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Investigation of the dual cascade algorithm in the diagnosis of antinuclear antibodies

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Abstract

Background: The dual cascade algorithm which involves screening and confirmation of antinuclear antibodies (ANAs) by further reflex testing is widely used in the detection of ANAs. We aimed to investigate this algorithm which is commonly used in many laboratories.
Methods: A total of 475 sera obtained from patients with a clinical suspicion of systemic autoimmune rheumatic diseases (SARDs) upon which three expert assessors agreed for interpretation in the indirect immunofluorescence (IIF) test were determined and tested by the line immunoassay (LIA) containing 16 antigens. The results of the tests were statistically compared and evaluated.
Results: In 141 of the sera (29.7%), there was an agreement between ANA-IIF(+) and LIA(+) results. The overall agreement rate between the two tests for positivity and negativity only was 85.5% with a Cohen's κ coefficient of 0.69. In 118 of these 141 sera (83.7%), pattern and associated ANA agreement was detected with an overall agreement rate of 80.6% and a Cohen's κ coefficient of 0.57. The highest agreement between the pattern and associated ANAs was seen in centromere, dense fine speckled (DFS) and cytoplasmic reticular patterns. In these patterns, the rate of anti-centromere-associated protein B (CENP-B), anti-DFS and anti-antimitochondrial antibody M2 (anti-AMA-M2) antibodies were 93.4%, 92.3% and 66.7%, respectively.
Conclusions: We found an overall moderate agreement between IIF screening and LIA confirmation tests. However, the level of agreement varies according to the pattern type. The discrepancy in agreement rates may cause false reflex test requests. Our results highlight the need for collaboration between clinical and laboratory professionals in selected cases instead of the reflex testing approach.

Keywords: antinuclear antibody; diagnosis; indirect immunofluorescence; line immunoassay.

Brief summary: We investigated the dual cascade algorithm that involves screening and confirmation of antinuclear antibodies (ANAs). Although there was a moderate agreement between the human epithelial carcinoma (HEp-2) pattern in indirect immunofluorescence (IIF) and associated antibodies in the line immunoassay (LIA), the level of agreement varies according to the pattern type. This discrepancy needs the collaboration between clinical and laboratory professionals in selected cases instead of the reflex testing approach.

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Introduction

Antinuclear antibodies (ANAs) are autoantibodies directed against nuclear, cytoplasmic and mitotic antigens, which play a critical role in the diagnosis of systemic autoimmune rheumatic diseases (SARDs) [1, 2]. Although there is no standard algorithm for the laboratory ANA testing, the dual cascade algorithm that involves ANA screening mostly by the indirect immunofluorescence (IIF) test and confirmation of the suspicious ANAs by further reflex testing is most commonly used [3]. The IIF test on human epithelial carcinoma (HEp-2) cells is the gold standard method for the detection of ANAs and is most widely used as a screening test [2, 4]. Despite its capability to detect

a large number of antibodies and high sensitivity, there are some troubles with standardization, with the main disadvantage of this method being the subjectivity of the assessor [4, 5]. Using the IIF assay, specific patterns on HEp-2 cells reflect the topographic distribution of the target autoantigens and have been associated with certain ANAs [6]. The greatest advantage lies in the fact that these patterns serve as indicators for these associated antibodies [5, 6]. The relationship between the patterns and certain ANAs allows for the selection of the ANAs to be tested in the second step (subserology testing) according to the pattern type or eliminates the need for reflex testing in certain patterns. In the second stage, associated antibodies against extractable nuclear antigens (ENAs) can be identified using a number of reflex testing such as the enzyme-linked immunosorbent assay (ELISA), Western blot, flow cytometry or line immunoassay (LIA) [7]. While associated antibodies indicated by the patterns have to be tested separately using methods other than LIA, LIA allows for testing of multiple antibodies simultaneously, but is a more expensive method compared to others. There may also be discrepancies between the ANA-IIF HEp-2 patterns and associated ANAs tested in reflex confirmatory tests in routine laboratory applications. Furthermore, a significant number of studies demonstrated variable degrees of agreement between the tests for patterns and associated ANAs [1, 8–12].

