Experience of an university hospital on the implementation of I and II Brazilian Consensuses for Standardization of ANA in HEp-2 Cells

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ABSTRACT

Objective: *To evaluate the prevalence of patterns and titers of antinuclear antibodies (ANA) detected by indirect immunofluorescence (IIF) technique on HEp-2 cells in a university hospital following the introduction of I and II Brazilian Consensuses for Standardization of ANA in HEp-2 Cells.* ***Methods:*** *A transversal study was performed between 2002 and 2005 during which all ANA orders to Serviço de Hospital de Clínicas de Porto Alegre (SPC/HCPA) and cognate results were reviewed.* ***Results:*** *12.095 tests of ANA were revised. The number of positive results during this period was 2.577 (21.30%), annual mean 644 (SD: 233). A marked increase in the number of positive results was observed following the introduction of the Consensuses ($p < 0.001$). Rheumatology was the medical specialty which requested the highest number of ANA testing per patient although a significant decrease of these numbers was observed after the introduction of the Consensus in 2004 ($p < 0,001$). Nuclear fine speckled immunofluorescence labeling was the most frequently ANA pattern observed, 52.3% (453/866), and low ANA titers (1/80 and 1/160) more commonly detected (27.8% and 29.4%, respectively).* ***Conclusion:*** *Following the introduction of the Brazilian Consensus for standardization of ANA in HEp-2 cells an increased number of positive results was observed, mostly in low titers and with nuclear fine speckled immunofluorescence pattern. Moreover, there were decreasing numbers of ANA orders by rheumatologists in the same period. Potential causes for these observations are discussed but the real impact in the clinical condition of the patient and therapy deserves to be better studied.*

Keywords: *autoantibody, antinuclear antibodies, HEP-2 cells, indirect immunofluorescent.*

INTRODUCTION

Antinuclear antibodies (ANA), whose name historically refers to the fact that the first autoantigens observed were directed to nuclear and nucleolar structures,[1] were initially discovered using the lupus erythematosus (LE) cell test.[2] In 1957, Holborow *et al.*[3] developed the indirect immunofluorescent (IIF) technique. The principle of this technique is antibody connection to antigenic epitopes of cells, and detection is done by a second antibody marked with fluorescent substances analyzed with fluorescence microscope. Later, Beck,[4] using histological cut-off of a rat's liver, demonstrated homogeneous, speckled and nucleolar immunofluorescent patterns in those cells' nucleus when incubated with the serum of patients with a variety of rheumatic diseases.

Using human cell's lineages as substrate in IIF became popular after the papers by Tan *et al.*[1,5] Among several cultures tested, HEp-2 cells had hegemony, and today they are the pattern of excellence used all around the world.[5,6] HEp-2 cells, whose origin is human larynx carcinoma (human epithelioma type 2

Received on 02/11/2008. Approved on 11/01/08.
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– clone CCl 23 ATCC), are cultured in monolayer, presenting a growing cycle of approximately 36 hours, which allows the observation of the cells in every phase of cell cycle in the same slide.[7] Even antigens preferentially identified in cell division are easily characterized, like centromere antigens[8] and mitotic spindle-related antigens.[9]

When reading ANA using IIF one should consider important characteristics of this methodology, such as topographic and intracellular distribution of autoantigen corresponding to immunofluorescent pattern, which provides an idea of the autoantigen nature and, in some cases, makes it possible to accurately identify the autoantibody specificity,[9] such as in anticentromere antibodies[8] and anti-PCNA.[10] However, the nature of this test permits some interferences, such as the type of microscope used (lens, objective and light), cell substrate, conjugate and, particularly, subjectivity in the observer's interpreting.

The lack of a defined terminology for report description has caused problems in the clinical and laboratory use of this test, due to difficulty in quality control and result standardization. Although some patterns were similar, they had received different names. In 2000, a initiative was taken to create the first Brazilian Consensus for Standardization of ANA in HEp-2 Cells. Brazilian experts in ANA got together and consensually defined opinions for different patterns: nuclear, nucleolar, cytoplasmatic and mitotic.[11] Recommendations about the criteria for reading a slide have been established, such as titrations and optical system used.

In 2003, there was a second consensus,[12] in which decision algorithms to read ANA patterns in IIF discussed in the first edition of Brazilian Consensus were validated, and a new algorithm related to mix patterns was added. They tried to establish possible associations of each pattern with a specific disease or clinical manifestation.

