RESEARCH

The influence of demography and referral medical specialty on the detection of autoantibodies to HEP-2 cells in a large sample of patients

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Abstract

Background: The prevalence of anti-cell autoantibodies detected by indirect immunofluorescence assay on HEp-2 cells (HEp-2-IIFA) increases with age and is higher in female sex. The number of medical specialties that use HEp-2-IIFA in the investigation of autoimmune diseases has increased lately. This study aimed to determine the prevalence and patterns of autoantibodies on HEp-2-IIFA according to demographics variables and referring medical specialties.

Methods: A retrospective analysis of the HEp-2-IIFA carried out between January and June of 2017 was performed. The International Consensus on Antinuclear Antibodies Patterns (ICAP) and the Brazilian Consensus on Autoantibodies were used for patterns definition on visual reading of the slides. Anti-cell (AC) codes from ICAP and Brazilian AC codes (BAC) were used for patterns classification.

Results: From 54,990 samples referred for HEp-2-IIF testing, 20.9% were positive at titer $\geq$ 1/80. HEp-2-IIFA positivity in females and males was 24% and 12%, respectively ($p < 0.0001$). The proportion of positive results in the 4 age groups analyzed: 0–19, 20–39, 40–59, and $\geq$ 60 years was 23.3, 20.2, 20.1, and 22.8%, respectively ($p < 0.0001$). Considering all positive sera (n = 11,478), AC-4 nuclear fine speckled (37.7%), AC-2 nuclear dense fine speckled (21.3%), BAC-3 nuclear *quasi*-homogeneous (10%) and mixed/composite patterns (8.8%) were the most prevalent patterns. The specialties that most requested HEp-2-IIFA were general practitioner (20.1%), dermatology (15%), gynecology (9.9%), rheumatology (8.5%), and cardiology (5.8%). HEp-2-IIFA positivity was higher in patients referred by rheumatologists (35.7% vs. 19.6%) ($p < 0.0001$). Moderate (46.4%) and high (10.8%) titers were more observed in patients referred by rheumatologists ($p < 0.0001$). We observed a high proportion of mixed and cytoplasmic patterns in samples referred by oncologists and a high proportion of BAC-3 (nuclear *quasi*-homogeneous) pattern in samples referred by pneumologists.

Conclusions: One-fifth of the patients studied were HEp-2-IIFA-positive. The age groups with more positive results were 0–19 and $\geq$ 60 years. AC-4, AC-2, BAC-3 and mixed/composite patterns were the most frequent patterns observed. Rheumatologists requested only 8.5% of HEp-2-IIFA. Positive results and moderate to high titers of autoantibodies were more frequent in patients referred by rheumatologists.

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Background

The use of the indirect immunofluorescence assay on HEp-2 cells (HEp-2-IIFA) as a reference method for anti-cell autoantibody detection has been subject of standardization initiatives worldwide [1, 2]. Global guidelines for the nomenclature, classification, codification and interpretation of HEp-2 IIF patterns and reporting of the results have been proposed by the International Consensus on Antinuclear Antibodies Patterns (ICAP) (www.anapatterns.org) [3]. In 2016, The V Brazilian Consensus on Antinuclear Antibodies (BCA) decided to adopt almost all ICAP anti-cell (AC) codes, keeping five Brazilian Anti-Cell (BAC) original codes (BAC-1-5), besides the mixed or composite patterns [3, 4]. ICAP and BCA initiatives represent a milestone in the procedures of anti-cell autoantibodies detection by IIF. Indeed, the history of reading and interpreting HEp-2 IIFA can be divided into periods before and after the ICAP and BCA guidelines.

A sustained increase in the prevalence of autoimmune diseases and autoantibodies in world population has been observed in recent decades [5–7]. Depending on demographic and genetic factors, environmental exposures, serum dilution, cutoff point used, and other technical variables, the presence of autoantibodies in population-based studies is observed in up to 33.3% of the samples [5–9]. Positive HEp-2 IIFA results can vary from 12.9% in apparently healthy individuals (HI) to 90.2% in patients with SARD [10]. Autoantibodies are also observed in 18.3% of patients with nonautoimmune diseases (NAD), such as infectious, neoplastic, and psychiatric disorders [11].

The demand for anti-cell antibody testing has also increased remarkably. Six decades ago, tests for the so-called Antinuclear Antibodies (ANA), a term considered outdated today, were ordered almost exclusively by rheumatologists and clinical immunologists [12]. With the recognition that systemic nonorgan-specific diseases and a plethora of organ-specific diseases can be associated with autoantibodies, a broader spectrum of medical specialists is now ordering tests for anti-cell autoantibodies. This change in the anti-cell antibody referral pattern has been associated with a decrease in the pretest probability and the diagnostic performance of the HEp-2 IIF assay in the context of SARD [12, 13]. This study aimed to describe the positivity and patterns of the HEp-2 IIF assay according to ICAP and BCA recommendations in large consecutive serum samples referred to the laboratory for anti-cell antibody testing. In addition, we also analyzed the relationship of these results with the requesting physician's specialty.

Methods

Study design and samples

This is a retrospective and cross-sectional study in which the results of HEp-2 IIFA performed between January and June 2017 were reviewed. In this period, 57,265 consecutive serum samples from 54,990 patients were referred to the laboratory for routine HEp-2 IIFA. If multiple serum samples from a single patient were referred, only the data for the first sample received were included in this analysis. For each sample, sex, age, and the referring medical specialty were identified.

