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The challenge of identification of autoantibodies specific to systemic autoimmune rheumatic diseases in high throughput operation: Proposal of reliable and feasible strategies

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ABSTRACT

Background: Autoantibodies to extractable nuclear antigens (ENA) are good biomarkers for systemic autoimmune rheumatic diseases (SARD), but no one assay for the detection of these antibodies provides satisfactory sensitivity and positive predictive value (PPV). Here we evaluate current assays and propose novel strategies to detect anti-ENA antibodies.
Methods: Diagnostic performance of double immunodiffusion (DID) and several enzyme immunoassays (EIA) for the detection of anti-ENA autoantibodies was determined using samples from 144 patients with a previous clinical diagnosis of SARD and 121 non-autoimmune individuals. A 2-step assay combining EIA and DID was developed and tested on 16,458 serum samples.
Results: EIA was more sensitive than DID for all anti-ENA antibodies, but yielded lower PPV (mean = 66%) than DID (mean = 96%) and a higher percentage of unexpected positive results. ROC-curve guided cut-off adjustments improved PPV for most EIA kits. Using the 2-step assay, over 80% of the samples were screened out by the first step (EIA), with results available within 24 h, leaving only about 20% to be confirmed by DID. 2.9% of the 16,485 samples were found to be positive.
Conclusions: A 2-step assay combining the speed and potential for automation of EIA with the high specificity and PPV of DID allows efficient and reliable detection of anti-ENA antibodies. Alternatively, improved PPV can be achieved by adjusting cut-off values for EIA assay results.

1. Introduction

Antinuclear antibodies represent a cornerstone in laboratory diagnosis of systemic autoimmune rheumatic diseases (SARD), such as systemic lupus erythematosus (SLE), Sjögren's syndrome (SjS), systemic sclerosis (SSc), and polymyositis (PM) [1–3]. Antibodies to several extractable nuclear antigens (ENA) are particularly relevant due to their strict clinical associations: autoantibodies to the Smith antigen (Sm) are strongly associated with SLE; anti-Scl-70 (DNA-topoisomerase I) with SSc; anti-Jo-1 (histidyl-tRNA synthetase) with PM; anti-U1-RNP (U1-ribonucleoprotein) with SLE, SSc, and mixed connective tissue disease (MCTD); and anti-SS-A/Ro and anti-SS-B/La with SjS and SLE. Antibodies to extractable nuclear antigens (ENA) were originally identified and characterized using gel-based immunoprecipitation techniques such as the double immunodiffusion (DID) assay [4–6]. All clinical associations of these autoantibodies have been derived from data obtained by means of the DID assay [7].

Although extremely specific, DID has limited sensitivity, is time-consuming, requires qualified personnel for interpretation, and is not appropriate for automation. Solid phase assays, such as enzyme immunoassay (EIA), in turn, are highly sensitive, ready for automation and high throughput operation, and do not require interpretation by expert personnel. Therefore, economic and operational pressures have progressively driven large clinical laboratories to adopt solid phase-based techniques for the identification of anti-ENA antibodies. However,

Abbreviations: ACR, American College of Rheumatology; ALBIA, addressable laser-based immunoassay; ANA-HEp-2, indirect immunofluorescence assay in HEp-2 cells; DID, double immunodiffusion; ENA, extractable nuclear antigens; Jo-1, histidyl-tRNA synthetase; PM, polymyositis; S, sensitivity; SARD, systemic autoimmune rheumatic diseases; Scl-70, DNA-topoisomerase I; SjS, Sjögren's syndrome; SLE, systemic lupus erythematosus; Sm, Smith antigen; Sp, specificity; SS-A, Sjögren syndrome type A; SS-B, Sjögren syndrome type B; SSc, systemic sclerosis; UNIFESP, Federal University of the State of Sao Paulo; U1-RNP, U1-ribonucleoprotein.

