantibody tests of autoimmune rheumatic diseases, ANA detection was suggested only in patients with clinical symptoms suspected of ARD. Determination of antinuclear specific antibodies should be done only in patients with ANA positive by IFA or in ANA negative patients but exhibit clear symptoms of ARD.[8] In addition, the international recommendations for detection of autoantibodies also advise to find out antibodies to extractable nuclear antigens (anti–ENA antibodies) in ANA positive cases.[9] Anti–ENA antibodies used in routine laboratories consist of seven main antibodies, namely anti–RNP, anti–Sm, anti–SSA (anti–Ro), anti–Ro52, anti–SSB (anti–La), anti–Scl70 and anti–Jo1. These specific autoantibodies could be found in and are related to many connective tissue diseases.[10,11]

The specificity of the anti–ENA test by line–blot immunoassay (LIA) has been reported to be higher than the ANA test by IFA (84.4% and 52.5%, respectively). This performance increases when both methods are used together.[12] Nevertheless, the different types of ANA staining patterns were also reportedly associated with several connective tissue diseases, which when combined with clinical symptoms were useful for identifying the specific autoantibodies.[13-15] At the screening dilution of 1:40 and 1:80 by using Hep–2 or Hep–2000 cells as substrate antigens, ANA titer and fluorescence intensity were also demonstrated to be beneficial in predicting specific antibodies against ENAs.[16-18]

However, these previous works on ANA were performed at different initial serum dilutions from what used in the laboratory of Khon Kaen Hospital and those on anti–ENA antibodies with different methods. Therefore, this study aimed to analyze ANA staining patterns from IFA at a single dilution of 1:100 which were positive with intensities 1+ to 4+, to find the frequency and probability of anti–ENA antibodies detection as found from line–blot immunoassay, to provide information for estimating the chance of more specific antibodies detection from a given ANA screening test.

METHODS

PATIENTS

This study was a retrospective descriptive study of laboratory records of patients who were clinically suspected of autoimmune diseases by clinicians' judgment. The laboratory data consist of 543 patient records with positive ANA by IFA and with anti–ENA results from January 2012 to January 2016. Ethical approval was obtained from the Khon Kaen Hospital Institute Review Board in Human Research (KIRB). The Ethical approval number is KE59047.

INDIRECT IMMUNOFLUORESCENCE ASSAY OF ANA DETERMINATION

The semi–quantitative ANA testing by IFA was performed in serum samples at a single dilution of 1:100 using phosphate buffer saline (PBS–Tween) pH 7.2 and staining on HEp–20-10 cells and primate liver cells (monkey's liver cells). Fluorescein–labeled anti–human IgG (goat) was used for monitoring the antigen and autoantibody reaction. The staining performed according to the instruction manual of the test kit (EUROIMMUN, Germany). Negative and positive controls with known ANA fluorescence pattern as homogeneous 4+ were used as internal quality control (IQC) in each slide. Fluorescence intensity was graded from 1+ to 4+ and reporting of negative and positive results performed as suggested in the guideline of the national committee for clinical laboratory standards (NCCLS).[19] ANA five major patterns of the cell nucleus, namely Homogeneous, Fine speckled, Coarse speckled, Nucleolar and Centromere patterns, were classified based on the Hep–2 cells–based nomenclature proposed by Wiik, et al.[20]

LINE–BLOT IMMUNOASSAY (LIA) OF ANTI–ENA ANTIBODIES DETECTION

Seven specific antigens of ENA were used: nRNP/Sm, Sm, SSA (Ro), Ro52, SSB (La), Scl70 and Jo1, which were coated on immunostrip of LIA commercial kit (Anti–ENA ProfilePlus 1, EUROIMMUN, Germany). As in the manufacturer's instruction, the 1:100 diluted serums was incubated with antigens on immunostrip. After washing unbound antibodies, alkaline phosphatase–labeled anti–human IgG (enzyme conjugate) was added and incubated with antigen–antibody complex. After washing steps, the substrate (NBT/BCIP) was incubated for color development on the antigen–antibody complex. The immunostrips were then scanned and analyzed using EUROLineScan software to get semi–quantitative results.

