Diagnostic Evaluation of ELISA and Chemiluminescent Assays as Alternative Screening Tests to Indirect Immunofluorescence for the Detection of Antibodies to Cellular Antigens

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ABSTRACT

Objectives: *Detection of antinuclear antibodies (ANAs) plays an important role in the diagnosis of systemic autoimmune rheumatic disease (SARD). Our goal was to evaluate the diagnostic accuracy of three commercially available enzyme-linked immunosorbent assay (ELISA) kits and one chemiluminescent assay for ANA detection, using the clinical diagnostic as the reference standard.*

Methods: *We evaluated serum samples from 143 patients with an established diagnosis of SARD (group 1), 166 patients with infectious diseases and other rheumatic diseases for which the ANA test is not useful in diagnosis (group 2), and 89 outpatients with suspicion of SARD (group 3).*

Results: *The sensitivity for ANA HEp-2, calculated in group 1, was 87.4% and varied between 62.9% and 90.0% for other tests. The specificity for ANA HEp-2, calculated in group 2, was 72.3% and varied between 45.2% and 90.4% for other tests. In group 3, the negative predictive value for ANA Hep-2 was 92.5% and varied between 89.3% and 100% for other tests.*

Conclusions: *Some ELISA kits have comparable or superior diagnostic sensitivity to ANA HEp-2 and could be used as an alternative method for ANA screening, therefore allowing the immediate report of the results with fewer false negatives than ANA HEp-2. Owing to the lower specificity, ELISA-positive samples should be submitted to ANA HEp-2 for confirmation of results.*

Autoantibodies to cellular antigens (anticellular antibodies), historically designated as antinuclear antibodies (ANAs), are pivotal tools to the diagnosis of systemic autoimmune rheumatic disease (SARD).[1,2] Since the 1950s, indirect immunofluorescence (IIF) has been the main method used to detect ANAs, initially using rodent tissues as substrates, and, from the 1980s onward, HEp-2 cells (ANA HEp-2).[2,3] The diversity of native human antigens distributed over all cellular compartments of the HEp-2 cells permits the identification of hundreds of different autoantibodies when this cellular lineage is used as an IIF substrate. Therefore, the ANA HEp-2 test is currently recommended as the gold-standard method for ANA screening.[1,4] In addition to the high sensitivity, this test provides two key aspects that can help discriminate ANA-positive healthy individuals and patients with SARD: the titer and the fluorescence pattern.[1,2]

ANA HEp-2 is an excellent screening test; however, it has some disadvantages. The assay is time-consuming and frequently carried out manually, and its interpretation is subjective and depends on qualified technicians.[1] Despite the efforts for standardization, differences among commercial kits result in high variability in terms of sensitivity, specificity, and IIF pattern reproducibility.[5] Finally, the test has low specificity for SARD, with a considerable proportion of individuals without any clinical evidence of autoimmune disease showing positive results.[6,7]

Over the past few years, other methods have been proposed as screening alternatives for laboratories processing a large number of ANA tests.[1,8] These tests may be fully automated and, since they do not require microscopy, they

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eliminate the subjectivity inherent to the interpretation of fluorescence patterns, which allows higher reproducibility. Nevertheless, the heterogeneity of these immunoassays regarding their antigenic composition and the choice of cutoff levels result in highly variable sensitivity and specificity.[9-13]

Several studies that demonstrated the superior performance of ANA HEp-2 have considered the results of this test as the reference standard for diagnostic purposes, instead of the clinical diagnosis.[4] In addition, the composition of the group with defined SARD diagnosis to assess test sensitivity, the inclusion of only healthy individuals in the control group to assess test specificity, the source of patient recruitment (primary, secondary, or tertiary care centers; prospective or cross-sectional study), and the evaluation of only one immunoassay method might bring some inconsistencies to these studies.[11,12,14]

On the other hand, well-designed studies, from a methodologic perspective, have shown that some enzyme-linked immunosorbent assay (ELISA) kits perform equivalently to the ANA HEp-2 test for ANA screening, offering an attractive alternative for high-volume laboratories.[9-13] The aim of this study was to determine the diagnostic properties of one chemiluminescent and three ELISA commercial kits for ANA screening, using clinical diagnosis as the reference standard, and compare their performance with the ANA HEp-2 test.