HEp-2 IIF testing for anti-cell antibodies

All anti-cell antibody tests were performed previously on commercially available slides of HEp-2 cells (Euroimmun Medizinische Diagnostika, Lübeck, Germany). When necessary, a confirmatory test was performed using slides from another manufacturer (INOVA Diagnostics, San Diego, CA, USA). The tests were carried out using the IIF technique according to instructions provided by the manufacturer. Serial dilution of positive samples was performed for endpoint-titer determination. Titers $\geq 1/80$ were reported as positive for fluorescent patterns staining either the nucleus, cytoplasmic or mitotic apparatus. Three experts performed visual pattern recognition on the HEp-2 cells slides. Pattern definitions were established according to ICAP and BCA recommendations.

Statistical analysis

Categorical data were presented using frequency distribution and were analyzed using the chi-square method. For quantitative variables, the Mann–Whitney test was used for comparisons between two groups, and the Kruskal–Wallis test was used for comparisons involving multiple groups. Comparisons between three or more groups involving parametric, quantitative data were made using analysis of variance (ANOVA) with additional procedures for comparing pairs (Bonferroni or Games-Howell, according to the case). Analyses were performed using statistical programs included in IBM version 22 of SPSS (Statistical Package for the Social Sciences, IBM, USA) for Mac. Statistical significance was established at $p < 0.05$.

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Results

From 57,265 consecutive serum samples referred for anti-cell antibody testing in the study period, 2275 were repeat samples, and were removed from this research. Therefore, 54,990 samples from different patients were included in the analysis procedures.

Demographic data

The median age of the 54,990 patients was 43 years, with a range of 1–102 years. According to age, patients were divided into 4 groups: 0–19 (n = 4570); 20–39 (n = 20,500); 40–59 (n = 20,085); and ≥ 60 years (n = 9835). The percentage of HEp-2 IIFA positivity, with titers ≥ 1/80, in these age groups was 23.3*, 20.2, 20.1, and 22.8*%, respectively. A higher positivity of the test was observed in the age groups of 0–19 and ≥ 60 years compared to the age groups of 20–39 and 40–59 years ($p < 0.0001$). The 0–19 and ≥ 60 years age groups did not differ from each other ($p = 0.49$).

Of the 54,990 participants, 74.3% were female, and 25.7% were male. HEp-2 IIFA was positive (titer ≥ 1/80) in 20.9% of the samples (n = 11,478). Among the positive samples, 85.1% came from female participants. When we compared the test positivity to sex, we observed a significant difference in positivity between females (24*%) compared to males (12%) ($p < 0.0001$). The greater positivity of HEp-2 IIFA in females was significant in any age group, as represented in Fig. 1.

HEp-2 IIFA fluorescence patterns according to ICAP and BCA proposals

During the period analyzed, the test reports were released following the IV BCA [14]. Later, they were coded according to ICAP recommendations. Some patterns that were not recognized by the ICAP were codified retrospectively using the BAC alphanumeric codes defined by the V BCA, a consensus that proposed harmonization between the two initiatives [4].

Based on the fluorescence pattern observed in the cell domains proposed by ICAP, the proportions of nuclear, cytoplasmic, and mitotic patterns reported were 85.7% (n = 9833), 4% (n = 464), and 1.5% (n = 176), respectively. The remaining 1005 samples (8.8%) had two or more fluorescence patterns or stained two or more cellular domains, being classified as mixed or composite and receiving multiple ICAP/BAC codes whenever indicated [4]. Patterns recognized only by BCA, such as nuclear *quasi*-homogeneous (BAC-3) and nuclear reticular coarse speckled (BAC-4), were found in 10% (n = 1152) and 0.9% (n = 103) of the sera, respectively (4, 14).

Considering all positive sera (n = 11,478), nuclear fine speckled (AC-4), nuclear dense fine speckled (AC-2),
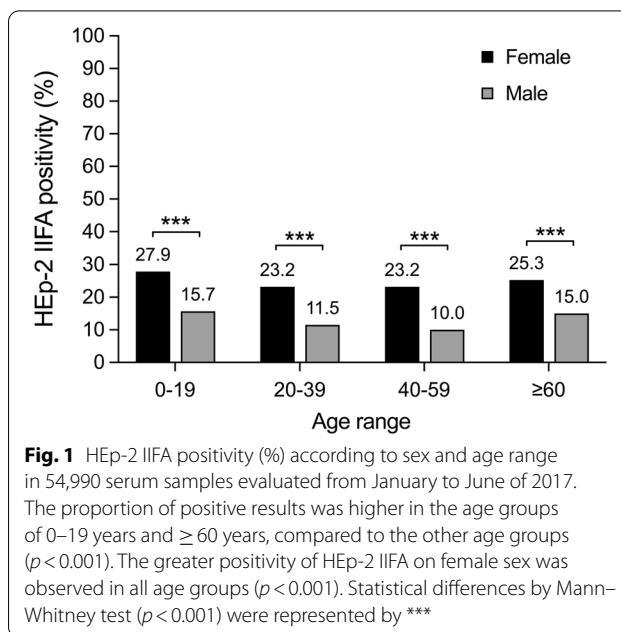

Fig. 1 HEp-2 IIFA positivity (%) according to sex and age range in 54,990 serum samples evaluated from January to June of 2017. The proportion of positive results was higher in the age groups of 0–19 years and ≥ 60 years, compared to the other age groups ($p < 0.001$). The greater positivity of HEp-2 IIFA on female sex was observed in all age groups ($p < 0.001$). Statistical differences by Mann–Whitney test ($p < 0.001$) were represented by ***

and nuclear *quasi*-homogeneous (BAC-3) were the most prevalent patterns, being observed in 37.7, 21.3, and 10% of the samples, respectively. The nuclear homogeneous (AC-1) and mixed patterns were observed in 6.8 and 8.8% of the samples, respectively. All other patterns were described at a frequency of ≤ 2.5%. The most prevalent cytoplasmic (reticular speckled, AC-21) and mitotic (nuclear mitotic apparatus–NuMA-like, AC-26) patterns were observed in only 1.8% and 0.9% of the total positive samples, respectively (Fig. 2).

