

Anti-double stranded antibody

Association with titers and fluorescence patterns of anti-nuclear antibody in systemic lupus erythematosus

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ABSTRACT

الأهداف: معظم المختبرات لا تقوم بإجراء اختبار الأجسام المضادة للنواة (ANA)، عن طريق الاختبار المناعي المتألق غير المباشر (IIF)، بالتوالي مع قياس الأجسام المضادة للحمض النووي (DNA) المزدوج التركيب. هذه الدراسة تهدف للتحقق من فعالية هذه الممارسة.

الطريقة: أجريت دراسة استرجاعية لتحليل بيانات نتائج قياسات الأجسام المضادة للنواة والأجسام المضادة للحمض النووي في 106 مريض تم تشخيصهم كحالات مرض الذئبة الحمراء (SLE) وبعض المرضى يشبه في إصابتهم بهذا المرض مستشفى الملك خالد الجامعي - الرياض - المملكة العربية السعودية. تم استخدام الاختبار المناعي المتألق غير المباشر (IIF) لقياس الأجسام المضادة للنواة والاختبار المناعي الإنزيمي لقياس الأجسام المضادة للحمض النووي (DNA) عن طريق اختبار أليسا.

النتائج: تفوق عدد المصابين من الإناث بنسبة عالية (96.3%) كما وأن النوع الخشن من نمط التآلق كان الأكثر شيوعاً (60.4%). لم يتم أي رصد للأجسام المضادة للحمض النووي (DNA) حتى بلوغ معدل تخفيف الأجسام المضادة للنواة (ANA) درجة (1:320). ويمكن رصد هذه الأجسام المضادة في أغلب العينات التي يكون فيها معدل تخفيف الأجسام المضادة للنواة في درجة (1:640) أو أكثر. الأنماط الأخرى للتآلق والتي تم فيها رصد الأجسام المضادة للحمض النووي في درجات تخفيف متدنية للأجسام المضادة للنواة (1:40) كانت على النحو التالي: تآلق ناعم (14.15%)، تآلق متجانس (9.4%)، تآلق النمط المضاد للميتوكوندريا (7.5%)، تآلق النمط الرايوسومي (4.7%) وتآلق النمط النووي (3.8%). أظهر التحليل الوبستي الخطي علاقة إحصائية واضحة بين معدلات قياس الأجسام المضادة للنواة (ANA) والأجسام المضادة للحمض النووي فقط في حالات النمط المتألق الخشن ($p=0.02$).

خاتمة: عدم وجود أجسام مضادة للحمض النووي (DNA) في حالة رصد معدلات واضحة إكلينيكية للأجسام المضادة للنواة (ANA) في النمط المتألق الخشن ووجود هذه الأجسام المضادة للحمض النووي بصورة ثابتة في معدلات التركيز العالية للأجسام المضادة للنواة ربما يؤدي لخطأ في تشخيص مرض الذئبة الحمراء (SLE). أيضاً عدم قياس الأجسام المضادة للحمض النووي في العينات التي بها معدلات قياس منخفضة للأجسام المضادة للنواة، ولا سيما في أنماط التآلق الأخرى.

Objectives: Many laboratories do not test antinuclear antibodies (ANA) by indirect immune-fluorescence (IIF) in parallel with anti-double stranded (ds) DNA antibodies. This study attempts to investigate the legitimacy of such practice.

Methods: A retrospective laboratory data analysis of simultaneous assessment of ANA and anti-dsDNA antibody results of 106 patients with either diagnosed or suspected systemic lupus erythematosus (SLE) was performed at King Khalid University Hospital, Riyadh, Kingdom of Saudi Arabia. The ANA was detected by IIF on HEp2 cells and anti-dsDNA antibodies were assessed by specific ELISA test.

Results: Among the patients, female preponderance (96.3%) was evident and a coarse speckled fluorescence pattern was commonly observed (60.4%). There was almost no detection of anti-dsDNA antibodies up to an ANA titer of 1:320. Anti-dsDNA antibodies were often detected at ANA titers of 1:640 and beyond. Other patterns of fluorescence observed at ANA titers as low as 1:40 and at higher dilutions were, fine speckled (14.15%), homogeneous (9.4%), anti-mitochondrial (7.5%), ribosomal (4.7%), and nucleolar (3.8%). Linear regression analysis revealed a statistically significant relationship ($p=0.02$) between ANA titers and anti-dsDNA antibodies only in the presence of a coarse speckled pattern.