STATISTICS ANALYSIS

Data were analyzed for the probability of anti–ENA antibodies detection by line–blot immunoassay in serum samples of patients with ANA positive results. The results were presented in percentage, with a 95% confidence interval. The frequency and probability of each type of anti–ENA antibody detection were also studied for each ANA positive pattern and intensity (1+ to 4+).

RESULTS

From the review of 543 ANA by IFA positive results of patients who had symptoms suspected of autoimmune diseases, 359 cases were found anti–ENA positive by LIA, the probability was 66.1% (95% confidence interval (CI); 62 to 70). At ANA screening dilution of 1:100, fluorescence intensities from 3+ to 4+ of homogeneous and

fine speckled patterns showed 23.4%–35.9% of anti–SSA and anti–Ro52 antibodies detection. Coarse speckle 3+ to 4+ could also detect anti–SSA and anti–Ro52 antibodies by up to 50.0%–63.6%. Coarse speckle 4+ obviously showed high probability of anti–RNP antibody detection at 66.2% (95% CI; 55.4 to 77.0) and anti–SSB at 20.8% (95% CI; 11.5 to 30.1). At the intensity of 3+, a coarse speckled pattern could detect anti–Sm antibody by 18.8%, and up to 33.8% (95% CI; 23.0 to 44.6) at intensity 4+. High intensity 4+ of the nucleolar pattern showed the highest probability of anti–Scl70 antibody detection at 77.8% (95% CI; 71.3 to 84.3). Similar tendencies of anti–Scl70 antibody detection can be found from low to high intensity of fine speckled and homogeneous patterns, which usually appear in mixed patterns. Centromere pattern 4+ showed 10.5%–26.3% of anti–SSA, anti–Ro52 and anti–SSB antibodies detection, and 15.8% of anti–Scl70 antibody detection. For low fluorescence intensities, fine speckle 1+ and homogeneous 1+ could detect anti–SSA antibody with notable probability of 17.5% (95% CI; 9.0 to 26.0) and 13.5% (95% CI; 2.0 to 25.1), respectively. Coarse speckle 1+ also showed 5.6% anti–SSA, anti–Ro52, and anti–SSB antibodies detection, while speckle 2+ had an increasing rate of anti–SSA, anti–Ro52, and anti–RNP antibodies detection from 15.1% to 33.3%, as shown in Table 1 and Table 2.

DISCUSSION

This study evaluated laboratory records of 543 patients who were suspected of autoimmune diseases and had ANA positive results by IFA from January 2012 to January 2016. Of these records, 359 (66.1%) showed anti–ENA positive by LIA. The

Probabilities of detection of anti–ENA antibodies for each ANA positive pattern with different fluorescence intensities were then analyzed.

SPECKLED AND HOMOGENEOUS PATTERNS AT HIGH INTENSITIES

For high fluorescence intensities from 3+ to 4+, ANA staining of fine speckled and homogeneous patterns, which are always present in mixed patterns, could yield anti–SSA and anti–Ro52 antibodies detection rate by 23.4%–35.9%. Moreover, a coarse speckled pattern of intensities 3+ to 4+ could also detect anti–SSA and anti–Ro52 antibodies by up to 50.0%–63.6%. These results were consistent with many previous studies which reported that high titers or high intensities of ANA speckled and homogeneous types demonstrated a high detection rate of anti–ENA antibodies.[16–18]

Since this study was retrospective, without information on clinical symptoms, it should be useful for applying the provided data to mention some related previous studies. Agustinelli et al. and Mariz et al. reported that a high titer of a fine speckled pattern (≥ 1:1,280) could be found in a high percentage of patients with autoimmune rheumatic diseases (ARDs).[5,6] Recently, Rodsaward et al. also demonstrated that fine–coarse speckled patterns had a significant association with anti–SSA and anti–Ro52 antibodies in juvenile SLE patients, and mixed patterns of homogeneous–fine speckled patterns also showed high percentage in SLE patients, especially in juvenile SLE patients.[15]

Therefore, the high fluorescence intensity of speckled and homogeneous patterns in the ANA screening test, which has a high probability of anti–SSA and anti–Ro52 antibodies detection, might be helpful in the diagnosis of ARDs, especially SLE.