In this study, we aimed to investigate the dual cascade algorithm which involves ANA-IIF screening and ANA-LIA confirmation tests which are frequently used complementary to each other in the detection of ANAs.

Materials and methods

This study was designed and conducted at the Serology Laboratory of Manisa Celal Bayar University Hospital, Manisa, Turkey. Between January 2017 and September 2018, 600 sera sent from the rheumatology department to the serology laboratory with a clinical suspicion of SARDs were aliquoted and stored at –70 °C until testing. The IIFT Mosaic Basic Profile 3A® assay (Euroimmun®, Lübeck, Germany) containing HEp-2 and monkey liver cells was used for the ANA-IIF test. The test was performed at 1:100 dilution in accordance with the manufacturer's recommendations. A total of 600 sera were interpreted in a double blind fashion by three medical microbiologists working in different centers with a minimum of 5 years of experience in ANA-IIF testing to minimize subjectivity of the assessor. Inter-rater agreement was defined as ≥1 pattern agreed among the assessors in case of multiple patterns. A total of 475 sera for which all assessors agreed on the interpretation of patterns and negativity were studied using LIA, excluding sera with ANA pattern diagnoses associated with those that are undetectable by not including in the LIA (e.g. mitotic patterns).

For 475 sera, the Euroline ANA Profile 3 Plus DSF70® LIA test (Euroimmun®) with 16 ENAs (dense fine speckled [DFS]-70, anti-mitochondrial antibody M2 [AMA-M2], ribosomal P protein, histone, nucleosome, double-stranded DNA [dsDNA], proliferating cell nuclear antigen [PCNA], centromere-associated protein B [CENP-B], Jo-1, PM-Scl, Scl-70, Sjögren's syndrome antigen A [SS-A], Sjögren's syndrome antigen B [SS-B], Ro-52, Smith antigen [Sm] and nuclear ribonucleoprotein [nRNP]/Sm) was applied at 1:100 dilution in accordance with the manufacturer's recommendations. The results were evaluated using the EuroLineScan® software (Euroimmun®). The agreement between the ANA-IIF patterns and associated ANAs in a serum sample was evaluated based on the study by Chan et al. [13] (Table 1). Only consensus patterns (single or multiple) of the three assessors were included in the IIF and LIA test comparisons. Inter-test agreement was defined as ≥1 agreement in the results of the ANA-IIF and ANA-LIA tests in case of multiple patterns and multiple antibodies.

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 15.0 software (SPSS Inc., Chicago, IL, USA). Descriptive statistics were expressed in number and frequency. Cohen's κ analysis was performed to evaluate the agreement between two tests, and the percentage of agreement was provided for all comparisons [14].

Results

Of the 475 sera, 151 (31.8%) were positive and 324 (68.2%) were negative for ANA-IIF, and 200 (42.1%) were positive and 275 (57.9%) were negative for ANA-LIA (Table 2). Both the tests showed agreement in 141 and 265 sera for only positivity and negativity, respectively. The agreement rates were 93.4% and 70.5% for the IIF(+) and LIA(+) results, and were 81.8% and 96.4% for the IIF(–) and LIA(–) results, respectively (Table 2). Accordingly, the overall agreement rate for positivity and negativity was 85.5% with a Cohen's κ coefficient of 0.69, indicating a moderate agreement.