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there is some concern about the clinical reliability of anti-ENA antibody results based on EIA, because of the heterogeneous performance of several commercially available kits and the high number of unexpected positive results obtained with these methods [8–10]. The reputation of anti-ENA antibodies as useful diagnostic biomarkers has been established based on their high clinical specificity and not on their sensitivity.

Studies comparing anti-ENA EIA kits from different manufacturers usually show good agreement rates [11–15]. However, studies analyzing these assays against clinical diagnosis as the gold standard usually show a poor diagnostic accuracy [9,16,17]. In general, EIA shows higher sensitivity and lower specificity than DID, and therefore yields many positive results in unexpected clinical context (false positive results). Considering that these autoantibodies are most useful for diagnostic definition in clinically ill-defined cases, a false positive result may be particularly troublesome, especially for disease-specific autoantibodies. Therefore, the critical question is how to balance diagnostic accuracy, turn-around time, cost, and effectiveness in the determination of anti-ENA antibodies in the clinical laboratory setting.

The present study explores the performance of current solid phase assays, including EIA and addressable laser-based immunoassay (ALBIA), from seven different manufacturers and the traditional DID assay for determination of anti-ENA antibodies in a series of 144 serum samples from patients with well-defined SARD consecutively selected according to the American College of Rheumatology (ACR) classification criteria and 121 non-autoimmune control subjects. As previously observed, EIA/ALBIA achieves higher sensitivity but lower PVP than DID, and also leads to higher frequency of unexpected positive results. We examine approaches to achieving high PVP while minimizing time and cost, and propose 2 alternative strategies for determination of anti-ENA antibodies in a medium to high throughput operation.

2. Materials and methods

2.1. Serum samples

The study included 265 serum samples from patients with well characterized clinical diagnosis: 144 SARD patients (45 SLE, 29 SSc, 17 PM, 29 RA, 17 overlap syndrome, and 7 primary SjS), and 121 non-autoimmune individuals (26 osteoarthritis (OA), 20 ankylosing spondilytis (AS), 28 chronic viral hepatitis (CH), and 47 healthy volunteers). All patients were consecutively selected from the outpatient clinic of Universidade Federal de Sao Paulo (UNIFESP) and met the appropriate ACR classification criteria [7,18–23]. Healthy blood donors were consecutively retrieved after completing a health questionnaire. Patients and healthy controls had ages varying from 27 to 58 years old, and female to male gender distribution varied from 3:1 to 8:1, with no relevant difference among groups. The study was approved by the UNIFESP ethics committee and all participants signed the informed consent form.

A reflex algorithm for an anti-ENA antibody detection combining a screening step by EIA and a confirmatory step by DID was applied to 16,485 consecutive serum samples for which individual anti-ENA antibody or anti-ENA antibody profile had been ordered at Fleury Health and Medicine Laboratories (certified on site by the College of American Pathologists — CAP) during an eight month period. The sole requirement for selection of samples for this analysis was the availability of appropriate serum volume for the assays.