Materials and Methods

Patients and Reference Serum Samples

In total, 371 individuals older than 18 years were initially selected. Among these, four were excluded, resulting in 367 individuals distributed in three groups:

- Group 1: 143 individuals with established diagnosis of systemic lupus erythematosus (SLE), primary Sjögren syndrome (SS), systemic sclerosis (SSc, including diffuse and limited cutaneous forms, mixed connective tissue disease [MCTD], and overlapping forms), undifferentiated connective tissue disease (UCTD), and polymyositis/dermatomyositis (PM/DM), according to specific classification criteria.[15-20] The mean age of the patients was 42 years (range, 20-70 years), the female to male ratio was 132/16, the median disease duration was 6.7 years (range, 0.1-36.5 years), and 46% were receiving immunosuppressive treatment.
- Group 2: 166 individuals with preestablished diagnosis of rheumatoid arthritis, spondyloarthritis, systemic vasculitis, osteoarthritis (OA), fibromyalgia, or gout, according to specific classification criteria.[21-26] A subgroup was composed of patients with the following acute and chronic infectious diseases: human immunodeficiency virus/AIDS, chronic hepatitis B, chronic hepatitis C virus (HCV), anti–HCV positive, pulmonary tuberculosis, Chagas disease, and dengue fever. The mean age of the patients was 50 years (range, 21-79 years), the female to male ratio was 92/38, the median disease duration was 5.0 years (range, 0.1-36.6 years), and 42% were receiving immunosuppressive treatment.
- Group 3: 89 individuals from the primary care who were referred for outpatient evaluation, with clinical suspicion of SARD. The mean age of the patients was 45 years (range, 20-81 years), the female to male ratio was 71/18, and the median duration of symptoms before the visit was 2.2 years (range, 0.1-20.1 years).

The following patients were excluded due to hemolysis of the samples: one patient with a likely diagnosis of UCTD but who did not fulfill the classification criteria (group 1), one patient with OA and a diagnosis associated with primary biliary cirrhosis (group 2), and one patient with a diagnosis of fibromyalgia and another with sciatica (group 3).

All patients in groups 1, 2, and 3 were recruited in the Rheumatology Service of "Hospital das Clínicas" of "Universidade Federal de Minas Gerais" and at "Santa Casa de Belo Horizonte," Minas Gerais, Brazil, between September 2010 and September 2011. After signing the informed consent, blood was collected and centrifuged, and serum aliquots were stored at −80°C. The study was approved by the Ethics Committees of "Universidade Federal de Minas Gerais" and "Santa Casa de Belo Horizonte."

Eleven Arthritis Foundation and Centers for Disease Control and Prevention (AF/CDC) reference serum samples were tested to evaluate the ability of the assays to detect different autoantibodies with defined clinical relevance.[27]

ANA HEp-2

Samples were analyzed according to the manufacturer's recommendations (BION Enterprises, Des Plaines, IL). Analyses were performed by two expert technicians (JG and LCC), with no previous knowledge regarding either the diagnosis or each other's readings, using an epifluorescence microscope (Eclipse E400; Nikon, Tokyo, Japan) at ×400 magnification, and the fluorescence patterns described according to recommendations of the Third Brazilian Consensus for Autoantibodies Screening in HEp-2 cells.[28] The screening dilution was 1:80. Samples were classified as positive when they showed a defined fluorescence pattern and the titer was determined by successive twofold dilutions up to 1:5,120. All cases with divergent results in titer and/or fluorescence patterns were reanalyzed and reclassified after consensus was obtained between the technicians.