In the analysis by cell domain, considering only the ICAP classification (n = 9218), among the nuclear patterns, the most prevalent were AC-4 (50.4%); AC-2 (28.6%); AC-1 (9.1%); AC-8,9,10 (nucleolar, 3.3%) and AC-5 (nuclear coarse speckled, 3.3%). Among the cytoplasmic patterns, we observed that reticular speckled–AMA (AC-21), dense fine and fine speckled (AC-19 and 20), and discrete dots (AC-18) were the most prevalent, being present in 44.2, 20.5 and 10.8% of the samples, respectively. Among the mitotic, nuclear mitotic apparatus type 1–NuMA-like (AC-26) was the most prevalent (56.3%), followed by the Spindle fibers (AC-25), the centrosome (AC-24), and intercellular bridge (AC-27), which were present in 28.4, 9.1 and 6.3% of the samples, respectively. BCA does not recognize the mitotic chromosomal pattern (AC-28). The proportion of each ICAP pattern by cellular domain is shown in Fig. 3.

HEp-2 IIF patterns and requesting physician specialties

Of the 54,990 samples evaluated, 2488 had no available information regarding the specialty of the requesting
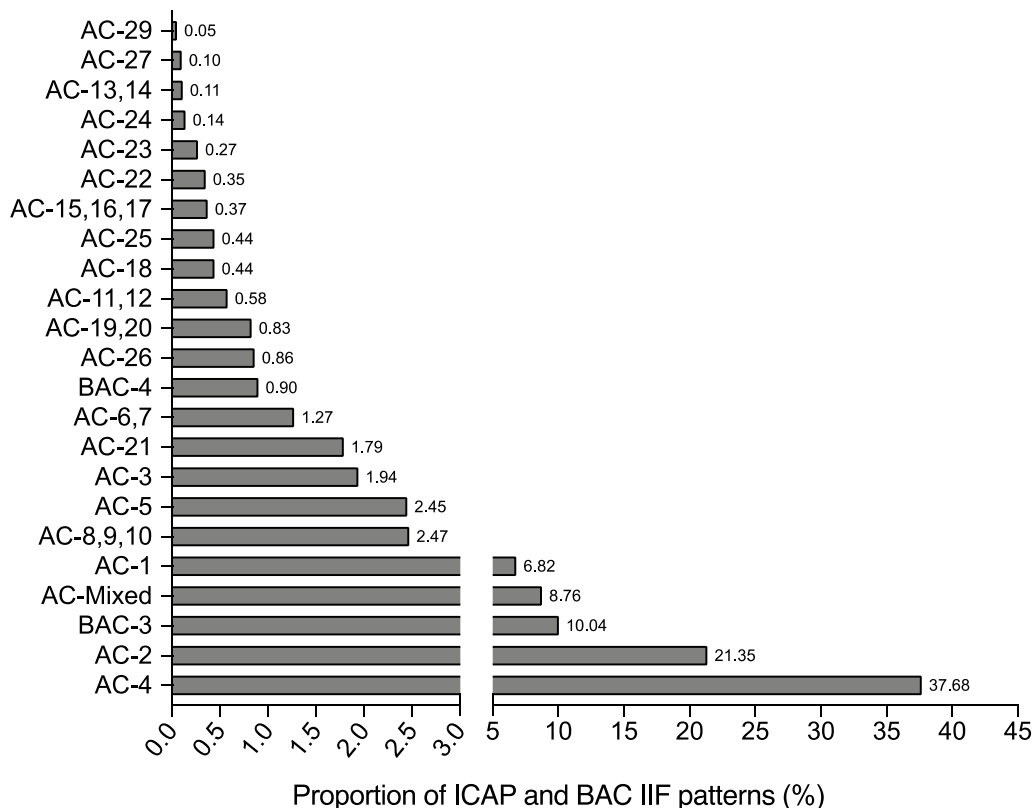
Fig. 2 Anti-cell antibodies-HEp-2 IIFA–Proportion (%) of ICAP and BAC IIF patterns in 11,478 positive samples analyzed from January to June 2017. BAC: Brazilian Anti-Cell code. BAC-3 = nuclear *quasi*-homogeneous. BAC-4 = nuclear reticular coarse speckled. AC-Mixed: mixed patterns (multiple ICAP/BAC codes). AC-28: not recognized by the Brazilian Consensus on Autoantibodies (BCA)
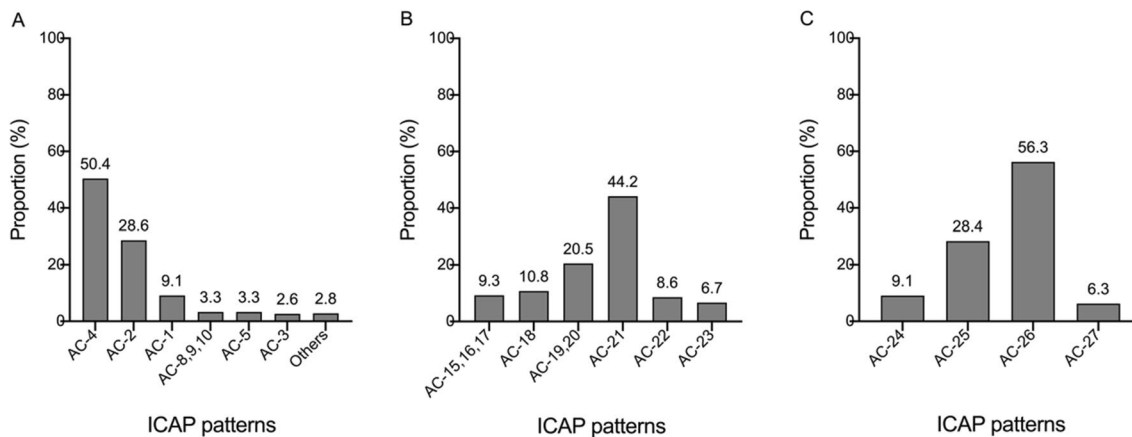

