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Original article

Detection of anti-nuclear antibodies by indirect immunofluorescence on HEp-2 cells: setting the appropriate screening dilution for the diagnosis of autoimmune rheumatic diseases

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ABSTRACT

Objective: To establish the abnormal title and the appropriate screening dilution for ANA (antinuclear antibodies) test by indirect immunofluorescence on HEp-2 cells (ANA HEp-2). **Methods:** An analysis of ANA Hep-2 in serum samples from 126 healthy individuals was performed. The samples were screened at a dilution of 1:80, and those positive were diluted to the title of 1:5120. The abnormal title of ANA was defined as that corresponding to the 95th percentile of the test in this population. The sensitivity of the different titles of antinuclear antibodies was determined in a group of 136 patients with a diagnosis of autoimmune rheumatic disease, and the specificity was determined in a group of 118 patients with other rheumatic diseases. The optimal cutoff value of the test was determined by ROC curve analysis.

Results: The frequency of ANA positivity in healthy subjects was 13.2%. There was no difference in the frequency of positive results according to gender or age. The abnormal title of ANA was defined as the dilution of 1:160. The 1:80 dilution had sensitivity of 87.7% and specificity of 67.8%, while the 1:160 dilution had sensitivity of 82% and specificity of 73.7%. By ROC curve analysis, a dilution of 1:160 corresponded to the optimal cutoff value.

Conclusion: The abnormal title and the optimal cutoff value of ANA HEp-2 in the population was 1:160. Therefore, the dilution of 1:160 is the optimal screening dilution, with better specificity but without significantly compromising the sensitivity of the diagnostic test.

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Detecção de anticorpos antinucleares por imunofluorescência indireta em células HEp-2: definindo a diluição de triagem adequada para o diagnóstico das doenças reumáticas autoimunes

R E S U M O

Palavras-chave:

Doença reumática autoimune
Anticorpos antinucleares
Imunofluorescência indireta
Valor de referência

Objetivo: Definir o título anormal e a diluição de triagem adequada para o teste de FAN (fator antinúcleo) por imunofluorescência indireta em células HEp-2 (FAN HEp-2).

Métodos: Realizamos a pesquisa do FAN HEp-2 em amostras de soro de 126 indivíduos saudáveis. As amostras foram triadas na diluição de 1:80, e aquelas positivas diluídas até o título de 1:5120. O título anormal de FAN foi definido como aquele correspondente ao percentil 95 do teste nesta população. A sensibilidade dos diferentes títulos do FAN foi determinada em um grupo de 136 pacientes com diagnóstico de doença reumática autoimune, e a especificidade em um grupo de 118 pacientes com diagnóstico de outras doenças reumáticas. O valor de corte ótimo do teste foi determinado pelo estudo da curva ROC.

Resultados: A frequência de FAN positivo em indivíduos saudáveis foi de 13,2%. Não houve diferença na frequência de resultados positivos de acordo com o gênero ou a idade. O título anormal do FAN foi definido como a diluição de 1:160. A diluição dos soros de 1:80 apresentou sensibilidade de 87,7% e especificidade de 67,8%, enquanto a diluição de 1:160 apresentou sensibilidade de 82% e especificidade de 73,7%. Pela análise da curva ROC, a diluição de 1:160 correspondeu ao valor de corte ótimo.

Conclusão: O título anormal e o valor de corte ótimo do FAN HEp-2 na população avaliada foram de 1:160. A diluição de 1:160 é, portanto, a diluição de triagem ideal, com melhor especificidade, porém sem comprometimento significativo da sensibilidade diagnóstica do teste.

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Introduction

Antinuclear antibodies are important diagnostic markers of some autoimmune rheumatic diseases (AIRD), especially systemic lupus erythematosus (SLE), Sjögren's syndrome (SS), systemic sclerosis (SSc), dermatomyositis/polymyositis (DM/PM) and mixed connective tissue disease (MCTD).^{1,2}

Known as antinuclear antibody (ANA), the test for antinuclear antibodies by indirect immunofluorescence (IIF) is now called Research of Antibodies against Cellular Antigens (RACA-IIF), since it allows the detection of a range of antibodies that react with antigens not only of the nucleus, but of the nucleolus, cytoplasm and cellular mitotic apparatus.³ However, the term ANA continues to be widely used in clinical practice for historical reasons; so we will keep its use in this manuscript.