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ELISA and Chemiluminescence

Three commercial ELISA assays—QUANTA Lite ANA ELISA (Inova Diagnostics, San Diego, CA), ORGENTEC ANA Detect (Orgentec Diagnostika GmbH, Mainz, Germany), and IMTEC ANA Screen (Human Diagnostics GmbH, Wiesbaden, Germany)—were evaluated. In addition, a chemiluminescent assay (LIAISON ANA Screen; DiaSorin, Saluggia, Italy) was also evaluated. These alternative immunoassays (AIs) were designated as kits 1, 2, 3, and 4, respectively. The ELISA tests were performed manually, the samples' absorbance was read on Stat Fax 2100 Microplate Reader (Awareness Technology, Palm City, FL), and the chemiluminescent assay was performed automatically on the LIAISON Analyser (DiaSorin). All tests were conducted according to the protocols provided by the manufacturers. Borderline results from kits 2 and 3 were considered positive. The antigenic composition of each kit is described in **Table 1**.

Precision Evaluation

The within-run coefficient of variation (CV) was assessed by testing the positive control of each kit in triplicate in one run.

Statistical Analysis

The agreement between ANA HEp-2 and the AI was calculated using the κ statistic. The diagnostic accuracy of each test was determined by the following parameters, with respective 95% confidence intervals: sensitivity, specificity, area under the receiver operating characteristic (ROC) curve (AUC), positive likelihood ratio (LR+), and negative likelihood ratio (LR–). Patients from groups 1 and 2 were enrolled in this evaluation. The optimal cutoff value for each test was established by the ROC curve analysis. The AUC of the tests was compared by the DeLong test, and statistical significance was assumed at $P < .05$.[29] In addition, distinct result intervals were constructed corresponding to different LR+ and LR–.[6,30] Finally, SARD frequency, sensitivity, specificity, positive predictive value, and negative predictive value (NPV) of each test were calculated in group 3.

All analyses were performed using the MedCalc statistical software version 12.1.3 (Medcalc Software, Mariakerke, Belgium).

Table 1
Antigenic Composition of Kits

Antigen	Kit 1	Kit 2	Kit 3	Kit 4
Hep-2 cell extract	Yes	No	No	Yes
Nucleolus extract	Yes	No	No	No
HeLa cell nucleus	No	No	Yes	No
dsDNA	Yes[a]	Yes	No	Yes[b]
ssDNA	No	Yes	No	No
Nucleosome	Yes	Yes	No	No
Histones	Yes[a]	Yes	No	No
SSA/Ro 52 kD[a]	No	Yes	No	No
SSA/Ro 60 kD[a]	Yes	Yes	No	Yes
SSB/La	Yes	Yes	No	Yes[c]
Sm/RNP	Yes	Yes	No	Yes
Jo-1	Yes	Yes	No	Yes[c]
Scl-70	Yes	Yes	No	Yes[c]
CENP B	Yes	Yes	No	Yes[c]
Ribosomal P	Yes	No	No	No
Mitochondria (M2)	Yes	No	No	Yes[c]
PM/Scl	Yes	Yes	No	No
PCNA	Yes	No	No	No

CENP B, centromere protein B; dsDNA, double-stranded DNA; PCNA, proliferating cell nuclear antigen; ssDNA, single-stranded DNA.
[a]DNA/histones complex (nucleosome).
[b]Synthetic oligonucleotides.
[c]Recombinant antigens; other antigens are native.

Results

Within-Run Precision

The CVs (mean concentration/index) for kits 1, 2, 3, and 4 were, respectively, 0.97% (104.3 U), 3.63% (1.89), 2.41% (209.3 U/mL), and 1.42% (4.07).

Sensitivity and Specificity of the Tests

The sensitivity of each test was initially determined in group 1 and specificity in group 2 **Table 2**. The sensitivity for each disease was determined for all kits **Table 3**.

κ Index and Agreement Study

The κ index varied from 0.395 (ANA HEp-2 vs kit 3) to 0.581 (ANA HEp-2 vs kit 2), showing a fair to moderate agreement between tests.

Some samples showed different results as follows: 53 samples, positive results in kit 1 and negative in ANA HEp-2; 56 samples, negative results in kit 2 and positive in ANA Hep-2; 71 samples, positive results in kit 3 and negative in ANA HEp-2; and 77 samples, negative results in kit 4 and positive in ANA HEp-2. Eighteen patients in group 1 were negative in ANA HEp-2. Among these, 14 were positive in kit 1, eight in kit 2, 12 in kit 3, and four in kit 4. Ten patients from group 1 had negative results in kit 1, 34 in kit 2, 10 in kit 3, and 53 in kit 4. Among these, four, 22, four, and 37, were positive in the ANA HEp-2, respectively.