Fig. 3 HEp-2 IIFA–Fluorescence staining patterns according to cellular domains and ICAP codes in 9218 serum samples analyzed from January to June 2017. **A** ICAP nuclear patterns; **B** ICAP cytoplasmic patterns; **C** ICAP mitotic patterns. BAC-3: nuclear *quasi*-homogeneous (n = 1152); BAC-4: nuclear reticular coarse speckled (n = 103) and mixed patterns (n = 1005) not represented
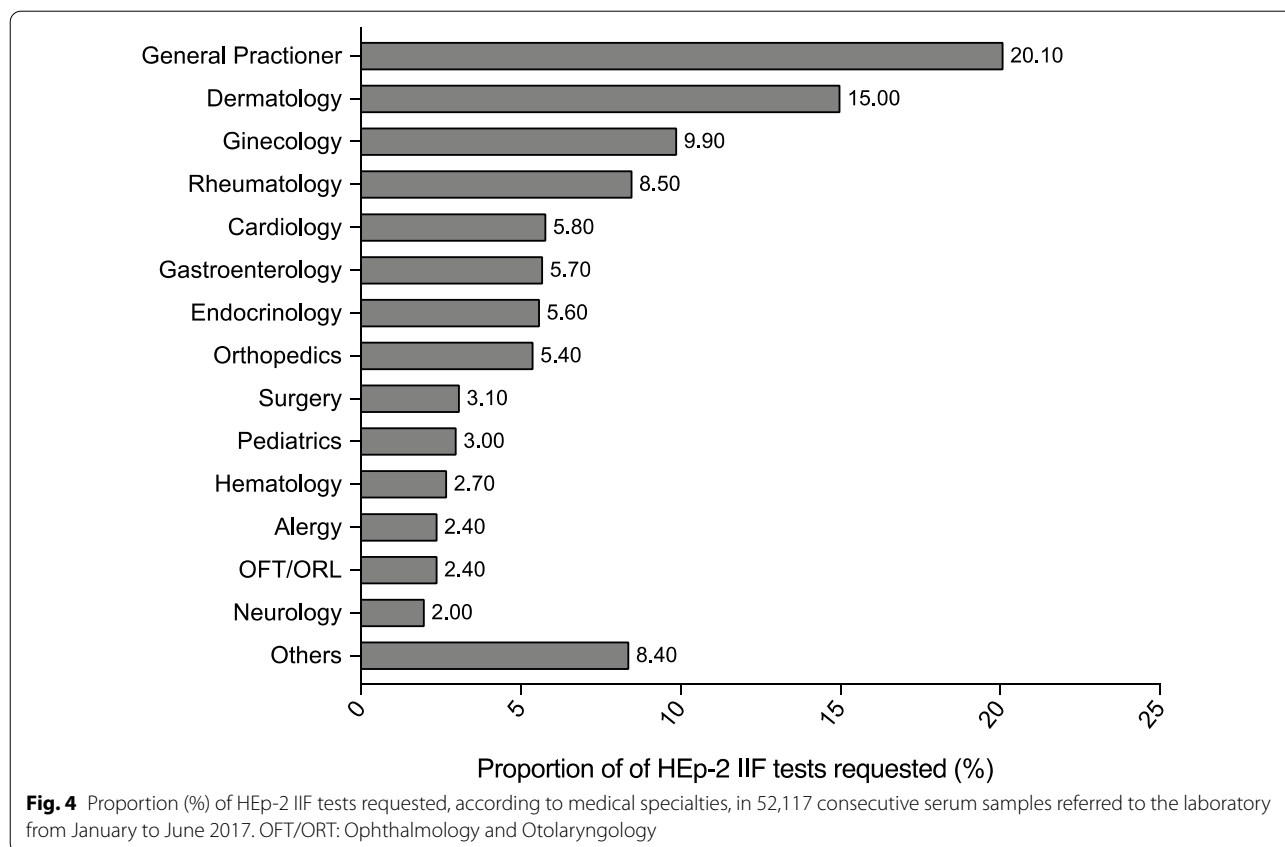
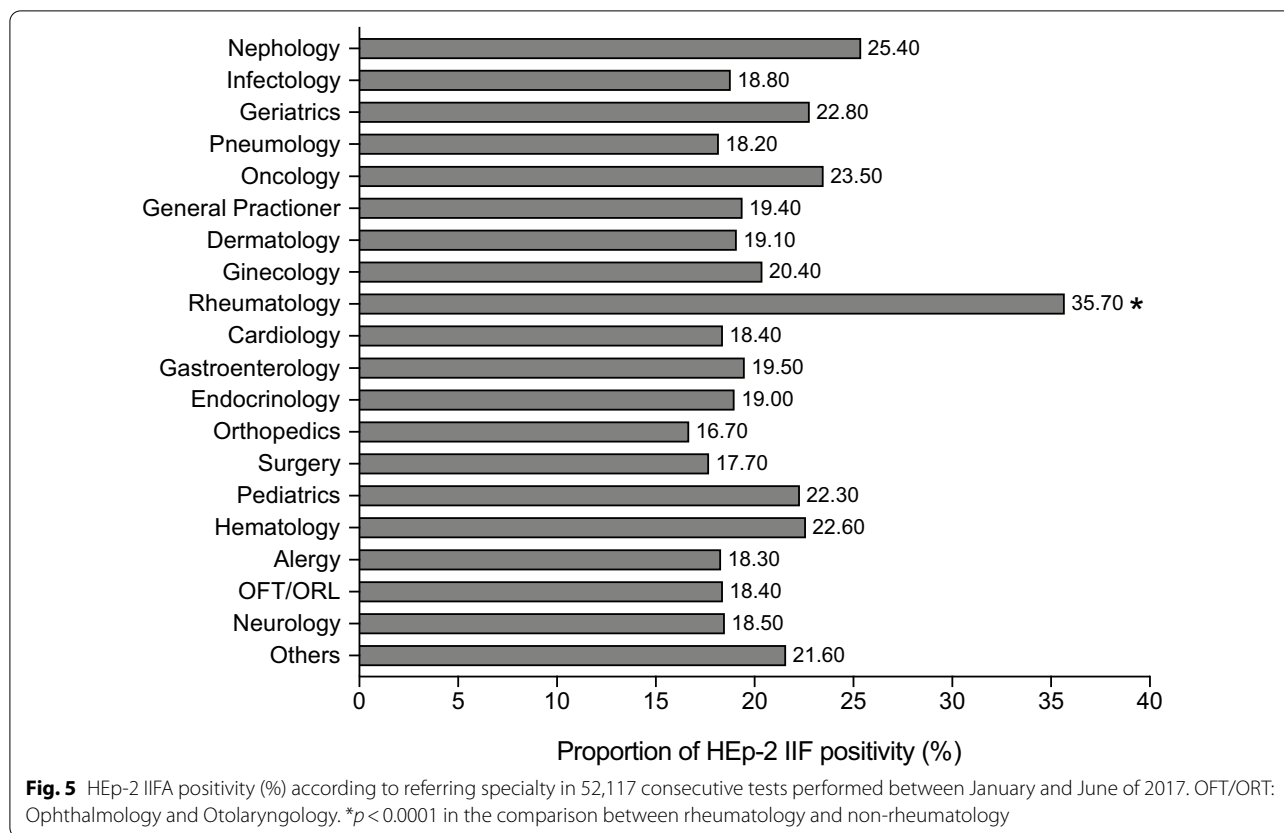physician. Nonphysicians made a total of 385 requests. Thus, the analysis of HEp-2 IIF results by requesting medical specialty was performed with the 52,117 remaining samples. Among these, the medical specialties that most requested HEp-2 tests were general practice (20.1%); dermatology (15%); gynecology (9.9%); rheumatology (8.5%);