Thanks to its high diagnostic sensitivity, IIF using HEp-2 cells as substrate is considered the gold standard method for research of ANA (ANA HEp-2).⁴

However, a significant percentage of patients with various other autoimmune diseases, for which the test has no diagnostic value, can provide positive results in the test.^{1,2} Positive results can also occur in the context of infectious or neoplastic diseases, or even in individuals without clinical and laboratory evidence of autoimmune disease.⁵

The presence of ANA in an abnormal title is one of the criteria for classification of SLE of the American College of Rheumatology (ACR).⁶ Recently, the Systemic Lupus International Collaborating Clinics (SLICC) Group reviewed and validated the ACR classification criteria, defining as one of the immunological criteria of SLE the presence of an ANA result above the laboratory reference value.⁷ However, neither ACR nor SLICC

published specific recommendations about what procedures should be followed, for the establishment of ANA test abnormal title or reference value.

Different dilutions of serum screening, i.e., 1:40, 1:80 and 1:160, have often been suggested as a cutoff value for ANA HEp-2 test, without taking into account the recommendation of ACR with respect to the definition of ANA's abnormal title.⁸⁻¹²

On the other hand, many manufacturers of commercial kits suggest that the test screening be made in the initial dilution of 1:40, usually based on studies on healthy European or American populations.

In our country, a study showed that 12.9% of healthy individuals may have positive ANA HEp-2 in the title of 1:80 – the screening dilution used by many clinical laboratories, and a fact that reinforces the importance of establishing an appropriate screening dilution for the diagnosis of AIRD.¹³

The objective of this study is to determine, in a Brazilian population, the ANA test abnormal title, when performed by IIF on HEp-2 cells, by studying apparently healthy individuals; to determine the measures of test diagnostic accuracy, such as sensitivity, specificity, positive (LR+) and negative (LR-) likelihood ratio, by studying a group of patients with AIRD and another group with other rheumatic diseases, for which ANA has no diagnostic significance; and to establish the optimal cutoff value for the test with an analysis by ROC (Receiver Operating Characteristic) curve.

Materials and methods

Patients

Two distinct groups of patients were investigated:

Group 1: To determine the sensitivity of ANA HEp-2, 139 patients with an established diagnosis of AIRD were evaluated, including 72 with SLE, 12 with SS, 31 with SSc (including limited and diffuse cutaneous forms, MCTD and the patterns overlapping with SLE, SS and PM), 10 with undifferentiated connective tissue disease (UCTD) and 14 with DM/PM, according to specific classification criteria.^{6,14-19}

Group 2: To determine the specificity of ANA HEp-2, 118 patients with an established diagnosis of other rheumatic diseases were evaluated, including 38 with rheumatoid arthritis (RA), 17 with spondyloarthritis, 26 with systemic vasculitis, 10 with osteoarthritis, 23 with fibromyalgia and 4 with gout, according to specific classification criteria.²⁰⁻²⁴

For the determination of reference intervals in the clinical laboratory, the Clinical and Laboratory Standards Institute (CLSI) recommends the assessment of at least 120 subjects considered healthy and the use of non-parametric statistical tests.²⁵ Following the CLSI recommendations, 126 healthy subjects living in the metropolitan region of Belo Horizonte, Minas Gerais, were evaluated, and matched according to gender and the following age groups: 18-30 years, 31-40 years, 41-50 years and 51-65 years.

Individuals aged between 18 and 65 years old, who were in full and regular practice of their work activities and did not present any physical or mental disabling condition, were considered as healthy subjects, according to the model proposed by Tan et al.⁸ The abnormal ANA HEp-2 title was defined as that corresponding to the 95th percentile of this population.²⁶

All patients in groups 1 and 2 were recruited consecutively in the Service of Rheumatology, Hospital das Clínicas, Federal University of Minas Gerais, and Santa Casa de Belo Horizonte, Minas Gerais, Brazil, between September 2010 and September 2011. All the subjects signed an informed consent, and then a sample of blood for centrifugation was obtained. After that, the resultant serum was aliquoted and stored at -80°C. The study was approved by the Ethics in Research Committees of the Federal University of Minas Gerais and Santa Casa de Belo Horizonte.