Conclusions: The rare occurrence of anti-dsDNA antibodies at clinically significant ANA titers associated with the coarse speckled pattern may mask the diagnosis of SLE. Similarly, the diagnosis of SLE may be overlooked if anti-dsDNA antibodies are not checked in the presence of clinically insignificant ANA titers associated with other patterns of fluorescence.

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Antinuclear antibody (ANA) tests are commonly performed on sera from patients with various connective tissue diseases (CTDs), including systemic lupus erythematosus (SLE). This test, apart from serving as an important diagnostic and prognostic tool, also helps in guiding therapeutic management.¹⁻³ On the basis of autoantibody detection, the ANA test has been categorized in 2 broad subtypes. These include a subgroup including anti-double stranded DNA (anti-dsDNA) antibodies and anti-histone antibodies,⁴⁻⁸ and a subgroup of auto-antibodies against extractable nuclear antigens (ENAs) that include antibodies against Smith antigen (Sm), nuclear ribo-nucleoprotein, Ro/Sjögren's syndrome antigen A (SSA), or La/ Sjögren's syndrome antigen B (SSB), Scleroderma-70 (Scl-70), Jo-1, and so forth.⁹⁻¹⁶ Most of these ENAs are disease specific; a significant overlap however exists.^{17,18} Anti-dsDNA and the Sm antibodies are considered highly specific for SLE,¹⁹⁻²¹ and the former has also been proposed as a marker of disease activity.^{22,23} The ANA test is based on an indirect immunofluorescence (IIF) technique for the detection of auto-antibodies. Several fluorescence patterns have been described in various autoimmune disorders, and because of the overlap between the fluorescence patterns and diseases it is difficult to assign a particular pattern to a specific disease condition.^{24,25} Similarly, patients suffering from a variety of diseases have been tested positive for ANA, thus undermining the specificity of the test. The sensitivity of the ANA test is however, more than 95% in patients with SLE, thereby increasing the chances of false positive results.²⁵ Several studies have attempted to determine the optimum screening dilution of sera for ANA testing. A titer of 1:160 is generally considered as significant for the diagnosis of CTDs in most laboratories.²⁶ Whereas a positive ANA test may suggest the presence of CTD, a negative test is associated with a high probability to rule out the presence of CTD. In the backdrop of symptoms and signs of CTD, a positive ANA test may warrant further investigations for a definitive diagnosis of an autoimmune disorder. The ANA test as a screening tool is commonly requested as an initial investigation for the diagnosis of CTDs. The clinician's decision to either rule out or further investigate the patient for evolving/presence of CTDs is often based on the result of the ANA test. Simultaneous testing for anti-dsDNA antibodies being highly specific, and ANA with its sensitivity may enhance the level of detection of SLE in suspected cases. This study was performed to investigate whether ANA test alone or in combination with assessment for anti-dsDNA antibodies has any impact on picking up cases of SLE.

Methods. This is a retrospective analysis of the data collected from laboratory reports for detection

of ANA and anti-dsDNA antibodies in the division of Immunology at King Khalid University Hospital, Riyadh, Kingdom of Saudi Arabia between November 2008 and June 2009. Approval from the ethical committee of College of Medicine Research Ethics Committee was obtained before the initiation of study. The patients were either diagnosed or suspected cases of SLE. Among the total of 218 requests, 106 patients in whom simultaneous assessment of ANA and anti-dsDNA antibodies had been requested were included in the study. There were 102 (96.3%) females with a mean age of 31±12 years (range 19-43 years) and 4 (3.7%) males with a mean age 18±3 years range (15-21 years). Out of these 106 patients, 73 were confirmed cases of SLE, and 33 had clinical suspicion of SLE. Data were extracted for ANA fluorescence pattern, ANA titer, and the anti-dsDNA antibody levels. Patients with missing information on any of these parameters were excluded from the study. Information regarding the ENAs was also recorded. Lack of access to the clinical data prevented confirmation of diagnosis in patients with suspected diagnosis of SLE. Indirect immunofluorescence was performed using HEP2 cells as a substrate and the results were reported as titer of ANA with the observed pattern of fluorescence. In the present study, an ANA test was regarded as negative if no fluorescence was observed, otherwise, fluorescence observed at titers as low as 1:40 dilution was regarded as positive. An ELISA test for anti-dsDNA antibody detection was performed using the Quanta Lite dsDNA ELISA Kit (INOVA Diagnostics, Inc, San Diego, CA, USA) in accordance with the instructions of the manufacturers and all detectable serum levels of anti-dsDNA antibody in IU/ml were recorded.