Correlation Between ANA HEp-2 Fluorescence Pattern and Alternative Test Positivity

The best performance of AI was observed in the identification of the patterns observed exclusively in samples from patients with SARD: nuclear homogeneous (100% for kits 1 and 3, 85.7% for kit 2, and 76.2% for kit 4), nuclear coarse speckled (93.3% for kits 1, 2, and 4 and 100% for kit 3),

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Table 2
Test Sensitivity and Specificity in Groups 1 and 2

Group	HEp-2	Kit 1	Kit 2	Kit 3	Kit 4
Sensitivity[a] (group 1) (n = 143)					
No. (%)	125 (87.4)	133 (90.0)	109 (76.2)	133 (90.0)	90 (62.9)
95% CI	82.0-92.8	85.1-94.9	69.2-83.2	85.1-94.9	55.0-70.8
Specificity[b] (group 2) (n = 166)					
No. (%)	120 (72.3)	99 (59.6)	150 (90.4)	75 (45.2)	145 (87.4)
95% CI	64.9-78.9	52.1-67.1	85.9-94.9	37.6-52.8	82.4-92.4

CI, confidence interval.
[a]Number of positive and indeterminate results in group 1 was divided by the total patient group.
[b]Number of negative results in group 2 was divided by the total patient group.

Table 3
Test Sensitivity According to the Diagnosis[a]

Disease	Sample, No.	No. (%) HEp-2	Kit 1	Kit 2	Kit 3	Kit 4
SLE	72	63 (87.5)	69 (95.8)	60 (83.3)	70 (97.2)	49 (68.1)
SSc	33	31 (93.9)	32 (97.0)	26 (78.8)	30 (90.9)	22 (66.7)
SS	15	15 (100)	15 (100)	13 (86.7)	14 (93.3)	12 (80.0)
UCTD	9	9 (100)	9 (100)	8 (88.9)	9 (100)	7 (77.8)
DM/PM	14	7 (50.0)	8 (57.1)	2 (14.3)	10 (71.4)	0 (0)

PM/DM, polymyositis/dermatomyositis; SLE, systemic lupus erythematosus; SS, Sjögren syndrome; SSc, systemic sclerosis; UCTD, undifferentiated connective tissue disease.
[a]Number of positive and indeterminate results in each disease was divided by the total number of patients with the disease.

nuclear centromeric (100% for all kits), and Scl-70 (100% for kits 1 and 3 and 80.0% for kit 4).[31,32] For the remaining patterns, the sensitivity of the kits was highly variable. In patients with SARD, the sensitivity for the cytoplasmic pattern was 100% for kits 1 and 3 and 50.0% for kits 2 and 4, whereas for the nucleolar pattern, the sensitivity was 88.9% for kits 1 and 3 (in nine patients, only one was not identified), 33.3% for kit 2, and 22.2% for kit 4. On the other hand, 86.6% of the samples showing the nuclear dense fine speckled pattern (DFS) was detected in kit 3 and 20.0% in kit 4. Among those 15 samples showing the DFS pattern, three belonged to patients with SARD, and all were reactive in kits 1 and 3, one in kit 2, and none in kit 4.

Accuracy of the Tests by Analysis of ROC Curve and Determination of Optimal Cutoff Values

Based on the ROC curve, all evaluated tests showed good performance in discriminating between the diseases of group 1 and the diseases of group 2 **Table 4**. Differences between the AUC of the ANA HEp-2 and the other tests were not significant. Among the AIs, only the AUC of kit 4 was considered different compared with kits 1 and 2.

The optimal cutoff value for ANA HEp-2 was a dilution of 1:160. With the exception of kit 2, all optimal cutoff values for the remaining kits were higher than those recommended by the manufacturers and were associated with a significant decrease in sensitivity (Table 4). In all kits except for kit 3, the optimal cutoff values established for the remaining kits resulted in LR+ of more than 5.

