Validation of a new screening strategy for anti-extractable nuclear antigen antibodies

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Abstract

Objective

Although indirect immunofluorescence (IIF) is the most widely applied screening test for antinuclear antibodies (ANA), it lacks specificity for the identification of specific diseases or antigen reactivities. The aim of the present study was to validate an anti-extractable nuclear antigen (ENA) screening strategy encompassing a three-step cascade whereby an ELISA with pooled specific ENA is positioned between the IIF and the final anti-ENA identification.

Methods

Sera from 4 populations were tested for anti-ENA using an automated ELISA (EliA Symphony) and a line immunoassay (INNO-LIA ANA update).

Results

At the manufacturer's cut-off, a 96% sensitivity (95% CI 94%–98%) and 96% specificity (95% CI 94%–98%) of EliA Symphony for anti-ENA was obtained in a consecutive selection of 328 IIF positive serum samples referred for ANA testing. In addition, a high sensitivity was demonstrated for anti-ENA reactivities in patients with SLE (99%, 95% CI 97%–101%) and SSc (100%), and for anti-ENA monoreactivities.

Conclusion

The EliA Symphony test was shown to be a sensitive second-line screening test for anti-ENA antibodies. In the context of a high clinical suspicion of connective tissue disease or autoreactivities not included in the EliA Symphony assay, third-line testing may be useful, even if the anti-ENA screening is negative.

Key words

Anti-extractable nuclear antigen antibodies, ROC curve, sensitivity and specificity, systemic lupus erythematosus, systemic sclerosis.

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Abbreviations:

ANA:	antinuclear antibodies
SLE:	Systemic lupus erythematosus
SSc:	systemic sclerosis
MCTD:	mixed connective tissue disease
SS:	Sjögren's syndrome
ENA:	extractable nuclear antigen
IIF:	indirect immunofluorescence
LIA:	line immunoassay

Competing interests: none declared.

Introduction

Antinuclear antibodies (ANA) are a hallmark of various connective tissue diseases, including systemic lupus erythematosus (SLE), systemic sclerosis (SSc), mixed connective tissue disease (MCTD) and Sjögren syndrome (SS) (1). The detection and identification of ANA may help the clinician to confirm the diagnosis. In some cases, ANA also offer prognostic information, as some specific antibodies are associated with disease manifestations (1, 2). The nuclear targets of ANA, apart from DNA, are usually referred to as extractable nuclear antigens (ENA). Indirect immunofluorescence (IIF) on a substrate of fixed and permeabilised human cells, such as HEp-2 or HEp-2000 cells, is the most widely applied screening test for ANA, including anti-ENA and anti-DNA antibodies. However, the test lacks specificity for the identification of specific diseases or antigen reactivities. Indeed, upon further testing with assays developed to identify anti-DNA or anti-ENA antibodies, the majority of ANA IIF positive samples turn out to be negative (nearly 80% with a 1:40 serum dilution) (3). A convenient method for identifying anti-DNA antibodies is IIF on a substrate of Crithidia luciliae. For anti-ENA antibodies, multiplex technologies have recently been developed, including line immunoassay (LIA) and laser-based beads arrays (4, 5). Both assays have the advantage of testing for multiple reactivities simultaneously.(6) This article focuses on the optimization of a screening assay for anti-ENA antibody detection.

Given the lack of specificity of ANA IIF for detecting anti-ENA, a substantial number of true negative anti-ENA samples are further worked out in a classical two-step approach, in which all ANA IIF positive samples are elaborated with a test designed to identify specific anti-ENA reactivities. This procedure, however, requires substantial and not optimally applied resources. A possible opportunity to overcome this problem lies in the introduction of a three-step algorithm to detect anti-ENA antibodies, whereby an automated ELISA (EliA Symphony) containing selected ENA as antigen substrate is positioned between the IIF and the final identification assay (Fig. 1). The study reported here was a validation of this three-step cascade for anti-ENA detection and identification. Although this algorithm has already been applied in clinical practice, no studies have addressed its accuracy. The antigens used in the EliA Symphony test are recombinant SSA/Ro (Ro52 and Ro60), SSB/La, U1-RNP (RNP-70k, RNP-A and RNP-C), Scl-70/Topo-I, Jo-1, CENP-B and purified Sm. In the current study, we pay particular attention to the sensitivity of the new strategy for anti-ENA detection in clinically well-defined serum samples and to the number of unnecessary identification assays that it avoided.