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cardiology (5.8%); gastroenterology (5.7%); endocrinology (5.6%); orthopedics (5.4%); surgery (3.1%); general pediatrics (3.0%); hematology (2.7); allergy (2.4%); ophthalmology (OFT) and otolaryngology (ORL) (2.4%); neurology (2.0%) and others (8.4%). Specialties with less than 2% of the requests included nephrology, infectology, geriatrics, pneumology, oncology, critical care medicine, homeopathy, psychiatry, physiatry, occupational medicine, nutrology, and orthomolecular medicine (Fig. 4). Patients referred by rheumatologists (n=4226) showed a percentage of HEp-2 IIF positivity (35.7*%) that was significantly higher than those of patients referred by the other areas analyzed together (n=50,564, positivity=19.6%, $p < 0.0001$) (Fig. 5).

Of the 52,117 tests, 10,869 were positive (20.9%). The analysis of the HEp-2 IIF patterns was made according to the specialties requesting the tests, considering the ICAP cellular domains (nuclear, cytoplasmic and mitotic) and the ICAP and BCA alphanumeric codes. The presence of a nuclear pattern varied between specialties from 63.3% (oncology) to 88.1% (others) (mean=84.4%). The proportion of cytoplasmic patterns ranged from 0.6 (pediatrics) to 11.7% (oncology), and mitotic patterns ranged from 0.0 (geriatrics) to 3.1% (pneumology) (means=4.6 and 1.6%,