Establishing Result Intervals According to Different LRs

Overall, SARD is associated with higher serum titers/concentrations of autoantibodies and, therefore, showed higher ANA levels in the screening tests compared with other diseases or healthy controls.[30,32] Since the interpretation of ANA results is made only in a dichotomous manner (negative or positive), it may lead to a loss of relevant clinical information, with higher results associated with a higher probability of SARD and lower results associated with a lower probability of disease. **Table 5** shows distinct intervals of ANA levels that correspond to different LRs.

Evaluation of the AF/CDC Reference Serum Samples Panel

Each reference serum sample, its specific antibody content, and the test results are described in **Table 6**.

Sensitivity, Specificity, and Predictive Values of the Tests in Group 3

The SARD frequency in group 3 was 28.1% (25 patients with a final diagnosis of SARD from 89 evaluated). SLE and UCTD were the most frequent SARDs, while cases of SS and DM/PM were not diagnosed.

The higher sensitivity of kits 1 and 3 resulted in a high NPV of 100% and 94.4%, respectively. The lower sensitivity of the ANA HEp-2 (92%) compared with kits 1 and 3 (100% and 96%, respectively) was due to the presence of two patients with a diagnosis of UCTD and ANA HEp-2 negative but positive in all the AIs **Table 7**.

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Table 4
AUC and the Optimal Cutoff Value, Sensitivity and Specificity, and LR+ and LR–[a]

Characteristic	HEp-2	Kit 1	Kit 2	Kit 3	Kit 4
AUC	0.859[b] (0.817-0.900)	0.893[b] (0.858-0.929)	0.897[b] (0.862-0.931)	0.856[b] (0.813-0.899)	0.807[b] (0.758-0.856)
Cutoff value	1:160 (≥1:40)[c]	51.4 (>20 U)[c]	1.0 (>1.2)[c]	75.3 (>55 U/mL)[c]	2.1 (>1.5)[c]
Sensitivity, %	82.5 (75.3-88.4)	76.9 (69.1-83.6)	75.5 (67.6-82.3)	79.7 (72.2-86.0)	58.4 (49.5-66.2)
Specificity, %	79.5 (72.6-85.4)	88.0 (82.0-92.5)	91.0 (85.5-94.9)	78.9 (71.9-84.9)	95.2 (90.7-97.9)
LR+	5.2 (4.7-5.8)	6.4 (5.7-7.1)	8.4 (7.5-9.3)	3.8 (3.4-4.2)	12.0 (10.4- 13.9)
LR–	0.29 (0.20-0.50)	0.26 (0.20-0.40)	0.27 (0.20-0.50)	0.26 (0.20- 0.40)	0.44 (0.20-0.90)

AUC, area under the receiver operating characteristic curve; LR+, positive likelihood ratio; LR–, negative likelihood ratio.
[a]Values in parentheses are 95% confidence intervals unless otherwise indicated.
[b]$P < .0001$.
[c]Manufacturer value.

Table 5
Intervals of Results Established According to Distinct Likelihood Ratios

	LR+			
Tests	≤2	>2 to ≤5	>5	LR– <0.2
HEp-2 (titer)	—	1:80-1:160	≥1:320	<1:80
Kit 1, U	Up to 18.8	>18.8-39.6	>39.6	<30.5
Kit 2 (index)	Up to 0.48	>0.48-0.83	>0.83	<0.62
Kit 3, U/mL	Up to 49.8	>49.8-92.0	>92.0	<47.2
Kit 4 (index)	Up to 0.57	>0.57-1.47	>1.47	—

LR+, positive likelihood ratio; LR–, negative likelihood ratio; — LR not achieved for this test.

Table 6
Results Profile of the AF/CDC Antinuclear Antibody Reference Serum Panel Analysis

Serum	Pattern/ Antibody	HEp-2	Kit 1	Kit 2	Kit 3	Kit 4
1	Homogeneous[a]	+	+	+	+	–
2	SSB/La	+	+	+	+	+
3	Speckled[b]	+	+	+	+	+
4	U1-RNP	+	+	+	+	+
5	Sm	+	+	+	+	+
6	Nucleolar[c]	+	–	–	+	–
7	SSA/Ro	+	+	+	+	+
8	Centromere	+	+	+	+	+
9	Scl-70	+	+	+	+	+
10	Jo-1	+	+	+	–	+
12	Ribosomal P	–	+	–	+	–

AF/CDC, Arthritis Foundation and Centers for Disease Control and Prevention.
[a]Double-stranded DNA.
[b]U1-RNP, SSA/Ro, and SSB/La.
[c]Fibrilarin.