Since 2004, the Department of Clinical Pathology at Hospital de Clínicas de Porto Alegre (SPC/HCPA) implemented the recommendations from the I and II Brazilian Consensuses for Standardization of ANA in HEp-2 cells,[11,12] following decision algorithms to demonstrate the data found, as well as morphological criteria observed during reading. The digital result for this test has been adapted to a new format, and examiners were trained to read the test during the period of one year.

Despite success in wide implementation of Consensus recommendations by clinical analysis laboratories, with a clear reduction in variability, we do not know any study assessing its performance to improve diagnosis and in clinical practice.

The purpose of this study is to describe the experience of implementing the Consensus at a university hospital in terms of prevalence of reagent results and its several IIF patterns found. We also tried to assess the influence in the request profile of the clinical specialties that most ordered this test.

MATERIALS AND METHODS

A transversal study had been carried out. All results of ANA performed by IIF sent to SPC/HCPA were reviewed, during the period from January 2002 until December 2005, using HCPA's computer system, applications for hospital management (AGH). In 2005, all patterns and titers of reagent result tests were reviewed, aiming to represent the one year period after professionals who read IIF slides for ANA at SPC/HCPA had been trained, according to new specifications suggested by the Consensuses.

Indirect immunofluorescent (IIF)

ANA research by IIF in HEp-2 cells (Wama Diagnóstica, Brazil) is done at SPC/HCPA, according to the following protocol: Cells are previously fixed and incubated for screening with the patient's serum diluted with 1/80 titer in phosphate buffered saline pH 7.2 (PBS) for 30 minutes in a humid chamber at room temperature. Then slides are washed twice for 10 minutes in PBS and incubated for 30 minutes with secondary anti gamma human globulin antibody conjugated with fluorescein isothiocyanate (FITC) in a dark chamber at room temperature. After incubation, slides are washed in PBS and mounted in buffered glycerin and cover slip. Reading is done with a fluorescence microscope, model Olympus BX 50, magnified at 500 times. Serum with reagent result in 1/80 is tittered in 1:80, 1:160, 1:320, 1:640, 1:1280, in the same buffer and according to the same conditions described before.[9]

Positive control serum and negative control serum, both provided by ANA kit, are used for tests.

Quality Control

For each ANA test, negative and positive internal controls are performed – such controls refer to centromere and homogeneous patterns, supplied by manufacturer. A selected serum database is also available, where serum present different patterns, whose antibodies were previously characterized by complementary tests, such as anti-ENA (extractable nuclear antigens) by *immunoblot,* anti-DNA by IIF in *crithidia luciliae*, as well as an image database with possible existing patterns as reference for identification.[7,11] Additionally, the Department is part of an alternative external quality program, with sample exchanges among laboratories. This program was introduced in 2004 and

today counts with three laboratories performing ANA test. Exchange periodicity is four samples per year, according to federal resolution RDC 202/2002.

Statistical analysis

A descriptive statistical analysis was used to present demographic data and positive test ratio in an Excel document (Microsoft, version 2003), and the chi-square test was used to analyze the relative frequencies of ANA results among the years and the specialties with the program SPSS® (version 14.0). A statistically significant difference was considered when $p < 0{,}001$.

RESULTS

A total of 12,095 ANA tests from the SPC at HCPA were performed from January 2002 to December 2005. Requests with positive result in this period were 2.577 (21.30%) with an annual average of 644 (±233). The number of ANA positive results according to the year was: 369 in 2002; 535 in 2003; 807 in 2004; and 866 in 2005. There was an addition of 11.6% in the positive result ratio after Consensus application (27.13% in 2004 to 2005 versus 15.54% in 2002 to 2003), and this difference was statistically significant by chi-square test ($p < 0.001$) (Figure 1).

Relative frequencies of ANA request by patient (inpatient and outpatient) referring to the ten clinical specialties that have requested ANA tests more frequently for screening from 2002 to 2005 are presented in Table 1. It has been observed that the specialty with higher average ANA request per patient was Rheumatology (average 4.5 ± 1.2%). This difference between Rheumatology and other specialties was statistically significant by the chi-square test for all years analyzed ($p < 0.001$). However, we observed a reduction in the number of test requests per patient at the Rheumatology Department from the first years evaluated ($p < 0.001$), to 2004 and 2005 (after the Consensus), a lower number of requests was registered as compared to previous years.