Three assessors reached a consensus on 153 patterns in 151 sera, with the diagnosis of double patterns in two sera. In 200 ANA-LIA (+) sera, 375 ANAs were detected, and 187 (49.9%) of them were antibodies associated with

Table 1: Hep-2 nuclear and cytoplasmic patterns, and associated antigens and testable antibodies in the ANA-LIA test.[a]

HEp-2 patterns	Antigen association	[b]Testable antibodies in the LIA test
Homogeneous	dsDNA, nucleosomes, histones	Anti-histone, anti-nucleosome, anti-dsDNA
Nuclear speckled	hnRNP, U1RNP, Sm, SS-A/Ro(Ro60), SS-B/La, RNA polymerase III, Mi-2, Ku, Mi-2, TIF1γ, TIF1β, RNA helicase A, Replication protein A	Anti-Ro-52, anti-Sm, anti-nRNP/Sm, anti-SS-A, anti-SS-B
Dense fine speckled	DFS-70/LEDGF	Anti-DFS-70
Centromere	CENP-A/B (C)	Anti-CENP-B
Nucleolar	PM/Scl-75, PM/Scl-100, Th/To, B23/nucleophosmin, nucleolin, No55/SC65, U3-snoRNP/fibrillarin, RNA polymerase I, hUBF/NOR-90	Anti-PM/Scl, anti-Scl-70
Pleomorphic	PCNA, CENP-F	Anti-PCNA
Cytoplasmic speckled	GW182, Su/Ago2, Ge-1, PL-7, PL-12, ribosomal proteins, Jo-1/histidyl-tRNA synthetase	Anti-ribosomal P protein, anti-Jo-1
Cytoplasmic reticular	PDC-E2/M2, BCOADC-E2, OGDC-E2, E1α subunit of P DC, E3BP/proteinX	AMA-M2

[a]Adapted from reference 13. [b]LIA test used in this study. ANA, anti-nuclear antibody; AMA, anti-mitochondria antibodies; BCOADC, branched chain 2-oxo acid dehydrogenase complex; CENP, centromere-associated protein; DFS, dense fine speckled; dsDNA, double stranded deoxyribonucleic acid; E3BP, E3-binding protein; hUBF, human binding upstream factor; Jo-1, histidyl-tRNA synthetase; LEDGF, lens epithelium-derived growth factor; LIA, line immunoassay; NOR, nucleolar organizer regions; OGDC, 2-oxo-glutarate dehydrogenase complex; PCNA, proliferating cell nuclear antigen; PDC, pyruvate dehydrogenase complex; PM, polymyositis; RNA, ribonucleic acid; RNP, ribonucleoprotein; RNP, ribonucleoprotein; Scl, scleroderma; SS, Sjögren's syndrome; TIF, transcription intermediary factor.

Table 2: Comparative numbers of ANA-IIF and ANA-LIA tests.

									ANA-LIA	
	P	%[a]	%[b]	%[c]	N	%[a]	%[b]	%[c]	Total	%[c]
ANA-IIF										
P	141	(93.4)	(70.5)	(29.7)	10	(6.6)	(3.6)	(2.1)	151	(31.8)
N	59	(18.2)	(29.5)	(12.4)	265	(81.8)	(96.4)	(55.8)	324	(68.2)
Total (%)[c]	200	(42.1)			275	(57.9)			475	(100)

[a]In total ANA-IIF test, [b]n total ANA-LIA test, [c]n total number of sera (n = 475), P, positive; N, negative; ANA, anti-nuclear antibody; IIF, indirect immunofluorescence; LIA, line immunoassay.

a pattern (Table 3). In the comparison of tests for ANA-IIF pattern and associated ANA in the LIA test according to the number of sera, at least one pattern and associated ANA was detected in 118 (83.7%) of 141 IIF(+)/LIA(+) sera (Table 3). The overall agreement rate for pattern and associated ANAs in 475 sera was 80.6% with a Cohen's κ coefficient of 0.57, which indicates a moderate agreement.

Discussion

We found that the overall agreement rate between the ANA-IIF screening test and the reflex ANA-LIA test was 85.5% with a Cohen's κ coefficient of 0.69, indicating a moderate agreement. However, variable results have been reported by previous studies including 69.2% (κ 0.40) [1], 76.3% (κ 0.21) [10], 83.4% (κ 0.45) [15], 77.3% [7], 83.7% [9] and 84.3% [12]. In this study, the disagreement rate was 6.6% for ANA-IIF(+)/LIA(−) and 18.2% for ANA-IIF(−)/LIA(+) (Table 2). In other studies, this rate was reported to be 39.5% [1], 18.5% [10], 44.2% [7] and 17.2% [9] for ANA-IIF(+)/LIA(−) and 14.5% [1], 38.0% [10], 14.4% [7] and 15.7% [6] for ANA-IIF(−)/LIA (+).