ANA HEp-2

Serum samples from all participants were analyzed for the presence of antibodies against cellular constituents by indirect immunofluorescence method, using slides with HEp-2 cells, according to the manufacturer's recommendations (BION Enterprises, Des Plaines, IL, USA) and using 1:80 as screening dilution. The analyzes were performed by two experienced observers (blinded to the patients' diagnosis and to the result issued by the other colleague) in an epifluorescence microscope (Nikon Eclipse E400) at 400× magnification. The fluorescence patterns were classified according to recommendations of the 3rd Brazilian Consensus for Research on Autoantibodies against Cellular Constituents - ANA HEp-2.³ The samples were classified as positive when they showed fluorescence intensity equal to or greater than the positive control of minimal reactivity (1+/4+) in any cellular compartment (nucleus, nucleolus, cytoplasm or mitotic apparatus). At the time of the screening, positive samples were successively diluted in phosphate buffered saline (PBS) to the titer of 1:5120. The final title was the one in which it was still possible

to identify a well-defined morphological pattern, with minimal fluorescence intensity.

All cases with divergent results of title and/or fluorescence pattern were reviewed and ranked after consensus among observers.

Statistical analysis

Continuous variables with normal distribution were expressed as mean \pm standard deviation, and those with non-parametric distribution were expressed as median and interquartile range. Categorical variables were presented as frequency and percentage. For analysis of categorical variables, we used the chi-square (χ^2) or Fisher's exact test. The level of statistical significance was 5% ($p < 0.05$). The study of interobserver agreement was conducted by kappa statistics. The sensitivity and specificity, with respective confidence intervals of 95%, were calculated as the percentage of positive results in group 1 patients and as the percentage of negative results in group 2 patients, respectively. LR_s were calculated by analysis of the results of group 1 patients, both in comparison with the results of group 2 and with those of healthy subjects. The discriminative ability of ANA HEp-2 was evaluated by ROC curve analysis in two distinct ways: as the ability to discriminate between patients in group 1 and in group 2, as well as among group 1 patients and healthy subjects. Through the study of ROC curve, the screening dilution of ANA HEp-2 with the best compromise between sensitivity and specificity (optimal cutoff value) was also established. Statistical analysis was performed by Medcalc for Windows, version 12.1.3 (Medcalc Software, Mariakerke, Belgium).

Results

The mean age of group 1 was 42 years (± 11.7), the proportion of female patients 88.5%, and the median for disease duration was 6.7 years (3.2-12.7). The mean age in group 2 was 50 years (± 13.8), the proportion of female patients 71.2%, and the median time after diagnosis was 5 years (2-10). The mean age of the healthy subjects was 39.7 years (± 10.8), and the proportion of female patients was 54%.

Initially, we evaluated the interobserver agreement for classifying samples as positive or negative at the time of screening, and observed that the concordance was good to very good ($\kappa = 0.783$, CI95% 0.640-0.925). In a second step, we have studied the agreement with respect to the final title and the fluorescence pattern. The kappa statistics for the title was 0.762 (linear weighted kappa, CI95% 0.681-0.844), and for the pattern was 0.894 (quadratic weighted kappa, CI95% 0.780-1.000), indicating that the degree of agreement was good to very good.

Assuming a change in the definition of the title until ± 1 dilution, the absolute agreement was 95.7%. Disagreements of definition between nuclear homogeneous and nuclear dense fine speckled patterns were the main cause of disagreement in the classification of fluorescence patterns.

Frequency of positive ANA in healthy population

Of 126 healthy subjects, 17 were positive for ANA HEp-2 (13.5%) (Table 1), with no difference in frequency between fe-

males and males (16.1% and 10.3% respectively, $p = 0.489$) or according to age ($p = 0.888$).

The distribution of titers in healthy subjects revealed a predominance of low titers (1:80 and 1:160), although 29.5% of positive results occurred in titers $\geq 1:320$, considered moderate to high (Table 1). The more prevalent fluorescence patterns were the nuclear fine speckled, present in 11 individuals (64.7%), and nuclear dense fine speckled, present in 3 subjects (17.6%) (Table 2).

Definition of abnormal ANA title

The 95th percentile of ANA HEp-2 in the healthy population corresponded to the dilution of 1:160.

Considering this dilution as the abnormal title and thus the reference value of ANA HEp-2, the frequency of positive test results in the group of healthy subjects decreased to 6.3%.

Sensitivity and specificity of ANA HEp-2

The sensitivity and specificity of ANA HEp-2, calculated for each dilution situated between 1:80 and 1:5120, are detailed in Table 3.

As the prevalence and diagnostic significance of ANA in the different AIRD are variable, the proportion of positive results of ANA HEp-2 was determined for each disease represented in groups 1 and 2 (Figs. 1 and 2).