respectively). The proportion of sera with mixed HEp-2 patterns (BCA) ranged from 5.0 (cardiology) to 23.3% (oncology) (mean=9.4%). Except for sera referred by pneumologists and oncologists, the ICAP patterns more frequently observed in all medical specialty samples were AC-4 (nuclear fine speckled) and AC-2 (nuclear dense fine speckled). In samples referred by oncologists, AC-4 and mixed patterns were observed in the same proportion (23.3%), with a relatively high proportion of cytoplasmic patterns (11.7%). Samples referred by pneumologists showed AC-4 and BAC-3 (nuclear *quasi*-homogeneous) as the most frequent patterns. When we compared the positive test results (n=10,869) between rheumatologists and non-rheumatologists, we observed that the most prevalent patterns in samples referred by rheumatologists (n=1580) were AC-4 (38.5%); AC-2 (15.4%); BAC-3 (11.2%); AC-1 (9.6%); mixed/composite patterns (9.1); AC-5 (4.1%); AC-3 (2.7%); and AC-8,9,10 (1.9%). For samples referred by non-rheumatologists (n=9289), the most frequent patterns were AC-4 (36.0%); AC-2 (21.1%); BAC-3 (10.3%); mixed/composite patterns (9.4%); AC-1 (6.4%); AC-8,9,10 (2.9%); AC-5 (2.3%); AC-21 (2.0%); and AC-3 (1.9%).

Fig. 4 Proportion (%) of HEp-2 IIF tests requested, according to medical specialties, in 52,117 consecutive serum samples referred to the laboratory from January to June 2017. OFT/ORT: Ophthalmology and Otolaryngology

Fig. 5 HEp-2 IIFA positivity (%) according to referring specialty in 52,117 consecutive tests performed between January and June of 2017. OFT/ORT: Ophthalmology and Otolaryngology. *$p < 0.0001$ in the comparison between rheumatology and non-rheumatology

HEp-2 IIF titles and requesting physician specialties

Of the 52,117 samples with identified referring specialties, 10,869 tested positive for anti-cell antibodies on HEp-2 IIFA. Titers of 1/80, 1/160, 1/320, 1/640, 1/1280 and ≥ 1/2560 were observed in 12.4, 40.7, 30.0, 11.5, 5.1 and 0.3% of patients, respectively. Antibody titers were stratified into low (1/80 and 1/160), moderate (1/320 and 1/640), and high (≥ 1/1280) according to the degree of fluorescence intensity. More than half of the positive samples (53.1%) had low HEp-2 IIF titers (1/80 or 1/160). Of the 10,869 positive samples, 1580 (14.5%) were requested by rheumatologists and 9289 (85.5%) by non-rheumatologists. There was a higher frequency of moderate (1/320–1/640) (46.4%) and high (≥ 1/1280) (10.8%) titers in the samples of patients referred by rheumatologists when compared to those referred by other medical specialists (moderate = 40.9%; high = 4.6%) ($p < 0.0001$). Conversely, a higher proportion of HEp-2-IIFA with low titers was observed in the samples ordered by other specialists (54.8%) than in the HEp-2 IIFA ordered by rheumatologists (42.8%) ($p < 0.0001$) (Table 1).

Table 1 HEp-2-IIF titers in 10,869 positive samples, according to referral specialty (rheumatology versus other specialties)

Titer	Rheumatology		Other specialties		p value (Chi-square test)
	n = 1580 (%)	Reactivity grade (%)	n = 9289 (%)	Reactivity grade (%)	
1/80	131 (8.3)	Low	1215 (13.1)	Low	$p < 0.0001$
1/160	545 (34.5)	676 (42.8)	3879 (41.8)	5094 (54.8)	
1/320	483 (30.6)	Moderate	2780 (29.9)	Moderate	$p < 0.0001$
1/640	251 (15.9)	734 (46.4)	998 (10.7)	3778 (40.7)	
1/1280	155 (9.8)	High	394 (4.2)	High	$p < 0.0001$
≥ 1/2560	15 (0.9)	170 (10.8)	23 (0.2)	417 (4.5)	

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Discussion

Approximately one-fifth of 54,900 serum samples included in this study were positive for anti-cell antibody tested on HEp-2 cells. This study reinforces the importance of demographic data on the positivity of HEp-2 IIFA results. In addition, the study details the medical specialties most involved in requesting the exam. Fluorescence patterns and antibody titer differences between patients referred by rheumatologists and non-rheumatologists were relevant. For an appropriate comparison of the present study results with those of other authors, the inclusion of literature published before the ICAP foundation in 2014 was avoided as much as possible.

Many factors can interfere with estimating the prevalence of HEp-2 test positivity. These factors range from aspects related to the sample population tested to technical issues. The technical issues could be related to the definition of the cutoff point, technicians' experience in reading and interpreting the HEp-2 cell staining patterns, and the cellular domains recognized as positive by the laboratory [15]. Substrate variability is an essential analytical aspect since many substrate-dependent patterns exist [16, 17]. The ICAP and BCA initiatives have made significant contributions toward standardizing technical aspects related to HEp-2 IIF procedures and issues related to the identification, description, reporting, and interpretation of the multiple HEp-2 staining patterns [4, 18, 19].

The prevalence of so-called ANA in healthy individuals (HI) in titers of 1/80 or 1/100 varied from 12.2 to 26.4% [5, 7, 11, 20]. In nonautoimmune diseases, comprising neoplastic, psychiatric, infectious, and chronic noninfectious diseases, anti-cell antibody positivity was described in 18.3% of the patients [11]. In patients with SARD, HEp-2-IIF positivity can be found in up to 90.2% of the cases [10]. In population-based studies in which a dilution of 1/80 or 1/100 was adopted as a criterion for HEp-2-IIF positivity, the prevalence of autoantibodies ranged from 15 to 33.3% [8, 9]. Some authors draw attention to the increase in the prevalence of HEp-2-ANA in the general population over time, which is more pronounced among males and among adolescents between 12 and 19 years of age [6, 7]. In this study, the prevalence of anti-cell autoantibodies, as detected on HEp-2 cells, was 20.9%. Clinical data for a more detailed analysis of this result were not available.