Discussion

The present study evaluated the diagnostic accuracy of four alternative assays with different principles and/or antigenic compositions and demonstrated that some are suitable for the screening of anticellular antibodies when we set the clinical diagnosis as the reference standard.

Recently, an international expert group endorsed the ANA HEp-2 test as the reference method for ANA screening.[1] However, recognizing the shortcomings of the IIF assay, the group has allowed the use of alternative assays for the screening of SARD-associated autoantibodies, provided that the platform being used is clearly specified on the test report, the result is not referred to as an "ANA test" or "ANA screen," and keeping in mind that the false-negative and false-positive ratio of these methods may be different. Therefore, before introducing such a method in the routine, the laboratory must perform a careful validation using clinically well-characterized biological samples to determine their diagnostic properties.[1,4]

In this study, the sensitivity of kits 1 and 3 was comparable to the sensitivity of ANA HEp-2. Both showed higher sensitivity than ANA HEp-2 in SLE, SSc, and DM/PM, although similar to it in UCTD and SS. The high sensitivity of kits 1 and 3 was reproducible in an additional independent group of patients who were referred for rheumatologic evaluation with a suspected diagnosis of SARD (group 3), thus confirming the robustness of the results. Kits 1 and 3 were similar to ANA HEp-2 in overall discriminatory capacity and also showed positive results ranging from 66.7% (kit 3) to 77.8% (kit 1) in patients with SARD and negative results in the ANA HEp-2. Therefore, the high sensitivity of kits 1 and 3 allows their use as an alternative to the ANA HEp-2 test.

The higher sensitivity of kit 1 may be explained by both the substrate, which is composed of various purified specific antigens associated with HEp-2 cell nucleus and nucleolus extracts, and the lower cutoff value set by the manufacturer, thus ensuring the lowest possible frequency of false-negative results. Kit 1 was evaluated in previous studies, with conflicting results regarding its diagnostic performance.[11-13] These discrepancies may be explained by differences in patient recruitment, as well as by changes in the antigenic composition.

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Table 7
Test Sensitivity/Specificity and Positive and Negative Predictive Values in Referral Patients: Group 3[a]

Test Property	HEp-2	Kit 1	Kit 2	Kit 3	Kit 4
Sensitivity (n = 25)[b]					
No. (%)	23 (92.0)	25 (100)	20 (80.0)	24 (96.0)	19 (76.0)
95% CI	68.8-97.5	86.3-100	59.3-93.2	79.6-99.9	54.9-90.9
Specificity (n = 64)[c]					
No. (%)	37 (57.8)	28 (43.8)	57 (89.1)	19 (26.6)	49 (78.1)
95% CI	44.8-70.1	31.4-56.7	78.8-95.5	16.3-39.1	66.0-87.5
PPV[d]					
%	44.9	41.0	74.1	33.8	57.6
95% CI	30.7-59.8	28.6-54.3	53.7-88.9	22.9-46.1	39.2-74.5
NPV[e]					
%	92.5	100	91.9	94.4	89.3
95% CI	79.9-98.4	87.7-100	82.1-97.4	72.7-99.9	78.1-96.0

CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value.
[a]PPV and NPV calculated based on systemic autoimmune rheumatic disease (SARD) frequency of 28.1.%.
[b]Calculated as number of positive and indeterminate results in patients with a final diagnosis of SARD.
[c]Calculated as number of negative results in patients with a final diagnosis of other diseases.
[d]Positive and indeterminate results in patients with SARD divided by the total number of positive and indeterminate results in group 3.
[e]Negative results in patients without SARD divided by the total number of negative results in group 3.