Statistical analysis. Simple linear regression analysis of the data for assessing the relationship between serum ANA titers with different patterns of fluorescence and anti-dsDNA antibody levels was performed using Smith's Statistical Package. Findings were considered statically significant when the *p*-value was either equal to or less than 0.05.

Results. The study population in this study predominantly comprised female patients (96.3%). The patterns of IIF staining observed were coarse speckled (60.4%), fine speckled (14.15%), homogenous (9.4%), anti-mitochondrial (7.5%), ribosomal (4.7%), and nucleolar (3.8%). **Figure 1** shows data of coarse speckled IIF ANA staining patterns observed at doubling dilution titers of ANA starting from 1:40 relating to the detection of anti-dsDNA antibody. Whereas no patient with ANA titers of 1:40 or 1:80 had any detectable anti-dsDNA antibody, only one patient each in 1:160 and 1:320 titers of ANA had detectable serum anti-dsDNA

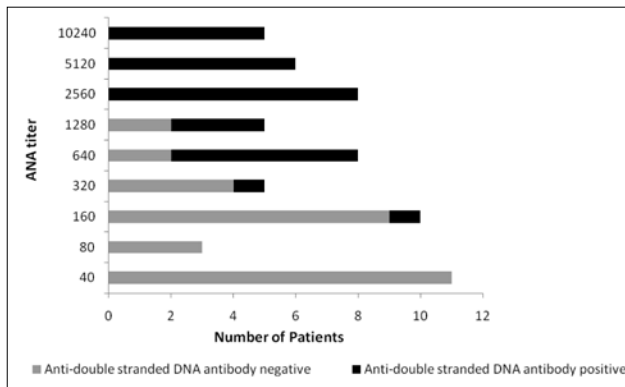


Figure 1 - Serum anti-double stranded DNA antibody levels related to ANA titers in 64 patients with a coarse speckled pattern of immunofluorescence. ANA - antinuclear antibody

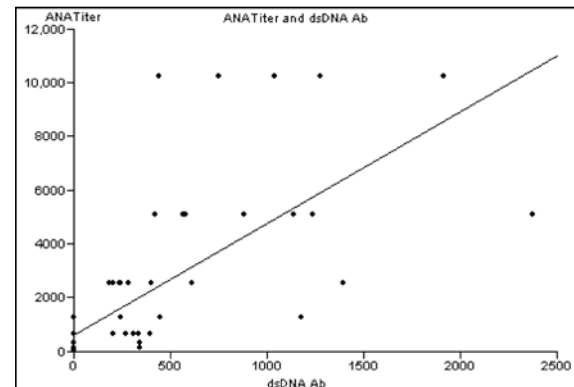


Figure 2 - Simple linear regression analysis of serum ANA titers with a coarse speckled pattern of immunofluorescence and anti-dsDNA antibody levels in IU/ml. ANA - antinuclear antibody, dsDNA Ab - anti-double stranded DNA antibody, coefficient of determination (r^2) = 0.5126, $p < 0.02$.