Compared with the 1:80 dilution, the use of the dilution of 1:160 as reference value for the test was accompanied by a reduction of sensitivity of ANA HEp-2, from 87.8% to 82.0%, and by an increase of specificity, from 67.8% to 73.8%.

The decrease in sensitivity was due mainly to a reduction in test positivity in patients with SLE and DM/PM. But in the patients with SSc, SS and DITC, the sensitivity remained practically unchanged.

Table 1 – Percentage of positive results of ANA Hep-2 in 126 healthy subjects, according to title.

	Title			
	1:80	1:160	1:320	1:640
Number	17	8	5	2
Percentage ^a	13.5%	6.3%	4%	1.6%

^aNumber of positive results considering the title as the cutoff value of the test, divided by the total number of healthy individuals.

Table 2 – Distribution of fluorescence patterns and ANA Hep-2 titles in 17 healthy subjects with a positive test.

Pattern	Title				Total
	1:80	1:160	1:320	1:640	
NFS	6	3	2	0	11 (64.7%)
NDFS	1	0	1	1	3 (17.6%)
Nucleolar	2	0	0	0	2 (11.8%)
Centromeric	0	0	0	1	1 (5.9%)
Total	9 (52.9%)	3 (17.6%)	3 (17.6%)	2 (11.9%)	17 (100%)

NFS, nuclear fine speckled; NDFS, nuclear dense fine speckled.

The sensitivity of ANA HEp-2 in patients with SLE was 87.5%, considering the dilution of 1:80 as the reference value. A decreased sensitivity to 81.9% was observed when only the test results in abnormal titer ($> 1:160$) were defined as positive.

In patients with DM/PM, the use of the reference value of 1:160 resulted in a decrease of diagnostic sensitivity, from 57.1% to 42.8%.

ROC curve analysis

The ROC curve analysis showed that the ANA HEp-2 test has a good performance to discriminate between group 1 and group 2 diseases. The area under the curve was 0.835 (CI95% 0.787-0.884, $p < 0.0001$), and the dilution of 1:160 was the one with the best compromise between sensitivity and specificity.

The use of the healthy subject population for determining the rate of false positive results of ANA HEp-2 resulted in better diagnostic performance of the test, with an area under the curve of 0.916 (CI95% 0.872-0.961, $p < 0.0001$). Also in this

Table 3 – Sensitivity and specificity of ANA HEp-2, according to the title (CI95%).

Title	Sensitivity	Specificity
1:80	87.7% (81.1-92.7)	67.8% (58.6-76.1)
1:160	82.0% (74.6-88.0)	73.7% (64.8-81.4)
1:320	74.8% (66.8-81.8)	80.5% (72.2-87.2)
1:640	57.6% (48.9-65.9)	85.6% (77.9-91.4)
1:1280	46.8% (38.3-55.4)	95.8% (90.4-98.6)
1:2560	27.3% (20.1-35.5)	96.6% (91.5-99.1)
1:5120	18.7% (12.6-26.2)	98.3% (94.0-99.8)

Sensitivity: calculated as the number of patients in group 1 with positive results, divided by the total number of patients in the group. Specificity: calculated as the number of patients in group 2 with negative results, divided by the total number of patients in the group.

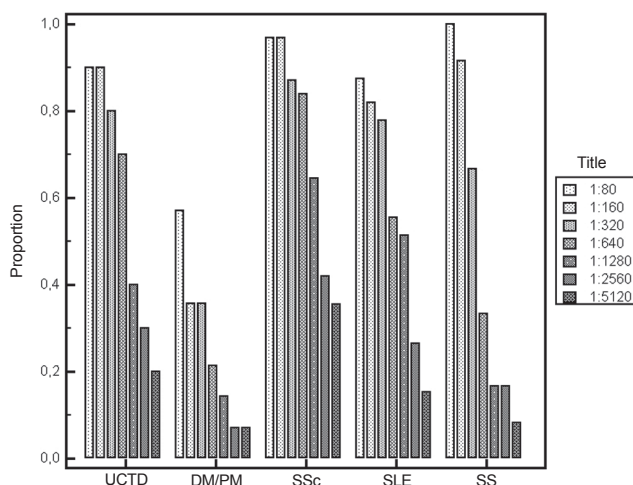


Fig. 1 – Proportion of positive results of ANA HEp-2 by Group 1 disease, according to the title. Proportion calculated as the number of positive results considering the respective title as the cutoff value of the test, divided by the total number of patients with the disease. UCTD, undifferentiated connective tissue disease; DM/PM, dermatomyositis/polymyositis; SSc, systemic sclerosis; SLE, systemic lupus erythematosus; SS, Sjögren syndrome.

population, the dilution of 1:160 was the one with the best compromise between sensitivity and specificity.