Table 1. Frequencies and probabilities of each anti–ENA antibody detection in ANA patterns with fluorescence intensity 1+ to 4+

ANA pattern and anti–ENA antibody	Frequency (Percentage) Fluorescence intensity of ANA			
	1+	2+	3+	4+
Homogeneous	37	47	55	220
anti–RNP	2 (5.4)	7 (14.9)	12 (21.8)	39 (17.7)
anti–Sm	1 (2.7)	2 (4.3)	5 (9.1)	11 (5.0)
anti–SSA	5 (13.5)	12 (25.5)	14 (25.5)	79 (35.9)
anti–Ro52	2 (5.4)	10 (21.3)	16 (29.1)	66 (30.0)
anti–SSB	1 (2.7)	1 (2.1)	4 (7.3)	20 (9.1)
anti–Scl70	2 (5.4)	3 (6.4)	13 (23.6)	134 (60.9)
Fine speckle	80	53	47	165
anti–RNP	5 (6.3)	8 (15.1)	11 (23.4)	14 (8.5)
anti–Sm	3 (3.8)	2 (3.8)	3 (6.4)	3 (1.8)
anti–SSA	14 (17.5)	10 (18.9)	11 (23.4)	46 (27.9)
anti–Ro52	6 (7.5)	11 (20.8)	13 (27.7)	39 (23.6)
anti–SSB	2 (2.5)	1 (1.9)	3 (6.4)	11 (6.7)
anti–Scl70	2 (2.5)	3 (5.7)	13 (27.7)	124 (75.2)
Coarse speckle	18	15	16	77
anti–RNP		4 (26.7)	4 (25.0)	51 (66.2)
anti–Sm			3 (18.8)	26 (33.8)
anti–SSA	1 (5.6)	5 (33.3)	8 (50.0)	49 (63.6)
anti–Ro52	1 (5.6)	3 (20.0)	8 (50.0)	44 (57.1)
anti–SSB	1 (5.6)	1 (6.7)	1 (6.3)	16 (20.8)
anti–Scl70				9 (11.7)
Nucleolar	29	25	30	162
anti–RNP		2 (8.0)	8 (26.7)	20 (12.4)
anti–Sm	2 (6.9)		2 (6.7)	3 (1.9)
anti–SSA	1 (3.5)	3 (12.0)	10 (33.3)	43 (26.5)
anti–Ro52	2 (6.9)	4 (16.0)	9 (30.0)	38 (23.5)
anti–SSB		1 (4.0)	2 (6.7)	8 (4.9)
anti–Scl70	1 (3.5)	3 (12.0)	8 (26.7)	126 (77.8)

ANA pattern and anti–ENA antibody	Frequency (Percentage) Fluorescence intensity of ANA			
	1+	2+	3+	4+
Centromere		1	2	19
anti–RNP				
anti–Sm				
anti–SSA		1 (100)	1 (50)	5 (26.3)
anti–Ro52			1 (50)	5 (26.3)
anti–SSB				2 (10.5)
anti–Scl70				3 (15.8)

Table 1. (continued.)

The presence of anti–SSA and anti–Ro52 antibodies as predicted by high intensity of speckled and homogeneous patterns is also supposed to be helpful in Sjögren's syndrome (SS) diagnosis, as the latest 2016 classification criteria for primary Sjögren's syndrome states that anti–SSA is the only specific antibody that indicates this disease when considered with clinical symptoms.[21,22] For the anti–Ro52 antibody, it was also reported to have the highest positive predictive value (100%) for primary SS.[23] Recently, the presence of anti–Ro52 antibody was reported as one of the factors associated with primary SS patients who had renal involvement.[24]

Moreover, the anti–Ro52 antibody could be found in several other autoimmune and non–autoimmune diseases such as SSc, RA, primary biliary cirrhosis (PBC) and idiopathic inflammatory myopathies (IIM).[25,26] Therefore, the presence of speckled and homogeneous patterns at high intensities suggest the presence of anti–SSA and anti–Ro52 antibodies, which in turn might indicate SLE, primary SS, and other diseases.