In our study, greater positivity of the HEp-2 IIF test was observed at the extremes of the age groups, which comprised individuals aged between 1–19 years and over 60 years. Positive results were observed in 23.3% and 22.8% of samples in these groups, respectively. The positivity of ANA in healthy Brazilian children and adolescents (6 months to 20 years old), considering the cutoff point of 1/80, was 12.6% [21]. In pediatric population-based studies, HEp-2 assay positivity ranged from 11.2 to 27.6% [22–25], which is consistent with our results. The opposite results, with a lower prevalence of autoantibodies at the extremes of age, were reported by Guo et al. [26] in a study involving 20,970 participants, using the 1/320 dilution as a cutoff point. Some authors emphasize a lower positivity of the so called ANA in children than in adults. However, this information is mainly supported by publications predating ICAP recommendations [27–29], which involved different cutoff point values and HEp-2 cell positivity criteria.

Dividing the group over 60 years old into two subgroups of 60–69 (n=5883) and ≥70 years (3952), HEp-2-IIFA positivity increases from 21.9 to 24.1%. This reinforces the knowledge that the prevalence of autoantibodies increases with aging. Similar to our results, many studies show greater ANA positivity in the elderly when compared to younger adults [6, 7, 9, 10, 22]. Accumulated exposure to environmental factors and the decline in adaptive and innate immune responses resulting from immunosenescence may be implicated in autoimmunity manifestations in old age [30, 31].

The striking female predominance in autoimmune diseases has been widely recognized [32]. Sex hormones, especially estrogens, seem to play a significant role in the development and predisposition of women to autoimmune disorders [33]. The presence of autoantibodies is considered an important biomarker of autoimmunity. Our research showed that the test's positivity was higher in women than in men in any age group. Similar results were observed by Akmatov et al. [8], who found higher positivity among women in all age groups, most significantly in the range of 50–59 years old. HEp-2 IIF positivity is higher in females in pediatric and adult populations [6, 7, 9, 22, 32].

In recent decades, a steady increase in the frequency of autoimmune diseases among adults and children has been observed [5, 34, 35]. ANA-HEp-2 testing has become the most requested test for initial autoimmunity screening worldwide. Currently, recognizing that several organ-specific and systemic diseases can be associated with anti-cell antibody positivity, practitioners of many medical specialties include autoantibody determination in routine clinical practice. Consequently, the specialty profile of physicians who order ANA has changed [36]. Unlike the 1960s, when ANA requests were almost exclusively made by rheumatologists and immunologists, today, these specialties are responsible for a minority of requests. However, it is worth noting that ANA tests obtained outside of the rheumatological setting are poorly predictive of ANA-associated SARD [13, 15]. Considering groups of patients and not any particular

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one, these changes in the HEp-2 assay referral pattern had a detrimental effect on test diagnostic properties, reducing pretest and posttest probabilities and generating possible unwanted effects on health care expenditures [12, 13, 36].

In our study, of 52,117 HEp-2 assays analyzed, only 8.5% were requested by rheumatologists. General practitioners, dermatologists, gynecologists, cardiologists, gastroenterologists, endocrinologists, and orthopedics were responsible for 67.5% of the requests. Notably, samples from patients referred by rheumatologists had almost twice as much HEp-2 test positivity (35.7%*) as those referred by non-rheumatologists (19.6%). It is worth mentioning that under similar technical conditions for performing the test described in the literature, apparently healthy individuals presented a HEp-2-IIFA positivity rate of 12.3–12.9% [10, 11] and patients with non-autoimmune diseases, 18.3% [11]. The higher test positivity in patients referred by rheumatologists probably reflects a more accurate indication of the exam and a better pretest probability. Ideally, HEp-2-IIF testing should be guided by clinical complaints that refer to early stage of anti-cell antibodies related diseases. Commonly, HEp-2-IIF testing by non-rheumatologists or non-immunologist is inappropriately ordered and misinterpreted. Possible false-positive results can be associated with misdiagnosis, potentially harmful treatments and unnecessary follow-up testing [13, 36].

Peene et al. [37], in a study involving 15,937 serum samples analyzed in a university hospital, observed that rheumatologists referred only 25% of the sera. The remaining samples were referred by other medical specialists, including internal medicine clinicians, gastroenterologists, dermatologists, neurologists, and nephrologists [37]. Similarly, Minz et al. [38], in a study performed in Northwest India with 3435 samples, found that rheumatologists were responsible for only 2.6% of the requests. Almost half of the requests came from the department of internal medicine (30.5%) and pediatrics (19.6%). Other samples were referred by nephrology (5.8%), gastroenterology (3.2%), neurosurgery (2.7%), dermatology (2.0%), and others specialty areas. In agreement with our research, positive results were more frequently observed among the samples referred by rheumatologists [38]. According to other authors, primary care practitioners were responsible for the most ANA requisition and referrals of patients with positive results to the rheumatologist [13, 39]. This change in the ANA request pattern and its diagnostic and financial implications are well addressed elsewhere [12, 13, 36].