The natural antigenic structure of HEp-2 cells is different from recombinant or purified antigens of LIA and this may affect the test results [16]. It has been suggested that multi-parametric detection of ANA using LIA may affect the optimal test performance of a single antibody [16]. Due to the deficiency of SS-A and Jo-1 antigens with a very high resolution on HEp-2 cells or fixation-induced denaturation, the appearance of ANA against these antigens in the IIF test leads to inaccurate results [4]. Although anti-SS-A, anti-SS-B, anti-Ro-52 and anti-Jo-1 antibodies were

Table 3: Comparative numbers of ANA-IIF test patterns and ANA-LIA test antibodies.

ANA-IIF	LIA																	
	dsDNA (n=49)[b] (%)[c]	**Histone (n=17)[b] (%)[c]**	**Nucleosome (n=28)[b] (%)[c]**	**SS-A (n=53)[b] (%)[c]**	**SS-B (n=24)[b] (%)[c]**	**Ro-52 (n=46)[b] (%)[c]**	**Sm (n=25)[b] (%)[c]**	**RNP/Sm (n=36)[b] (%)[c]**	**CENP-B (n=20)[b] (%)[c]**	**Ribosomal P (n=6)[b] (%)[c]**	**PM/Scl (n=10)[b] (%)[c]**	**PCNA (n=11)[b] (%)[c]**	**Scl-70 (n=6)[b] (%)[c]**	**DFS-70 (n=23)[b] (%)[c]**	**AMA-M2 (n=18) [b](%)[c]**	**Jo-1 (n=3)[b] (%)[c]**	**Negative (n=10)[d] (%)[c]**
Nuclear speckled (n=54)[a]	7 (13.0)	4 (7.4)	4 (7.4)	**26** (48.1)	**13** (24.1)	**25** (46.3)	**9** (16.7)	**24** (44.4)	1 (1.9)	0 (0)	0 (0)	1 (1.9)	0 (0)	1 (1.9)	0 (0)	0 (0)	0 (0)
Homogeneous (n=39)[a]	**23** (59.0)	**10** (25.7)	**18** (46.2)	14 (35.9)	5 (12.8)	10 (25.6)	9 (23.1)	6 (15.4)	2 (5.1)	5 (12.8)	2 (5.1)	0 (0)	4 (10.3)	2 (5.1)	0 (0)	0 (0)	1 (2.6)
Centromere (n=15)[a]	1 (6.7)	0 (0)	1 (6.7)	1 (6.7)	1 (6.7)	1 (6.7)	0 (0)	0 (0)	**14** (93.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (13.3)	0 (0)	1 (6.7)
Cytoplasmic reticular (n=15)[a]	0 (0)	0 (0)	1 (6.7)	3 (20.0)	1 (6.7)	1 (6.7)	1 (6.7)	0 (0)	1 (6.7)	0 (0)	0 (0)	0 (0)	0 (0)	2 (13.3)	**10 (66.7)**	0 (0)	2 (13.3)
Nucleolar (n=13)[a]	4 (30.8)	1 (7.7)	1 (7.7)	1 (7.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**2** (15.4)	1 (7.7)	**0 (0)**	1 (7.7)	2 (15.4)	0 (0)	5 (38.5)
Dense fine speckled (n=13)[a]	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (7.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**12** (92.3)	1 (7.7)	1 (7.7)	0 (0)
Cytoplasmic speckled (n=4)[a]	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (25.0)	0 (0)	0 (0)	1 (25.0)	0 (0)	0 (0)	1 (25.0)	**1** (25.0)	1 (25.0)
Negative (n=59)[d]	14 (23.7)	2 (3.4)	3 (5.1)	8 (13.6)	4 (6.8)	8 (13.6)	6 (10.2)	6 (10.2)	1 (1.7)	1 (1.7)	6 (10.2)	8 (13.6)	2 (3.4)	4 (6.8)	3 (5.1)	1 (1.7)	