Methods

Patients and samples

Four groups of serum samples were used. Population A was drawn from a consecutive collection of sera sent for ANA testing by in-house rheumatologists (from December 2006 until March 2007). From each patient for whom more than one ANA test was ordered in the evaluation period, only the first sample was included. All ANA IIF positive sera were retained (n=328). Median age was 50 yrs (range 7–90) and 79% were female. To identify the clinical relevant samples, all of population's medical charts were reviewed. The following combinations of a clinical diagnosis with an anti-ENA reactivity were considered clinically relevant: anti-SSA/Ro or anti-SSB/La and RA, SLE, SS or SSc; anti-Scl-70/Topo-I or anti-CENP-B and SSc; anti-Jo-1 and polymyositis (PM); anti-RNP and MCTD, SLE or SSc; and anti-Sm and SLE.

Population B was selected from a previously described cohort of SLE patients (2) (all fulfilled the ACR revised criteria for SLE)) (7, 8). From the original cohort of 235 patients, sera that were still available for testing (n=186) were used. Median age was 39 yrs (range 17-77) and 83% were females.

Population C consisted of a consecutive cohort of 93 SSc patients (all fulfilled LeRoy and Medsger's criteria for SSc (9). Median age in this population was 53 yrs (range 15–82) and 72% were females.

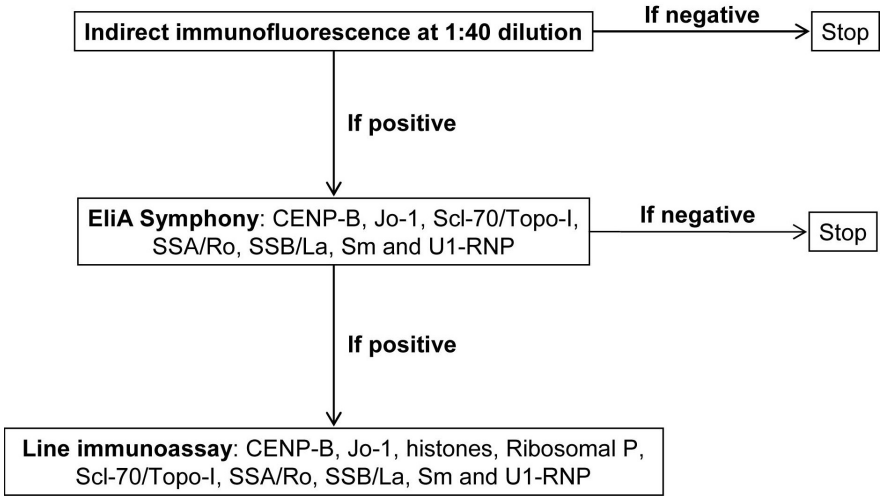

Fig. 1. Schematic depiction of the new screening strategy for anti-ENA antibodies.

Population D consisted of a cohort of clinically documented sera with anti-ENA monoreactivity on LIA. This cohort was derived from a consecutive collection of sera sent to the laboratory for ANA testing by in-house rheumatologists (Ghent University Hospital). If more than one sample from the same patient was sent to the laboratory during the period of inclusion (May 2002 to July 2006), only the first sample was used for this study. Samples displaying monoreactivity on LIA were selected (n=397). After reviewing all medical charts, only patients with an established connective tissue disease typically associated with an anti-ENA antibody included in EliA Symphony (see above) and not undergoing therapy known to induce ANA (anti-TNF, sulphasalazin or D-penicillamine) at the time of the blood collection were retained (n=72). Median age of patients in this population was 48 yrs (range 12–77) and 75 % were females.

All the serum samples of populations B, C and D were kept frozen (-20°C) after initial testing with IIF and LIA until the EliA Symphony was performed.

This study was conducted after approval by the ethics committee of Ghent University Hospital.

Identification of anti-ENA by LIA

The INNO-LIA ANA Update (Innogenetics NV, Zwijnaarde, Belgium) was conducted as described previously (2). The nylon strips used in this procedure contain the following antigens: recombinant SSA/Ro52, SSB/La, SmB, RNP-A, RNP-C, RNP-70, CENP-B, Scl-70/Topo-I, Jo-1; synthetic peptides of SmD and Ribosomal P; and SSA/Ro60 and histones as natural antigens. According to the manufacturer, a positive test for anti-U1-RNP is defined as the presence of at least 2 of the 3 anti-U1-RNP autoreactivities. For a positive anti-Sm result, the presence of anti-SmD antibodies is required. Either the presence of anti-Ro52 or anti-Ro60 is sufficient for the test to be positive for anti-SSA/Ro.

Detection of ANA by IIF

IIF was performed at a 1:40 or 1:160 serum dilution on HEp-2000 cells (Immunoconcepts, Sacramento, CA, USA) as described previously (3).