The fluorescence pattern and antibody titer levels are essential factors for the correct interpretation of the HEp-2 IIF assay. In the present study, 11,478 of 54,990 sera were HEp-2-IIFA positive. Fluorescence patterns recognized in any cellular domain (nucleus, cytoplasm, or mitotic

apparatus) with titers ≥ 1/80 were reported as positive. Considering the ICAP and BCA proposals, the proportions of nuclear, cytoplasmic, mitotic, and mixed patterns were 85.7, 4, 1.5, and 8.8%, respectively. Chhabra et al. [40] found a similar proportion of stained cellular domains in 1656 ANA-positive sera, where 4.9% presented with cytoplasmic and 0.4% with mitotic fluorescence. The five nuclear patterns more frequently observed in our study were AC-4, AC-2, BAC-3, AC-1, and mixed. Only the Brazilian consensus recognizes the BAC-3 code (named nuclear *quasi*-homogeneous). It is an intermediate pattern between the nuclear homogeneous and dense fine speckled patterns and has an undefined clinical association. BAC-3 and Mixed/composite patterns are represented in the BCA decision tree, but not in the ICAP one. These two patterns together represent almost a fifth of all positive results, which points to the need for greater convergence between ICAP and BCA initiatives. The most frequently reported cytoplasmic pattern was AC-21. Furthermore, the most frequently observed mitotic pattern was AC-26. In this study mixed or composite patterns were assigned to cases with more than one fluorescence pattern detected or more than one stained cell domain in the same sample. We observed a high relative proportion of cytoplasmic and mixed patterns in patients referred by oncologists and also a high relative proportion of nuclear *quasi*-homogeneous (BAC-3) in patients referred by pneumologists.

Some population-based studies published in recent years do not specify ANA-HEp-2 patterns or use automated or semiautomated ANA reading systems, with limited fluorescent patterns, making comparisons with our findings difficult [6–8, 22]. Wei et al. [20], in a patient-based study with 4583 individuals (3510 with SARD and 1073 HI), found AC-4, AC-1, AC-5, AC-8-9, and mixed patterns to be the most frequent results in the SARD group (HEp-2 test positivity = 78.7%). In the HI group, 12.2% of HEp-2 tests were positive, and AC-2, AC-4, AC-1, and AC-8-9 were more frequently observed [20]. Augustinelli et al. [11], in a study involving 1969 Brazilian individuals divided into three groups (HI; nonautoimmune disease – NAD; and SARD), found HEp-2 IIF positivity equal to 21.4%. The patterns most frequently observed were AC-4, AC-2, AC-1, AC-21, AC-5 and AC-8-10. Patterns such as AC-1, AC-3, and AC-5 were significantly more prevalent in the SARD group. Moreover, AC-2 and AC-21 were more frequent in the HI and NAD groups [11]. Krzemień et al. [9] analyzed 1731 HEp-2 test results from a general Polish population and found anti-cell antibodies in 15% of the cases. The most frequent patterns observed were AC-2, AC-21, AC-4,5, AC-9,10 and AC-1. Hadalwar and Sodani [41] found 18.9% mixed patterns in 280 ANA-positive samples from 650 subjects. Speckled and homogeneous patterns were the most frequent single ANA patterns observed.

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Autoantibody titers are another relevant criterion for the interpretation of HEp-2 IIF results. Higher antibody titers are more associated with SARD and more likely to identify antigen specificity [5, 10, 15, 18, 42]. As demonstrated by Vulsteke et al. [43], depending on the fluorescence pattern, the positive predictive value (PPV) of the HEp-2 test for some autoimmune diseases increases as the titer increases. In this study, we observed that anti-cell antibodies in moderate (1/320–1/640) and high titers (≥ 1/1280) were significantly more frequent in the samples referred by rheumatologists compared to those referred by non-rheumatologists. Higher titers of autoantibodies seen on HEp-2 cells were significantly more frequent in SARD patients than in NAD or HI patients [10, 11]. Wang et al. [44] conducted a prospective study involving 355 symptomatic individuals with an initial positive ANA test divided into two groups, a high- (≥ 1/640, n = 118) and a low-titer group (< 1/640, n = 237). After six months of follow-up, 167 patients had developed an autoimmune disease, 81 developed a NAD, and 107 had no defined diagnosis. The majority of patients who developed autoimmune diseases were in the high-titer ANA group. Most patients who developed NADs were in the low-titer group [44]. However, it is essential to note that even with high titers of ANA-HEp-2-IIFA, the positive predictive value for developing ANA-associated autoimmune diseases is low in the absence of clinical suspicion or symptoms [13, 45]. Currently, the HEp-2-IIFA is requested for the diagnostic investigation of a broad spectrum of autoimmune disorders, and rheumatologists have lost their leading role in ordering the exam. This change in the HEp-2-IIFA referral pattern has had negative repercussions on the test's diagnostic properties [12, 13, 36].

Conclusions

Anti-cell antibodies detected on HEp-2 cells were present in approximately one-fifth of a large serum sample referred to the laboratory for autoantibody testing. The age groups for which HEp-2 assays were more positive were 0–19 years and ≥ 60 years. HEp-2 IIFA positivity in samples from female patients was higher regardless of the age group analyzed. Rheumatologists were responsible for less than 10% of the HEp-2 IIF exam requests. HEp-2 IIFA positive results and moderate to high titers of autoantibodies were more frequent in the samples of patients referred by rheumatologists. AC-4, AC-2, BAC-3 and the mixed/composite patterns were the most prevalent HEp-2-IIF patterns observed. There were relatively high proportions of mixed and cytoplasmic patterns in samples referred by oncologists and also a high proportion of BAC-3 nuclear pattern in samples referred by pneumologists.

Declarations

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