antibody. At 1:640 and 1:1280 dilutions, anti-dsDNA antibodies were being frequently detected, and beyond this limit of ANA titer, anti-dsDNA antibodies were present consistently. Simple linear regression analysis revealed a significant relationship between ANA titers and anti-dsDNA antibody levels only for a coarse speckled pattern of fluorescence. Figure 2 shows the linear regression analysis depicting a significant relationship ($p=0.02$) between the 2 parameters in 64 patients with a coarse speckled pattern, where the coefficient of determination (r^2) value was 0.5126. In contrast to these findings, patients with other patterns of IIF staining were found to have detectable levels of

anti-dsDNA antibodies in their sera at ANA titers as low as 1:40 and above. Table 1 shows other IIF staining patterns observed at ANA titers starting from 1:40 and corresponding serum anti-dsDNA antibody levels. Interestingly, all the patients with a homogenous pattern of IIF staining were tested positive for anti-dsDNA antibodies notably at ANA titers of 1:40. Similarly, significant levels of anti-dsDNA antibodies were frequently detected at ANA titers that are considered clinically as negative ANA test. Comparative analysis of the data shows that in the presence of a coarse speckled fluorescence pattern of IIF ANA test, anti-dsDNA antibodies may not be detected in the sera of patients

Table 1 - Anti-double stranded DNA antibody detection by ELISA related to ANA titers and other patterns of immunofluorescence observed in SLE.

FS		Homo		AM		Ribo		Nu	
ANA Titer	dsDNA Ab IU/ml	ANA Titer	dsDNA Ab IU/ml	ANA Titer	dsDNA Ab IU/ml	ANA Titer	dsDNA Ab IU/ml	ANA Titer	dsDNA Ab IU/ml
1:40	266	1:40	189	1:40	-	1:80	243	1:40	-
1:40	296	1:40	223	1:160	-	1:80	528	1:40	1811
1:40	244	1:160	229	1:320	473	1:160	362	1:160	286
1:40	-	1:160	362	1:640	-	1:320	305	1:2560	252
1:80	-	1:1280	280	1:1280	1826	1:320	308		
1:80	-	1:2560	908	1:1280	1090				
1:80	453	1:2560	1175	1:2560	-				
1:160	777	1:5120	415	1:2560	348				
1:320	276	1:5120	2236						
1:320	461	1:5120	275						
1:320	1423								
1:640	225								
1:1280	1481								
1:1280	193								
1:1280	440								

n - 42, - - negative test, ANA - antinuclear antibody, dsDNA Ab - anti-double stranded DNA antibody, FS - Fine speckled, Homo - homogenous, AM - anti-mitochondrial, Ribo - ribosomal, Nu - nucleolar, SLE - systemic lupus erythematosus

at ANA titers that are considered clinically significant. Anti-dsDNA antibodies in patients demonstrating a coarse speckled pattern may be sought when ANA titers are at or beyond 1:640, in case these were not detected at lower dilutions in rising ANA titers. Similarly, ANA titers as low as 1:40 may warrant further investigation and should not be ignored especially in the presence of other fluorescence patterns as there may be associated evidence for diagnosis of CTDs. Other ENAs detected were anti-SSA (16%), anti-SSB (3%), anti-Sm (11%), anti-RNP (2.1%), anti-nucleosome (2.1%), and anti-histone (0.9%) (data not shown).

Discussion. Based only on the retrospective laboratory data analysis, this study focuses on the interplay between ANA titers and the presence of anti-dsDNA antibodies. It was shown that ANA titers and fluorescence pattern exhibit a definite relationship in patients either with confirmed diagnosis or clinical suspicion of SLE. There are insufficient data comparing ANA test with anti-dsDNA antibody levels, however, one study²⁶ has attempted to examine these parameters and failed to detect any relationship between ANA titers and anti-dsDNA antibody levels in patients with rheumatic diseases. Since the substrate used for ANA detection in this and the previous study was HEp2 cells, it might not have contributed to the observed disparity. The difference in study populations might have been an important factor in the 2 studies. Inclusion criteria of patients in the present study specifically with requests for simultaneous assessment of ANA test and anti-dsDNA antibodies might have increased the likelihood to detect the observed relationship.