Screening for anti-ENA by ELISA

The EliA Symphony assay was performed on the ImmunoCAP 250 instrument (Phadia GmbH, Freiburg, Germany) according the manufacturer's instructions. The antigens used in the EliA Symphony are recombinant SSA/Ro (Ro52 and Ro60), SSB/La, U1-RNP (RNP-70k, RNP-A and RNP-C), Scl-70/Topo-I, Jo-1, CENP-B and purified Sm. The manufacturer's cut-off value was used (negative if ratio<0.7). Since the assay was used as a screening tool, borderline test results (defined by the manufacturer as a ratio ≥0.7 and <1.0) were considered positive. The inter-test variability, expressed as a CV percentage, was calculated to be 13.5% using a positive control sample provided by the manufacturer (mean ratio = 27.0, n=75).

Statistical analysis

Sensitivities and specificities, including their 95% confidence intervals, and positive and negative likelihood ratios were calculated as described previously (10). ROC curve analysis and the calculation of area under the curve, with 95% confidence intervals, was performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Accuracy of EliA Symphony for detection of anti-ENA in IIF positive samples

In order to get insight in the accuracy of the EliA Symphony assay, a ROC curve was constructed using 328 consecutive IIF positive samples, all of which were positive at a 1:40 serum dilution. Both the INNO-LIA ANA Update, used as reference assay for anti-ENA, and the EliA Symphony, the test assay, were performed on all samples. The medical charts of the corresponding patients were reviewed to obtain the clinical diagnosis. An overview of the diagnoses is shown in Table I.

Serum samples of patients with an established connective tissue disease and anti-ENA typically associated with the disease (for definition, see *Methods*) were regarded as 'true' positive (n=66). The diagnoses of these patients were: SSc (n=33), SLE (n=27), RA (n=2) and SS (n=4). As a negative control population, we selected the serum samples of the patients in whom a connective tissue disease was excluded and who did

Table I. Overview of diagnoses in cohort A (n=328).

Diagnosis	Number (%)
Polymyositis	1 (0.3)
Reumatoid arthritis	29 (8.8)
Sjögren syndrome	5 (1.5)
Systemic lupus erythematosus	46 (14)
Systemic sclerosis	56 (17)
No connective tissue disease	191 (58)

not have anti-ENA (n=183). All other samples were withdrawn from the analysis. ROC curve analysis yielded an area under the curve of 0.973 (95%CI 0.939–1.006) (Fig. 2). At the cut-off of the manufacturer, a 96% sensitivity (95% CI 94%–98%) and 96 % specificity (95% CI 94%–98%) were obtained. These correspond to a positive likelihood ratio of 24, and a negative likelihood ratio of 0.042. In addition, the manufacturer's cut-off proved to be the most optimal, since it was the closest point to the upper left corner of the ROC curve.

Sensitivity of EliA Symphony for detection of anti-ENA reactivities in patients with SLE or SSc

The EliA Symphony assay and the INNO-LIA ANA Update were conducted on consecutive cohorts of patients with SLE (n=186) or SSc (n=93) who fulfilled the classification criteria for the disease. The samples containing anti-ENA reactivities detected by the reference assay and which are typically associated with the SLE or SSc (see *Methods*) were considered positive. In the SLE cohort, 93 samples were positive (50%), 99% of which were also detected by the EliA Symphony (95% CI 97%–101%). In the SSc cohort, 79 samples were positive (85%), all of which were captured by the EliA Symphony. In summary, the EliA Symphony demonstrated excellent sensitivity for anti-ENA reactivities in SLE and SSc patients.

Sensitivity of EliA Symphony for detection of monoreactive anti-ENA positive sera

Although the EliA Symphony assay showed high overall sensitivities for anti-ENA in three consecutive cohorts, a minority of the samples was missed. To exclude a deficiency of the test assay for the detection of a particular anti-ENA reactivity, the EliA Symphony was conducted on a consecutive series of clinically relevant monoreactive sera (n=72). Diagnoses of this cohort were: MCTD (n=1), PM (n=2), RA (n=3), SLE (n=21), SS (n=4), SSc (n=37) and overlap syndrome between RA and SLE (n=4). The results of these samples were pooled with those of the monoreactivities from the three other consecutive cohorts (n=99). The diagnoses of the 99 additional samples were: RA (n=1), SLE (n=52), SS (n=1) and SSc (n=45). For most reactivities, optimal sensitivity was found (Table II). Nevertheless, the non-detection of 5% of the anti-Scl-70/Topo-1 and 20% of the anti-Sm positive monoreactive samples is worrying, as these reactivities are not infrequently present as an isolated autoantibody.