As the titles of the ANA HEp-2 are an important parameter for the interpretation of test results, and considering that the high titers are more frequent in patients with AIRD, LRs specific for the different titles of ANA HEp-2 were calculated (Table 4).

Discussion

The use of IIF HEp-2 as a method of choice for the investigation of ANA, the optimization of commercial kits for the detection of many autoantibodies of clinical importance, and the technological evolution of microscopes resulted in significant increase in the sensitivity of this test. These factors, combined with the use of the test by many physicians with the objective of to exclude the diagnosis of AIRD in patients with nonspecific signs and symptoms, had the effect of re-

ducing the specificity of ANA HEp-2, with the identification of positive results in a significant proportion of individuals without clinical evidence of AIRD.^{2,3,5}

Several studies have investigated the frequency and distribution of titles and fluorescence patterns of ANA HEp-2 in healthy subjects and in the general population.^{8,13,27-30} It is important to emphasize that different definitions were used in these studies to classify an individual as healthy or normal, besides different recruitment criteria.

Despite these conceptual disagreements, the frequency of ANA HEp-2 positivity in the healthy population was 13.5%, similar to that reported by studies conducted in Brazilian and in other populations, using different criteria of selection and definition of healthy individuals.^{8,13,27,28,29}

In the study by Tan et al., the frequency of positive results of ANA HEp-2 in 125 healthy subjects, recruited by fifteen international reference centers in search of autoantibodies, was 31.7% in the title of 1:40, 13.3% in the title of 1:80, 5% in the title of 1:160 and 3.3% in the title of 1:320.⁸

In the other hand, Mariz et al. considered as healthy only individuals without previous or current evidence of AIRD, chronic infection and neoplastic disease, with negative serology for HIV, hepatitis B and C, and that were not under regular use of glucocorticoid, immunosuppressive, antimicrobial or anti-inflammatory agents. Of the 918 individuals analyzed, 13.3% were positive at a dilution of 1:80, 7% at a dilution of 1:160, 6% at a dilution of 1:320, 4.4% at a dilution of 1:640 and 2,1% at dilutions \geq 1:1280.¹³

Studying an ethnically heterogeneous population sample of 4,754 U.S. civilian non-institutionalized individuals aged from 12 years and without employing any specific criteria of recruitment or exclusion, Satoh et al. found a frequency of ANA HEp-2 of 13.8% at a screening dilution of 1:80.²⁷

Fernandez et al. investigated 500 Brazilian blood donors and found the following frequencies of positive results of ANA HEp-2: 22.6% at 1:40 dilution, 8% at 1:80 dilution, 3.4% at 1:160 dilution and 1.4% at dilutions \geq 1:320.²⁸

In contrast to the results described above, none of the 100 healthy blood donors from predominantly Caucasian ethnicity in Copple et al. study showed ANA HEp-2 positivity in the screening dilution of 1:40.³⁰

Such discrepancies in the prevalence of ANA may be a result of both genetic-environmental and sociodemographic

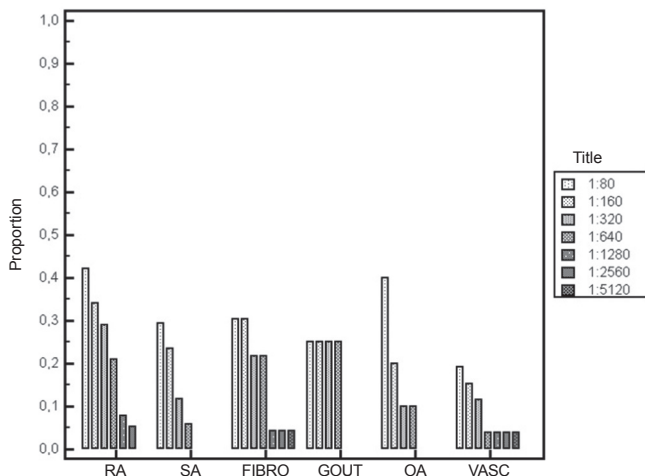


Fig. 2 – Proportion of positive results of ANA HEp-2 by Group 2 disease, according to the title. Proportion calculated as the number of positive results considering the respective title as the cutoff value of the test, divided by the total number of patients with the disease. RA, rheumatoid arthritis; SA, spondylarthritis; FIBRO, fibromyalgia; OA, osteoarthritis; VASC, vasculitis.