COARSE SPECKLED PATTERN AT HIGH INTENSITIES

The Coarse speckled pattern of intensity 4+ showed an outstanding probability of anti–SSB antibody detection among all ANA patterns (20.8%). This appears to be comparable to the study of Yang et al., where speckled pattern and high fluorescence titer were found to suggest anti–SSA and anti–SSB antibodies detection with a high percentage. Clinically, anti–SSA and anti–SSB antibodies could be found with a higher rate in SLE and SS patients.[27] Moreover, Frodlund et al. reported that speckled and homogeneous mixed pattern had the highest anti–SSA and anti–SSB antibodies detection rate in SLE patients.[28]

Obviously, the coarse speckled pattern at intensity 4+ in this study showed a high probability of anti–RNP antibody detection (66.2%). This is similar to the result of an autoantibody study of Rayes et al. on mixed connective tissue disease (MCTD) patients, where high titer of ANA speckled pattern and a high frequency of anti–RNP antibody were detected in

Table 2. Classification and probabilities

ANA pattern	Fluorescence intensity	Percentage	Anti–ENA antibody
Homogeneous	1+ and 2+	13.5 and 25.5	SSA
	1+ and 2+	5.4 and 21.3	Ro52
	2+ and 3+	14.9 and 21.8	RNP
	3+ to 4+	25.5–35.9	SSA and Ro52
Fine speckle	1+ and 2+	17.5 and 18.9	SSA
	1+ and 2+	7.5 and 20.8	Ro52
	2+ and 3+	15.1 and 23.4	RNP
	3+ to 4+	23.4–27.9	SSA and Ro52
Coarse speckle	1+	5.6	SSA, Ro52, and SSB
	2+	33.3 and 20.0	SSA and Ro52
	3+ to 4+	50.0–63.6	SSA and Ro52
	2+ and 3+	26.7 and 25.0	RNP
	4+	66.2	RNP
	4+	20.8	SSB
	3+ and 4+	18.8 and 33.8	Sm
Nucleolar, Fine speckle, Homogeneous	1+	3.5, 2.5, 5.4	Scl70
	2+	12.0, 5.7, 6.4	Scl70
	3+	26.7, 27.7, 23.6	Scl70
	4+	77.8, 75.2, 60.9	Scl70
Centromere	4+	10.5–26.3 and 15.8	SSA, Ro52, SSB and Scl70

all cases.[29] This means the two features tend to befound together, although the present study only found the same tendency in the coarse speckled pattern, but not in fine speckled. In other studies, the anti–RNP antibody was reported to be detected with a higher rate in MCTD.[30,31]

Moreover, the anti–Sm antibody could also be detected with relatively high probability in the

coarse speckled pattern of intensity 3+ (18.8%) and it even increased at intensity 4+ (33.8%). In a study by Rodsaward et al., the detection of anti–Sm and anti–RNP antibodies in the coarse speckled pattern was also reported associated with SLE patients.[15] These two anti–ENA antibodies had high significance of classification between SLE and non–SLE.[32] Their presence was also associated with a risk of lupus nephritis.[33-35] For anti–Sm antibody, in particular, it was the only anti–ENA antibody included in the 2019 European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR) classification criteria for SLE as an additive criterion.[36]

FINE SPECKLED AND HOMOGENEOUS PATTERNS AT LOW INTENSITIES

At fluorescence intensity 1+ (titer 1:100), this study found that fine speckled and homogeneous patterns showed detection of the anti–SSA antibody with the highest probability among all specific antibodies (17.5% and 13.5%, respectively). This is similar to a study of Peen et al., where anti–SSA and anti–SSB antibodies were reported altogether to be predominantly detected at low intensities of speckled pattern, except that anti–SSB did not show up in the present study.[16] Interestingly, in the present study, speckled and homogeneous patterns of intensity 2+ had increasing rate of anti–SSA, anti–Ro52, and anti–RNP antibodies detection compared with intensity 1+. The Low intensity of ANA might still be meaningful in the screening of some ARDs, as previous studies have reported that mothers who had babies with neonatal lupus erythematosus (NLE) or congenital heart block (CHB) could be

detected to have anti–SSA, anti–Ro52, anti–SSB, anti–RNP and anti–Sm antibodies.[37-41] Especially, Adelowo, et al. reported that a mother and her child, an NLE patient with complete heart block, who had moderate and low ANA titers (1:320 and 1:160, respectively) were detected to have anti–SSA autoantibody.[39] Moreover, Wisuthsarewong, et al. reported anti–SSA or anti–SSB positive in NLE patients who had ANA speckled pattern with moderate to high titer, but some cases with low titer (less than 1:100) could still be detected to have anti–SSA and anti–SSB antibodies as well.[37] Hence, as fine speckled and homogeneous patterns at low intensity 1+ are frequently found in routine laboratory, attention should still be paid on the screening results of patients who have clinical symptoms suspicious of autoimmune rheumatic diseases because there is still a low chance of presence of anti–ENA antibodies as shown in this study.