2.2. Anti-ENA assays

Anti-ENA antibodies were determined by DID and by EIA kits from 6 different manufacturers: 1) Euroimmun Medizinische Labordiagnostika AG, Anti-ENA PoolPlus ELISA, Anti-SS-A ELISA, Anti-SS-B ELISA, Anti-Sm ELISA, Anti-Jo-1 ELISA, and Anti-Scl-70 ELISA, using native antigens purified by affinity chromatography; 2) HUMAN GmbH, IMTEC-ENA Screen, IMTEC-SS-A/Ro, IMTEC-SS-B/La, IMTEC-U1-snRNP, IMTEC-Jo-1, and IMTEC-Scl-70 using purified native antigens; 3) INOVA Diagnostics, Inc., Quanta Lite™ ENA E6, Quanta Lite™ SS-A, Quanta Lite™ SS-B, Quanta Lite™ Sm, Quanta Lite™ RNP, Quanta Lite™ Jo-1, and Quanta Lite™ Scl-70 using purified native antigens; 4) ORGENTEC Diagnostika GmbH,, ORG 506 ENAscreen, ORG 508 Anti-SS-A, ORG 509 Anti-SS-B, ORG 510 Anti-Sm, ORG 511 Anti-RNP/Sm, ORG 513 Anti-Jo-1, and ORG 512 Anti-Scl-70 using purified native antigens; 5) PHADIA GmbH, Freiburg, Germany, Varelisa ReCombi ANA Screen, Varelisa SS-A/Ro, Varelisa SS-B/La, Varelisa Sm, Varelisa U1-RNP, and Varelisa Scl-70 from using human recombinant antigens and purified native SmD protein; and 6) The Binding Site, Bindazyme™ ENA screen, Bindazyme™ anti-SS-A/Ro, Bindazyme™ anti-SS-B/La, Bindazyme™ anti-Sm, Bindazyme™ anti-Sm/RNP, Bindazyme™ anti-Jo-1, and Bindazyme™ anti-Scl-70 using purified native antigens. In addition, samples were also assayed in the multiplexed AtheNA Multi-Lyte® ANA II plus addressable laser-based immunoassay (ALBIA) system, using recombinant antigens (SS-A/Ro, SS-B/La, Sm, U snRNP B/B′, U1 snRNP68, U1 snRNP A, U1 snRNP C, Jo-1, Scl-70, dsDNA, CENP-B, histones) from ZEUS Scientific Diagnostic, Inc., Raritan, USA. All kits have received Food and Drug Administration clearance and were labeled for in vitro diagnostic use.

Samples were processed in all kits strictly according to the manufacturer's instructions in order to emulate the actual operation in clinical laboratories. Results from kits from different manufacturers were coded A to G in order to blind potential commercial interest. Each manufacturer offers one screening multiple-antigen EIA kit and individual single-antigen kits for antibodies to SS-A/Ro, SS-B/La, Sm, RNP, Scl-70 or Jo-1, originally comprehended in the screening multiple-antigen EIA kit. Therefore, all samples were screened using the multi-antigen EIA kits from each manufacturer and the positive samples were further tested in the single-autoantibody kits from each manufacturer. For the multiplex ALBIA platform, there was a single step in which samples were simultaneously assayed against the individual autoantigens. All samples were tested by DID against calf spleen extract as previously described [24]. In addition, we developed a 2-step high throughput strategy, in which 16,485 samples were screened using the most sensitive multi-antigen kit (kit A) and the positive samples were further tested in the standard DID assay.

2.3. Indirect immunofluorescence

The presence of antinuclear antibodies (ANA) in serum samples was evaluated by indirect immunofluorescence in HEp-2 cells (ANA-HEp-2) (Bion Enterprise Ltd., BioRAD Laboratories Inc.) according to the manufacturer's instructions. The serum samples were diluted 1:80 in sample buffer and incubated with HEp-2 cells for 30 min at room temperature in a moist chamber. After washing twice in 0.15 mol/l NaCl and 10 mmol/l phosphate buffered saline (PBS), pH 7.4, for 10 min, cells were incubated with anti-human IgG goat immunoglobulin labeled with fluorescein isothiocyanate (provided by the manufacturer) for 30 min at room temperature in a moist chamber. After washing twice as before, slides were assembled with buffered glycerol pH 9.5 and cover slips. All slides were analyzed in an Olympus BX50 fluorescence microscope at ×400 magnification by a blinded expert observer.

2.4. Statistical analysis

The diagnostic performance of each anti-ENA autoantibody under the perspective of the various assays (DID and each EIA/ALBIA kit) was determined using clinical diagnosis as the gold standard. Sensitivity (S), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) for each test were determined with 2-by-2 contingency tables [25]. ROC (Receiver Operating Characteristic) curve analysis for each test was performed by EP Evaluator® 10 Version (Data Innovations, LLC). The comparability of results obtained with EIA kits from