Table 4 – Positive and negative likelihood ratios of different titles of ANA HEp-2, considering as “not ill” those patients in group 2 and the healthy population (CI95%).

Title	GROUP 2		HEALTHY	
	LR+	LR-	LR+	LR-
1:80	2.73 (2.4-3.1)	0.18 (0.1-0.3)	6.5 (15.9-7.1)	0.14 (0.08-0.3)
1:160	3.12 (2.7-3.6)	0.24 (0.2-0.4)	20.67 (19.0-22.5)	0.19 (0.07-0.5)
1:320	3.84 (3.4-4.4)	0.31 (0.2-0.5)	18.85 (17.0-20.9)	0.26 (0.1-0.6)
1:640	3.99 (3.4-4.7)	0.5 (0.3-0.8)	36.26 (31.4-41.9)	0.43 (0.1-1.7)
1:1280	11.04 (9.2-13.2)	0.56 (0.2-1.3)		
1:2560	8.06 (6.1-10.6)	0.75 (0.3-2.0)		
1:5120	11.04 (7.8-15.6)	0.83 (0.2-3.3)		

LR+: number of positive results in group 1 patients, divided by the number of false-positive results in patients from group 2 or in healthy individuals.

LR-: number of false-negative results in group 1, divided by the number of negative results in group 2 patients or in healthy subjects.

factors as technical ones. Differences between microscopes and commercial reagents, as well as the heterogeneity in the training of observers, confer high interlaboratory variability in the results of IIF HEp-2.^{31,32}

Unlike other studies that showed a higher frequency of ANA HEp-2 positivity in females and older people, we found no differences in the frequency of ANA HEp-2 in healthy subjects by gender or age, dispensing thus the necessity of using specific screening dilutions, according to these variables.²⁶⁻²⁸

It is possible that our findings may be explained by the small number of subjects with positive ANA in each gender and age extract, thus limiting the statistical power to detect significant differences between groups, as well as by the absence of septuagenarian or octogenarian subjects. However, Mariz et al. also found no significant differences in the prevalence of ANA according to gender or age.¹³

A small proportion of healthy subjects (18 subjects, 14.3%) consisted of health professionals such as physicians, lab technicians and nursing technicians. Despite some studies implying that this occupational profile constitutes a risk factor for presence of anti-dsDNA antibodies or ANA HEp-2 in high titers, especially for those handling blood samples from patients with SLE, only two individuals of that subgroup had a positive test, which makes it unlikely that the recruitment of healthy volunteers with this profile did result in some sort of bias.^{8,29,33}

The distribution of the fluorescence patterns and of the titles of ANA HEp-2 was similar to that described by Mariz et al.¹³ The only exception was the occurrence of a nuclear centromeric speckled fluorescence pattern in a healthy individual. In the Mariz et al. study, this occurrence was observed exclusively in patients with AIRD.

Following the determination of ACR and SLIC, and adopting the CLSI guidelines for the definition of reference intervals in the clinical laboratory, the present study established a dilution of 1:160 as abnormal title or reference value of ANA HEp-2.^{6,25} Employing this dilution as a reference value for the test, the frequency of ANA in abnormal titers in SLE patients was 81.9%. Even lower frequencies of HEp-2 ANA in abnormal titles, i.e., 76.0%, can be observed in patients with established SLE.²⁶

Besides not sacrificing the diagnostic sensitivity of the test, the use of the 1:160 dilution for an initial screening of autoantibodies produced a 53.0% reduction in the number of positive results of ANA HEp-2 in the healthy population. As probable benefits of increasing the specificity of the test, we can list: the reduction of the number of incorrect diagnoses and of therapeutic prescriptions potentially harmful to the patient, as well as the reduction of requests for additional exams and unnecessary referrals to the rheumatologist.