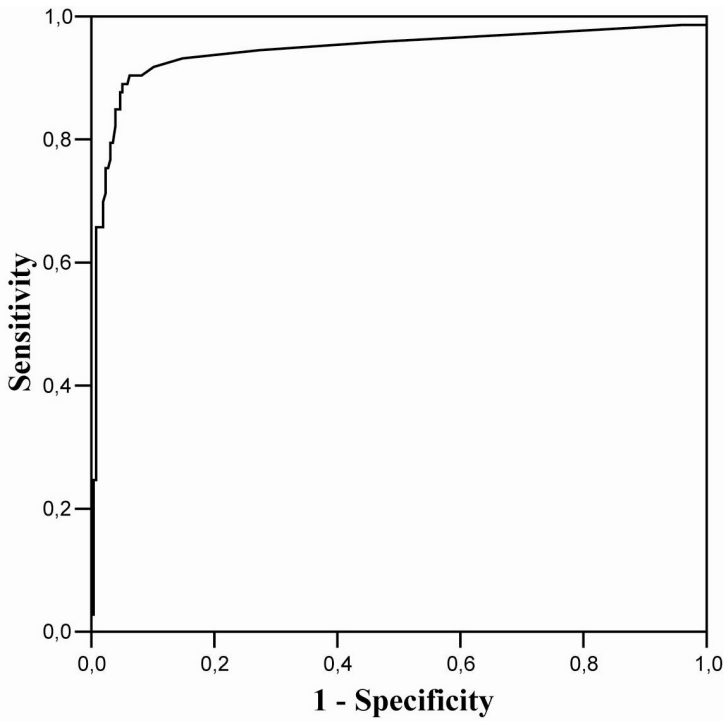

Fig. 2. ROC curve analysis of the EliA Symphony assay (population A). The area under the curve is 0.973 (95% CI 0.939-1.006).

Table II. EliA Symphony results in patients with anti-ENA monoreactivities* (n=171).

Anti-ENA reactivity (LIA)	EliA Positive (ratio ≥ 0.7)
CENP-B (n=51)	51 (100%)
Jo-1 (n=2)	2 (100%)
U1-RNP (n=27)	27 (100%)
SSA/Ro† (n=53)	53 (100%)
SSB/La (n=6)	5 (83%)
Scl-70/Topo-I (n=22)	21 (95%)
Sm† (n=10)	8 (80%)

*Values indicate number of samples.
†According to the INNO-LIA ANA Update manufacturer, either the presence of anti-Ro52 or anti-Ro60 is sufficient for the test to be positive for anti-SSA/Ro. For a positive anti-Sm result, the presence of anti-SmD antibodies is required. A positive test for anti-U1-RNP is defined as the presence of at least 2 of the 3 anti-U1-RNP autoreactivities.

Logistical advantage of the new algorithm

A potential advantage of the use of EliA Symphony is a reduction in the number of final identification assays to be performed. In contrast, multiplex final identification assays are more labour intensive and non-automated, whereas laboratories using individual ELISA for final identification face the costs of conducting several assays. We explored the reduction in the number of final assays using cohort A. Of the consecutive samples that were positive on IIF with a 1:40 serum dilution, only 107 had to be further worked out, corresponding to a 67% (95% CI 65%–70%) decrease in the number of final assays to be performed. In some auto-immune laboratories, however, IIF is performed at a 1:160 dilution, or both the results of 1:40 and 1:160 dilutions are used in decision-making (11). Therefore, we conducted the IIF at a 1:160 dilution on all samples of cohort A. 45% of the cohort was positive at this dilution (n = 149). As 87 samples had a positive EliA Symphony test, a 42% (95% CI 39%–44%) decrease in the number of final assays to be performed was observed.

Discussion

In most laboratories, screening for anti-ENA is performed with IIF. Not only is this the most sensitive screening test available: in some countries reimbursement for further anti-ENA detection is only provided if IIF yields a positive result. Unfortunately, the specificity of this screening test is low, which is reflected in the low percentage of IIF positive sera in which specific anti-ENA reactivities can be detected.(3) To enhance the efficiency of this process, we propose that, following a positive IIF test, an anti-ENA ELISA screening containing a pooled group of antigens (SSA/Ro, SSB/La, Sm, U1-RNP, CENP-B, Scl-70/Topo-I and Jo-1) should be performed, thus converting the classical two-step staged approach into a three-step detection algorithm (Fig. 1). Although this strategy has already been implemented in some autoimmune laboratories, it has never been validated. Previous studies on the EliA Symphony assay have focussed on its use as a first-line screening test for ANA (12, 13). This study was designed to investigate the sensitivity of the three-step algorithm and to explore the performance of the EliA Symphony in the algorithm by means of a ROC curve analysis.