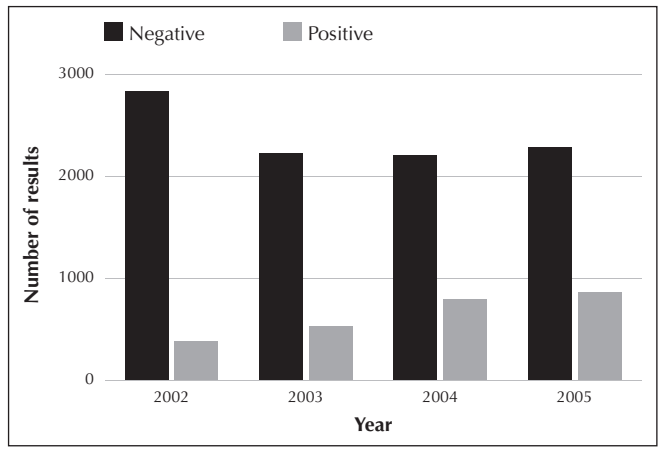

Figure 1. Distribution of ANA by indirect immunofluorescent results at HCPA during each year.

Table 1
ANA requests according to clinical specialties from 2002 to 2005*

Especialidades clínicas	2002			2003			2004			2005		
	ANA requests	Patients	ANA (%)**	ANA requests	Patients	ANA (%)**	ANA requests	Patients	ANA (%)**	ANA requests	Patients	ANA (%)**
Internal Medicine	533	41666	1.3	400	36953	1.1	441	35508	1.2	436	36865	1.2
Gastroenterology	276	11727	2.4	319	10956	2.9	403	11362	3.5	402	10967	3.7
Rheumatology	401	6983	5.7	283	5366	5.3	199	5597	3.6	186	5359	3.5
Occupational Medicine	293	15002	2.0	189	17990	1.1	313	20211	1.5	279	17993	1.6
Dermatology	199	15296	1.3	186	14381	1.3	189	14035	1.3	323	14385	2.2
Hematology	199	11532	1.7	125	10445	1.2	145	11213	1.3	242	10506	2.3
Neurology	202	7898	2.6	144	7135	2.0	131	6564	2.0	143	7178	2.0
Nephrology	176	8627	2.0	162	7981	2.0	138	8177	1.7	126	8053	1.6
Orthopedics/ Traumatology	136	20858	0.7	117	19819	0.6	124	19601	0.6	158	20030	0.8
Emergency	79	51819	0.2	88	53282	0.2	176	57810	0.3	63	53282	0.1
Total	3.878	191.408		3.473	191.408		3.755	190.273		3960	184.618	

* Medical care (outpatients and inpatients); **Percentage of appoitments where ANA was requested

Relative frequencies of ANA patterns for IIF in 2005 are represented in Table 2. The most frequent ANA Immunofluorescent pattern was nuclear fine speckled, representing 52.3% of the samples (453/866) (Figure 2), followed by mixed pattern on 11% of the samples (95/866).

ANA titers in year 2005 were mostly (57.2%) low titers (1/80 to 1/160). Frequencies of different titers found are shown in Table 3. A similar situation was found when analyzing nuclear fine speckled immunofluorescent pattern titers, where 63.4% were found to be 1/80 and 1/160.

Table 2
Frequency of ANA patterns using IIF in 2005

IIF patterns	n	Relative frequency (%)
Nuclear		
Nuclear fine speckled	453	52.3
Dense nuclear fine speckled	55	6.3
Nuclear coarse speckled	46	5.3
Speckled nuclear with isolated dots <10	31	3.6
Homogeneous nuclear	22	2.5
Centromere nuclear	13	1.5
Nuclear membrane	12	1.4
Nucleolar	50	5.8
Cytoplasmatic		
Speckled cytoplasmatic	56	6.5
Reticular cytoplasmatic	20	2.3
Polar cytoplasmatic	3	0.3
Fibrilar Cytoplasmatic	9	1
Mitotic apparatus		
Mitotic apparatus type mitotic spindle	1	0.1
Mixed	95	11
TOTAL	**866**	**100**

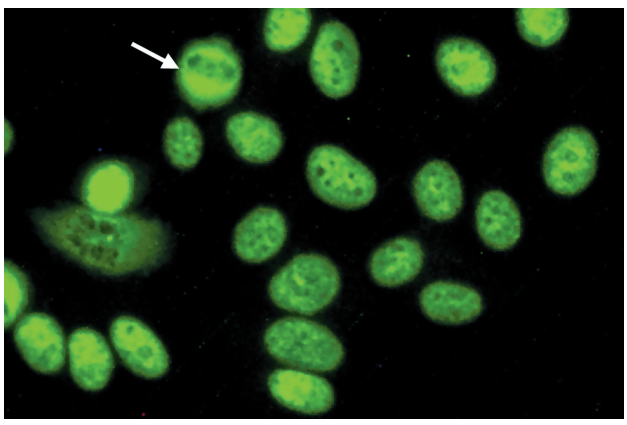

Figure 2. Indirect immunofluorescent. Nuclear fine speckled immunofluorescent pattern, with negative metaphase plate – arrow (diluted serum 1:80; 50x objective lens).