Using HEp2 cells as a substrate, a titer of 1:160 is generally regarded as a cut off point for interpreting an ANA test as clinically significant.²⁶ A separate study on healthy individuals using the same substrate for ANA titer determination recommended a cut-off point of 1:320 dilution.²⁷ A large number of cut-off points determined for a variety of other laboratory tests have been based on the observations in a normal healthy population. The findings of the present study indicate that ANA titers observed in the otherwise normal population may not be applicable to define a reliable cut-off point for ANA titers. This was clearly evident from the fact that anti-dsDNA antibodies could be detected in ANA titers as low as 1:40 with fluorescence patterns other than coarse speckled, and was rarely detected at ANA titers that are considered clinically significant when coarse speckled fluorescence was observed.

A homogenous pattern of fluorescence has been strongly associated with the diagnosis of SLE based on the high probability of detecting anti-dsDNA antibodies.²⁸ The homogenous pattern of fluorescence in

the present study was also shown to have detectable anti-dsDNA antibodies even at low titers. On the contrary, it has been difficult to associate a specific fluorescence pattern to a particular disease condition because of the overlap.²⁴ A coarse speckled pattern of fluorescence was most frequently detected in the present study. Since the diagnosis of SLE could not be confirmed in a significant number of patients in the present study due to the lack of access to the relevant information, it is difficult to claim that all the patients in whom a coarse speckled pattern was observed had SLE. It has also been suggested that a particular fluorescence pattern is patient specific and not the disease. In addition, when using HEp2 cells as a substrate, the fluorescence pattern of anti-DNA antibodies can vary and is not restricted to any specific type.²⁹ Reliance on a particular fluorescence pattern alone may be confusing, especially when HEp2 cells are used as a substrate. It is therefore, important to interpret the ANA test based not only on the fluorescence pattern but the titer along with other relevant investigations and clinical evidence in SLE.

There is sufficient evidence to indicate that the ANA titers and the pattern of fluorescence are important to predict the presence of anti-dsDNA antibodies.^{28,30} This was evident in the present study, particularly for a coarse speckled pattern of fluorescence where high titers of ANA were associated with consistent detection of anti-dsDNA antibody. Regression analysis between the 2 parameters revealed a statistically significant relationship, which could not be observed in other fluorescence patterns. It is therefore, important that when a coarse speckled pattern on HEp2 cell line is reported, especially in clinically significant titers up to 1:320, the appearance of the anti-dsDNA antibodies may occur as a late event in SLE, especially in rising ANA titers. Similarly, ignoring clinically insignificant titers as low as 1:40 may have important bearing as the presence of anti-dsDNA antibodies may be overlooked.

The findings of this study were based on anti-dsDNA antibody assessment by specific ELISA test. This being a highly sensitive test might have increased the chances of false positive results. A specific ELISA test for anti-dsDNA antibody detection has also been reported to be capable of detecting anti-dsDNA antibodies in other rheumatic diseases.³¹ In addition, detection of anti-dsDNA antibodies using the *Crithidia luciliae* assay is considered to be more specific for diagnosing SLE.³¹ This study was limited by the lack confirmation of the diagnosis of SLE in a significant number of patients and the availability of the *Crithidia luciliae* assay data. It would be interesting to perform a similar study investigating these parameters with data generated by a *Crithidia luciliae* assay in a larger group of patients with confirmed diagnosis of SLE.

The association of ANA and anti-dsDNA antibodies reported in this study was entirely based on the laboratory data. Although, simultaneous presence of ANA and anti-dsDNA antibodies increased the likelihood of the diagnosis of SLE, the lack of access to the clinical information denied confirmation of diagnosis in patients suspected to have SLE. For the same reason, it was not possible to relate the findings of this study with the clinical course of the disease. Further studies involving correlation of laboratory data to clinical findings in SLE may help in gaining a better understanding of the observed relationship.

In conclusion, interpretation of the ANA test as a screening test may not be as simple as it appears. This study clearly shows that there is no direct relationship of the titer or pattern of fluorescence staining and the presence of anti-dsDNA antibodies, except when a coarse speckled fluorescence pattern is present. In the presence of signs and symptoms of SLE, ANA testing should be accompanied by anti-dsDNA antibody assessment along with other relevant laboratory investigations. If a coarse speckled pattern of fluorescence is reported, appearance of anti-dsDNA antibodies may occur at a later date with rising ANA titers and deserves re-evaluation in suspected SLE.

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