On the other hand, the high sensitivity of kit 3 may be explained by the use of the whole HeLa cell nucleus, bound covalently to the microplate, keeping the antigens in their topographic distribution and native conformation. Tonutti and colleagues[11] also observed similar results with kit 3.

However, due to lower specificity, positive results obtained in kits 1 and 3 should be complemented with ANA HEp-2, both to confirm the results and to determine the titer and fluorescence pattern. Considering that most ANA results are negative in clinical laboratories associated with primary care services, the use of the strategy mentioned above would allow rapid, accurate, and safe report of negative samples.[11-13]

The low sensitivity of kits 2 and 4, particularly in SSc and DM/PM, precludes its use as an ANA screening test. Perhaps the choice of antigens by the manufacturers may be responsible for this. The only specific antigen associated with DM/PM present in the kits is histidyl-tRNA synthetase (Jo-1). In SSc, most false-negative results occurred in patients with a nucleolar fluorescence pattern in HEp-2 IIF. Therefore, the absence of antigens such as RNA polymerase, fibrillarin, PM/Scl, and Th/To might explain the false-negative results with kits 2 and 4 in SSc.[33]

The low sensitivity of kit 4 was unexpected, since in addition to specific native and recombinant antigens, the test also uses HEp-2 cell nuclear extract, a strategy associated with high diagnostic sensitivity.[9-13] Callado and colleagues[34] validated this kit in a two-step ANA screening strategy, after discussion of the clinical impact of the false-negative results with the medical community. On the other hand, the high AUC of kit 3 allows its use as an ANA screening assay in settings with a low pretest probability of SARD or in combination with ANA HEp-2, since this last approach was associated with a better diagnostic accuracy, particularly for SARDs such as SLE and SS.[35,36]

The optimal cutoff values for kits 1 and 3 resulted in a loss of sensitivity. This fact, associated with low specificity if keeping the manufacturer's cutoff, prevents their use as the only ANA screening method and supports the use of ANA HEp-2 to confirm positive results. However, the optimal cutoff values of each test corresponded to higher concentrations of antibodies with positive LRs. Since higher ANA concentrations or titers are more likely to occur in patients with SARD, specific reference intervals for different LRs would be very useful.[6,30,32] In addition, it is known that the ANA HEp-2 may produce false-negative results with monospecific serum samples for anti-SSA/Ro, anti–Jo-1, and anti–ribosomal P antibodies.[1,37] In this study, two patients with UCTD in group 3 had this profile. These patients had ANA results in all alternative immunoassays tested with LR+ above 5. Thus, it is important to take into account the quantitative value of ANA, even when the ANA HEp-2 test is negative. Specific autoantibody tests may be ordered in these cases.

Regarding the analysis of AF/CDC reference serum samples, negative results deserve further discussion, particularly if the tested kit contained the antigen intended to be recognized by the reference serum. The reference serum 12 (anti–ribosomal P) was negative in ANA HEp-2. As shown in other studies, ANA HEp-2 may produce false-negative results in samples containing only anti–ribosomal P antibodies, a feature that is seen with kits from different manufacturers.[38] Reference serum 6 (nucleolar pattern ANA) was negative in kit 1, and the recognition of this serum by ANA HEp-2 and kit 3 illustrates that the supplementation with nucleolus extract may not be enough to detect antinucleolar antibodies, probably due to alterations in antigen conformation and/or

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antigen paucity in the substrate.[39] However, kit 1 was the most sensitive in patients with SSc, with positive results even in patients with a nucleolar fluorescence pattern in ANA HEp-2, confirming that its cutoff value was optimized to prioritize sensitivity. Kit 4 did not recognize reference serum 1 (homogeneous pattern ANA), which contains single- and double-stranded anti-DNA antibodies. This kit has synthetic DNA oligonucleotides (40 base pairs) as antigens. Some serum samples of patients with SLE contain anti–double-stranded DNA (dsDNA) antibodies that require longer DNA fragments to bind, and thus, synthetic oligonucleotides may not cover all relevant binding epitopes of anti-dsDNA antibodies.[40]

All immunologic reactions are based on the classic antibody-antigen curve, and the manufacturer should adjust the antigen amount by varying the dilution of the patient's serum or standard during titration.[41] Therefore, the concentrations of antigens in the solid phase might affect the diagnostic performance of the kit. Unfortunately, this information is not provided in the package insert of the kits. However, the analysis of the performance of AF/CDC serum samples shown by the alternative assays allows us to speculate that this may be difficult to accomplish, particularly in kits that use as substrate a mix of various antigens and HEp-2 cell extracts. Scarcity of antigens in HEp-2 cells extracts that have not been properly supplemented in the solid phase, as well as competition among the various antigens for binding to the solid phase, could also contribute to different kit sensitivities in the detection of specific antibodies.