The screening dilution of the test and how the patients are recruited are factors that may influence the frequency of ANA positivity.^{26,34} We did a cross-sectional evaluation of patients with established SLE, comprising a heterogeneous group with respect to activity and duration of illness, ethnic composition and use of corticosteroids and immunosuppressants. Therefore, it is important to emphasize that in all SLE patients with a positive ANA HEp-2 test, the disease was in remission only at a dilution of 1:80. As such, it is important to emphasize that in all SLE patients with a positive ANA HEp-2 test, the disease was in remission only at a dilution of 1:80.

By studying the ROC curve, the optimal cutoff value of ANA HEp-2 for both comparison groups evaluated (group 2 and healthy subjects) was defined as the dilution of 1:160. Beck et al. analyzed by ROC curve the results from ANA HEp-2 of 47 patients with SLE and 27 patients with other autoimmune diseases, and also found a dilution of 1:160 as the optimal cutoff value of the test.³⁵ However, in that study the use of dilution of 1:160 as the cutoff value was associated with a significant reduction in the diagnostic sensitivity of HEp-2 ANA for SLE.³⁵ Moreover, in the study by Tan et al., only the dilution of 1:160 was able to adequately discriminate among normal subjects and patients with SSc, SLE and SS, with sensitivities of 87.0%, 95.0% and 74.0%, respectively, and a specificity of 95%.

There is great variability in the literature as for what screening dilution to be used in the ANA HEp-2 test; some authors suggest the 1:40 dilution and others suggest dilutions of 1:160 and 1:80.⁸⁻¹² However, universally they recommend that only titers >1:160 should be considered positive or relevant from a diagnostic point of view, due to its higher positive predictive value for AIRD and to the presence of specific autoantibodies. The titles \leq 1:80 are considered indeterminate, because they usually do not have diagnostic significance and are not associated with presence of specific autoantibodies (anti-dsDNA and anti-ENA).⁸⁻¹²

Because this is a test for autoantibodies screening, positive ANA HEp-2 results must be complemented by ordering of specific autoantibodies. There is a well defined association between the titles of ANA HEp-2 and the probability of presence of specific autoantibodies.^{11,12} In this sense, González et al. suggested 1:160 as the ideal screening dilution for ANA HEp-2, since this dilution has the highest positive predictive value for presence of anti-dsDNA and anti-ENA antibodies, when compared with the lower dilutions, and without compromising the sensitivity of the test for the diagnosis of SLE.¹²

In the healthy population, the cutoff value of 1:80 was associated with LR+ > 5, which situates a diagnostic test as very useful. However, only from the dilution of 1:160 a LR+ > 10 was obtained, considered as able to significantly modify the post-test probability of the disease. In the population of individuals with other rheumatic diseases, only the dilution of 1:1280 was associated to a LR+ > 5. In both populations, titles under 1:160 showed LR- < 0.2, considered as very useful to exclude the diagnosis of AIRD.

Our study has some limitations. We evaluated only patients with an established diagnosis of AIRD. However, except in SLE, the immune profile of most patients with SSc, DITC, MD/MP and SS tends to be stable over time.³⁷⁻³⁹

The results of our work were obtained with slides from the same manufacturer. Although the frequency of positive results in the healthy population was similar to studies that used slides from other manufacturers or *in house*, there are important qualitative differences among the commercial HEp-2 slides.^{8,13,27-29}

As a result of the lack of standardization with regard to cell culture conditions, type of fixative used (acetone, alcohol/acetone), characteristics of the conjugate (polyvalent or specific IgG, fluorescein/protein ratio), etc., such differences produce significant inconsistencies in the reproduction of titles and fluorescence patterns, when analyzing the same sample of

serum on slides from different manufacturers (even with the use of a single microscope and the same observer).⁴⁰

Finally, other factors of technical nature, such as the microscope, the lamp power and the observer experience, also contribute to the great interlaboratory variability of ANA HEp-2 test.^{31,32} Thus, our findings cannot be extrapolated to other laboratories; it is advisable that each laboratory establish its own ANA abnormal title, analyzing healthy individuals from the local population and using their own instruments.

In conclusion, the dilution of 1:160 was defined as the abnormal title of ANA HEp-2 and as the optimal cutoff value of the test. Therefore, it is important to note that in situations where ANA is required outside of the clinical setting, with the main objective to exclude the presence of AIRD in patients with nonspecific clinical signs and symptoms, as has occurred in today's medical practice, and due to the high frequency of positive results in healthy subjects, the reference value of 1:160 would bring the benefit of a significant reduction in the frequency of "false-positive" results without significant loss of diagnostic sensitivity.

Conflicts of interest

The authors declare no conflicts of interest.

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