[a]Number of patterns in the ANA-IIF test, [b]number of ANAs in the ANA-LIA test, [c]percentage of ANAs detected by ANA-LIA in the sera including the pattern, [d]number of sera (associated ANAs are emphasized in bold). AMA-M2, anti-mitochondrial antibodies; ANA, anti-nuclear antibody; CENP-B, centromere-associated protein-B; DFS-70, lens epithelium-derived growth factor (LEDGF); dsDNA, double-stranded deoxyribonucleic acid; IIF, indirect immunofluorescence; Jo-1, cytoplasmic histidyl-tRNA synthetase; LIA, line immunoassay; nRNP, nuclear ribonucleoprotein; PCNA, proliferating cell nuclear antigen; PM/Scl, polymyositis/scleroderma; Ro, Sjögren's syndrome antigen; Scl-70, topoisomerase 1; Sm, Smith antigen; SS-A, Sjögren's syndrome antigen A; SS-B, Sjögren's syndrome antigen B.

reported to be responsible for ANA-IIF(−)/LIA (+) results, we found a higher frequency of anti-dsDNA in our study [1, 17]. With regard to ANA-IIF(+)/LIA(−) results, anti-dsDNA which may be undetectable by LIA has been suggested to play a role [18]. In addition, our study method which required the consensus of three assessors to minimize the subjectivity for the ANA-IIF test may have led to a decrease in ANA-IIF(+) results.

It is well established that ANA-IIF patterns are closely associated with certain antibodies, and reflex testing is recommended for these ANAs in routine practice [6]. In this study, at least one pattern and associated ANA agreement was detected in the 118 sera (83.7%) tested. However, this agreement varied depending on the pattern type (Table 3). Pattern and associated ANA agreement were highest in centromere, DFS and cytoplasmic reticular patterns. In these patterns, the rate of anti-CENP-B, anti-DFS and anti-AMA-M2 antibodies were 93.3%, 92.3% and 66.7%, respectively, using the ANA-LIA test. Therefore, it should be noted that this moderate level of pattern agreement does not apply to all patterns. In the literature, some authors have reported an agreement rate for pattern and anti-CENP-B of over 90%, while others have shown lower rates such as 85% [9], 60% [10] and 25% [19]. In our study, the highest rate for nuclear speckled pattern-associated ANAs was found in anti-SS-A (48.1%), indicating a relatively moderate correlation. Consistent with previous studies, the agreement rate for speckled pattern-specific antibodies was higher than other ANAs [1, 17, 20]. Although anti-dsDNA and nucleosome were the most frequent antibodies in the homogeneous pattern, agreement rates were similar to the speckled pattern, and also had a high detection rate for anti-SS-A, anti-SS-B, anti-Ro-52, anti-Sm and anti-RNP/Sm. Similarly, others studies reported that these antibodies were the most frequently detected antibodies in the homogeneous pattern [1, 8, 10, 11]. In our study, anti-dsDNA was detected in 13% of the speckled pattern and in 30.8% of the nucleolar pattern, consistent with other studies [11, 21]. There are several studies suggesting that the agreement between the ANA-IIF testing and anti-ENA is only valid for the homogenous pattern [10, 22]. In our study, the lowest rate was found in the cytoplasmic speckled and nucleolar patterns. This finding is also consistent with previous studies, showing lower anti-Jo-1 and anti-PM/Scl antibodies in these patterns and no strong agreement [1, 8–10]. In addition, unlike LIA testing, we were unable to detect anti-PCNA and anti-Scl-70 antibodies-specific patterns by ANA-IIF testing. Although we used the consensus results by three assessors, particular technical difficulties in the diagnosis of these antibodies-specific patterns might have led to this result. Similarly, in their study, Lee et al. reported that the detection of PCNA-specific pleomorphic patterns could be difficult in mixed patterns [1]. As described in the present study, the agreement between the patterns and associated ANAs may vary according to the pattern type, and it was revealed that some of the ANAs were difficult to predict in the ANA-IIF test. Likewise, Verstegen et al. reported that the IIF pattern can be used to some extent to predict ANAs and is not useful for most patterns, except for centromere [19]. In another study, Kang et al. highlighted that the IIF pattern did not provide information on specific ANAs [22].