DISCUSSION

Interpretation of indirect immunofluorescent patterns in HEp-2 cells has significantly evolved in the last years. This methodology presents the capacity of tracing a wide range of known and unknown autoantibodies, providing location and probably identity of autoantigen. As a limitation, it presents variations in interpretation of immunofluorescent patterns.[13] Today there is a plurality of patterns. According to the Brazilian Consensus for Standardization of ANA, there are more than 25 possibilities of patterns described. Each one may reflect a certain antigen expression recognized by its autoantibody.[11, 12, 14]

In this study, it was not possible to evaluate patient's reports previous to the Consensus application, due to the variety in terminology used to establish the same pattern of IIF.

During the study, a significant increase in the number of requests with positive result was registered. Despite the fact that the Consensus do not intend to increase sensitiveness of ANA technique, but to make a standard reading, they stated that the IIF presence in cytoplasm should be considered as a positive result, while previous report said "negative ANA with presence of IIF in cytoplasm", which might have favored other clinical specialties besides Rheumatology.

An interesting observation was that, in the Rheumatology department, there was a reduction in the number of ANA requests along this period. ANA is not considered a monitoring test of disease, so a new serum assessment is not necessary in case of a positive result. Therefore, reduction in the number of requests may be related to an increment of the technical sensitivity, with reduction in repetition of tests with negative result in patients

Table 3
Prevalence of ANA titers by IIF in 2005

Titer	*n*	Prevalence (%)
1/80	241	27.8
1/160	255	29.4
1/320	139	16.1
1/640	75	8.7
1/1280	41	4.7
>1/1280	115	13.3
Total	**866**	**100**

with suspected autoimmune disease. Another possible association to the increased positive result ratio may be to the training carried out with ANA observers at SPC/HCPA according to the Consensus. With a computerized hospital system, it was not possible to identify the positive result ratio for each specialty, what could confirm this hypothesis.

After the Consensus, non-nuclear patterns that had not been classified before began to be considered. Such patterns may be relevant in certain circumstances, such as autoantibodies against cytoplasmic compartment and mitotic apparatus, also considered autoimmune disease markers.[9,15] However, until today no study has been carried out to assess clinical impact and doctor's satisfaction with this new ANA report proposed by the Consensus, regarding such patterns.

According to Phan *et al*.,[13] the ANA test has a difficult standardization. The Brazilian initiative for ANA standardization favored test interpretation and may be an important tool in quality control to reduce differences among patient's reports.

The most common ANA immunofluorescent pattern was nuclear fine speckled, mostly with titers considered low (1/80 and 1/160), as reported in other populations.[11,12,16,17] Our data showed a low prevalence of dense nuclear fine speckled immunofluorescent pattern (nuclear fine speckled with positive metaphase plate). This positive plate with a fine speckled pattern may be related with anti-protein antibody p75 kDa; and negative metaphase plate with fine speckled, to anti-SSA/Ro and anti-SSB/La.[7,12] Nuclear fine speckled immunofluorescent pattern with positive metaphase plate is described in the literature as having a higher prevalence in patients without clinical evidence of autoimmune diseases.[17]

Due to its high sensitivity, IIF ANA technique has autoantibody tracking as its primary role, especially concerning antinuclear antibody, anti-nucleolus antibodies and anti-cytoplasm antibody.[1,6,9,18,19] The clinical importance of ANA test is its association with autoimmune diseases, presenting positive results in 95%-98% of LES patients,[20] 90% of them with systemic sclerosis and 89% with Sjögren's syndrome.[5] During diagnostic investigation of such diseases, negative ANA test is associated with a strong negative predictive value.[21] However, this test may present positive results in patients with other diseases, such as inflammatory diseases, infectious diseases or neoplasia, and also in healthy individuals.[14,22]

It is important to highlight that this test request should be based on each patient's clinical context. According to Tampoia *et al*.,[15] in a study analyzing ANA test requests before and after an application of a protocol for rational use of secondary ANA tests (anti-ENA and anti-DNA), it was seen that rheumatologists would request ANA more frequently to patients presenting with two or more criteria for autoimmune disease classification, different from physicians in other specialties, who requested this test for patients presenting only a non-specific inflammatory condition.

From this study, we consider the accomplishment of the Brazilian Consensus for standardization of ANA in HEp-2 cells tangible at a large university hospital. The impact of this implementation in support quality for patients under suspicion of autoimmune disease should be better explored.

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