NUCLEOLAR PATTERN

High fluorescence intensity 4+ of the nucleolar pattern showed detection of the anti–Scl70 antibody with the highest probability (77.8%), while low intensity 1+ and 2+ still did with low probability. In previous studies, Bernstein et al. reported finding of nucleolar pattern with fairly high percentage in progressive systemic sclerosis (PSS) patients with at least 1+ intensities, at a dilution of 1:40.[42] Nevertheless, presence of nucleolar pattern is not specific to SSc, as it has also been reported on other diseases.[43] However, anti–Scl70 is the specific antibody that had higher prevalence of detection with immunoblot assay in PSS than in other ARDs like SLE and MCTD.[44] In

addition, this study found a similar tendency of anti–Scl70 antibody detection from low to a high intensity of nucleolar, nuclear fine speckled and homogeneous patterns, which usually show up in mixed patterns. A similar result had been found in a study of Khan et al., in which patients who were detected to have the anti–Scl70 antibody using immunoblot assay showed a mixture of ANA homogeneous, speckled and nucleolar patterns.[43] These IFA staining patterns were part of the Scl70 pattern which was reported in a study of Dellavance et al. to be associated with anti–DNA topoisomerase I (anti–Scl70) antibodies.[45] And the Scl70 pattern has later been designated as an AC–29 pattern in the international consensus on antinuclear antibody patterns (ICAP) classification algorithm.[46]

CENTROMERE PATTERN AT HIGH INTENSITIES

For intensity 4+ of centromere staining pattern, this study found 10.5%–26.3% of anti–SSA, anti–Ro52, anti–SSB, and anti–Scl70 antibodies detection. High frequency of anti–centromere detection and its association with CREST syndrome has been reported in a study of Bernstein, et al.[42] Pakunpanya et al. reported that anti–centromere antibodies were detected with higher frequency in autoimmune patients but its titer could not discriminate SSc from other autoimmune diseases.[47] However, centromere pattern, anti–centromere and anti–Scl70 antibodies were included in ACR–EULAR classification criteria for SSc[48] and in preliminary criteria for the very early diagnosis of SSc.[49]

LOW INTENSITY OF ANA PATTERNS

In many previous studies of ANA levels, Perilloux et al. reported that autoimmune patients had higher ANA titers (≥ 1:160) than non–autoimmune patients.[50] Mariz et al and Agustinelli et al. reported that high titer of ANA test using Hep–2 IFA had a higher detection rate in patients with systemic autoimmune rheumatic diseases (SARDs), especially nuclear fine speckled pattern. However, the nuclear fine speckled pattern at low titers as 1:80 and 1:160 could also be found in SARD patients.[5, 6] In 2019 EULAR/ACR classification criteria for SLE, ANA positive result at titer ≥ 1:80 on HEp–2 cells was even approved as the entry criterion before applying additive clinical and immunologic criteria.[36] In the present study, IFA of ANA detection using HEp–20–10 cells at low fluorescence intensity 1+ (titer 1:100) of homogeneous, fine speckled, coarse speckled and nucleolar patterns still showed low probabilities of anti–ENA antibodies detection as in the data provided. Therefore, in diagnosing SARDs, low fluorescence intensities of ANA detection should not be overlooked, in which case clinical symptoms should also be considered as additional criteria. And as IFA relies on subjective judgment, one should take special caution when judging between negative and low intensity 1+, to avoid ruling out potential patients.

In conclusion, high levels of fluorescence intensity of ANA showed a high detection rate of anti–ENA antibodies, while low intensities could still suggest the detection of some kinds of anti–ENA antibodies. The probabilities reported in this study might be able to supply additional information for diagnosis when combined with the patient's clinical symptoms. In addition, it is important to be cautious in reporting the fluorescence intensity, as low intensities may still provide some hint on the presence of anti–ENA antibodies.

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