Along with many others, this study demonstrated that the reflex testing approach may not be always useful in the detection of ANAs [1, 8, 10, 11, 19, 22]. Kavanaugh et al. reported that the reflex testing approach in positive ANA-IIF results is not supported by scientific evidence [23]. The term reflex testing refers to a cascade diagnostic approach which is based on predefined rules and automatism, and requires no intervention by the laboratory professional. In the detection of ANAs, testing only antibodies associated with the HEp-2 pattern can cause many ANAs to be missed out. It is a known fact that multiple antibodies may be involved in the pathogenesis of SARDs [24]. Only strong clinical suspicion may trigger the testing of ANAs other than HEp-2-associated antibodies, which is only possible if there is an effective collaboration between clinicians and laboratory professionals. This approach is referred to as the reflective algorithm. This algorithm involves a clinical judgment (reflection) by a laboratory specialist in interpreting the results before further testing [25, 26]. As demonstrated by our results, it is essential that in case of strong clinical suspicion, not only HEp-2-related antibodies, but also other ANAs should be tested in the second step. Detecting ANAs by the ANA-LIA profile, however, is relatively expensive and many laboratories routinely use ELISA which tests ANAs separately. The reflective approach becomes even more important in laboratories which implement this system. Likewise, effective collaboration between laboratory professionals and clinicians may ensure that a confirmation test is performed in the next step even in case of a negative ANA-IIF test. Furthermore, some factors associated with the ANA-IIF test may require a review of clinical information when interpreting the test results. Firstly, the ANA-IIF test has low predictive value [11]. Clinical utility of this testing method mainly depends on pre-test probability and hence, the patient selection by the clinician. If used appropriately by the clinician, the predictive value of the ANA test may be improved [27]. There are, however, certain challenges involved; Abeles and Abeles reported

that ANAs were not requested appropriately by the clinicians and 90% of the test results fail to predict SARDs [28]. In addition, an international recommendation statement on ANA testing highlighted the importance of more deliberate utilization of ANA testing in the clinical practice [5]. Yet another confounding factor is the presence of ANAs in patients with infectious diseases, malignancies, in the elderly and even in up to 25–30% of healthy individuals [2, 4]. In addition, the ANA-positivity may be detected months or years before the signs of the disease appear, and certain antibodies such as anti-DFS-70 may not be associated with SARDs [29]. Assessor subjectivity is another challenge that interferes with ANA-IIF testing. To overcome the difficulties in distinguishing positivity and negativity and the challenge presented by multiple patterns, the laboratory specialist must be equipped with extensive knowledge of the disease before reporting and performing the confirmation tests. Some publications also highlight the importance of data sharing between clinicians and laboratory specialists, which ultimately leads to more satisfactory results in the detection and use of ANAs [11, 30–32].

Conclusions

We found an overall moderate agreement between IIF screening and LIA confirmation tests in the cascade algorithm used in the detection of ANAs. However, this agreement shows variability according to the pattern type. The agreement between ANA-IIF patterns and associated ANAs was higher for centromere, DFS and cytoplasmic reticular patterns, and the lowest for nucleolar and cytoplasmic speckled patterns. The discrepancy in the level of agreement between the ANA-IIF and anti-ENA tests may lead to inappropriate anti-ENA test requests. Our results highlight the need for collaboration between clinical and laboratory professionals in selected cases instead of the reflex testing approach.

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