Despite the absence of patients with a diagnosis of DM/PM or SS and the limited number of patients with SSc, the results obtained from group 3 confirmed the diagnostic test performance as previously determined in groups 1 and 2. The high negative predictive values of kits 1 and 3 (100% and 94.4%, respectively), which are even superior to that of the ANA HEp-2 test, indicate that an ANA negative result would exclude the diagnosis of SARD, unless a clinical picture highly suggestive of SARD is present, in which case the ANA HEp-2 test must be performed.

Our study had some limitations. The diagnostic performance of the tests was determined primarily in patients with an established diagnosis of SARD, most of them under treatment or with an inactive disease. Some diseases were represented by only a small number of patients, while others, such as Raynaud syndrome, were absent. However, it is unlikely that the study of patients with an established diagnosis of SARD influenced the relative performance of each test. In addition, the same diagnostic performance was observed in group 3, which represents a real clinical scenario, supporting the strength of our conclusions. An additional limitation is the absence of data concerning tests for the detection of specific autoantibodies, and thus the ability of each kit to identify them was not established. However, the analysis of the reference serum samples and the ability of the tests to identify the main ANA HEp-2 fluorescence patterns minimized this limitation.

Another limitation is that our group did not perform a cost-effectiveness analysis. We recognize that the best way to do this is through processing the routine samples sent to the laboratory in each AI and estimating the number of positive samples that will further require ANA HEp-2 confirmation. Notwithstanding, three aspects related to this deserve attention. First, the best option for ANA screening is the most sensible and specific test, in this case kit 1, as a lower number of confirmatory ANA HEp-2 tests would be necessary, resulting in more cost savings and lower turnaround time. Second, the source of the samples does matter. Our specificity analysis was performed in patients with well-defined rheumatic/infectious diseases or attending a tertiary rheumatology center. The two-step analysis would likely not be cost-effective for laboratories treating these kinds of patients, in whom a high pretest probability of SARD is associated with a high background ANA prevalence. On the other hand, the use of an AI could be an appropriate strategy for ANA screening in laboratories that process a large number of samples of patients referred by primary care physicians, in whom the frequency of SARD and ANA-positive results is lower. For example, in a previous study, kit 1 demonstrated a specificity of 78% in such a population.[11] As a matter of fact, the two-step strategy is less labor-intensive if compared with the ANA HEp-2 screening, since fewer IIF tests will be performed, thus allowing cost reduction by means of staff reduction.

In conclusion, our data show that the diagnostic sensitivity of some alternative immunoassays may be comparable to or even higher than the sensitivity of ANA HEp-2, similar to results published by other groups.[9-13] This allows their use as an alternative for ANA screening, particularly in laboratories that conduct a large number of tests, once full automation is available, leading to the optimization of the workflow and improvement in turnaround time. A positive result obtained from kits 1 or 3 is suggested to be followed by ANA HEp-2 testing to confirm and define the titer and fluorescence pattern. In addition, in a scenario of strong clinical suspicion of SARD and a negative result, ANA HEp-2 should be performed. On the other hand, a sample that is positive in some of these alternative assays but ANA HEp-2 negative, if showing concentrations associated with LR+ above 5, should be reported. In the presence of signals and symptoms that suggest an SARD, these results may be clinically relevant, thus justifying the search for specific autoantibodies.

Different tests may present variable sensitivity and specificity based on the heterogeneity of antigen composition or methodologic principle of each test. Therefore, before

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being introduced into the diagnostic routine, they should be submitted to a rigorous clinical validation, with the aim of establishing operational characteristics and possible technical limitations.

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