In a cohort reflecting typical patients for whom the test assay is ordered, we found a high sensitivity for clinically relevant anti-ENA positive samples. As criteria for the identification of positive and negative samples, we used the clinical diagnosis obtained from the medical record and the result of a validated and optimised reference assay (INNO-LIA ANA Update). In a large multicenter study, the overall sensitivity and specificity of this LIA was shown to be similar to or higher than combined conventional techniques, and the cut-offs for the different reactivities were optimised to obtain a 98% specificity (4, 14). Only patients with the clinical diagnosis of a connective tissue disease and a corresponding anti-ENA were considered as 'true' positive. Although this combination can serve as an obvious gold standard, these criteria may be subject to bias as diagnostic errors of both the clinician and the reference assay can occur. This potential bias was minimised by including only samples sent by in-house rheumatologists, who it might be supposed have a higher diagnostic accuracy than non-connective tissue disease specialists. In addition, by using only patients without connective tissue disease and no anti-ENA as a negative control population, we aimed to avoid false negative reference tests. Finally, as a complimentary validation of the sensitivity of the new algorithm, the EliA Symphony test was performed on consecutive cohorts of SLE and SSc patients, all of which fulfilled the disease classification criteria and both containing patients in early (<1 year, ~25%) as well as late disease stages.

Although the proposed anti-ENA strategy showed good sensitivity for the antibodies against most antigens included in the EliA Symphony test, 5% of the anti-Scl70/Topo I and 20% of the anti-Sm monoreactivities were not detected. In addition, the EliA Symphony assay does not fully cover the spectrum of antigens in the INNO-LIA ANA Update: Ribosomal P and histones are missing. However, from the clinical point of view, the non-detection of these two autoantibodies is only a minor disadvantage. Peene *et al*. report that 0.5% of 1986 consecutive IIF positive samples, screened at a 1:40 serum dilution, contained anti-Ribosomal P antibodies.(3) In cohort A in our study, none of the 328 consecutive IIF positive sera contained an anti-Ribosomal P monoreactivity. Since approximately one third of the SLE patients in cohort B of this study with an anti-Ribosomal P reactivity had no other autoreactivities, it can be estimated that anti-Ribosomal P monoreactivities can be found in less than 0.2% of all IIF positive samples. Although the specificity of anti-histones is low, the detection of this reactivity could be of importance in the case of a clinical suspicion of drug-induced lupus. It is notable that the algorithm for ANA testing used in the in the Statens Serum Institute in Copenhagen does not include anti-histones (15). Apart from observed lower sensitivity for anti-Scl/Topo I and anti-Sm monoreactivities, and the absence of Ribosomal P and histones in the test assay, anti-ENA against several nucleolar proteins, such as fibrillarin and PM/Scl, are also not picked up by the cascade. These considerations indicate that when one of these anti-ENA is suspected, specific assays should be added to the proposed strategy, independently from the EliA Symphony result.

Of particular interest for laboratory management is whether the implementation of the EliA Symphony is a more economic diagnostic approach for the detection of anti-ENA. As calculated in cohort A, the new algorithm led to a 68% reduction in the number of final assays to be performed when a 1:40 dilution is used for the IIF test. Even when only the serum samples from cohort A which were positive at a 1:160 dilution were considered, a 42% reduction in the number of final assays was found. The cost-efficiency of the algorithm will differ across laboratories because the cost of reagents varies and the number of avoided test depends on the a priori chance for anti-ENA in the test population. Indeed, the number of avoided test could even be higher in a collection of samples from patients referred by general practitioners. It should be noted that, as described above, the narrower spectrum of the EliA Symphony may require additional tests in cases of high clinical suspicion of one of the excluded anti-ENA reactivities.

The limitations of this study were the low number of tested serum samples for certain monoreactivities, and the exclusion of samples with anti-ENA from patients without a typical associated clinical diagnosis. Recent studies have shown that autoantibodies can be present in early stages of connective tissue disease and can even appear before the clinical symptoms (16). However, the validation of an anti-ENA test assay for predictive purpose requires large prospective cohort studies of patients with non-specific symptoms with long-term follow-up. This issue should be addressed by future studies.

In summary, the EliA Symphony test was shown to be a sensitive second-line screening test for anti-ENA antibodies. In addition, the use of a three-step anti-ENA cascade reduced the number of final identification assays substantially.

In the context of a high clinical suspicion of connective tissue disease or autoreactivities not included in the EliA Symphony assay, third-line testing is useful, even if the anti-ENA screening is negative.

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