REVISTA BRASILEIRA DE REUMATOLOGIA

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SOCIEDADE BRASILEIRA DE REUMATOLOGIA

Original article

IV Brazilian Guidelines for autoantibodies on HEp-2 cells

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http://dx.doi.org/10.1016/j.rbre.2014.02.006

ARTICLE INFO

Article history:
Received 4 September 2013
Accepted 13 October 2013

Keywords:
Autoantibodies
HEp-2 cells
Antinuclear antibodies
Indirect immunofluorescence
ANA consensus

ABSTRACT

Objective: The Fourth Brazilian Consensus for Autoantibodies Screening in HEp-2 Cells (ANA) was held in Vitória, Espírito Santo, and aimed to discuss strategies and recommendations about the technique, standardization, interpretation and quality control of the indirect immunofluorescence reaction on HEp-2 cells.
Methods: Twenty three ANA experts from university centers and private laboratories in different areas from Brazil discussed and agreed upon recommendations for the fourth edition of the Brazilian Consensus for Autoantibodies Screening in HEp-2 Cells.
Results and conclusion: The 4th ANA Consensus included three novel patterns into the existing algorithm (cytoplasmic Rods and Rings, nuclear *Quasi*-homogeneous, and CENP-F). Emphasis was given to the need of attention in describing the peculiar mixed pattern elicited by anti-DNA topoisomerase I (Scl-70) autoantibodies, comprising nuclear fine specked, nucleolar homogeneous pattern, NOR staining in metaphase plates, and cytoplasmic fine speckled patterns. The group also emphasized the need for continuous quality control in indirect immunofluorescence assays, the establishment of screening dilutions, as well as conjugate titration. An alert was made regarding the heterogeneity of commercial kits in defining patterns and the use of solid phase methodologies to determine the presence of autoantibodies.

IV Consenso Brasileiro para pesquisa de autoanticorpos em células HEp-2

Palavras-chave:
Autoanticorpos
Células HEp-2
Anticorpos antinúcleo
Imunofluorescência indireta
Consenso de FAN-HEp-2

RESUMO

Objetivo: O IV Consenso Brasileiro para Pesquisa de Autoanticorpos em Células HEp-2 (FAN) realizado em Vitória (ES), no dia 18 de setembro de 2012, objetivou discutir estratégias e recomendações relacionadas ao procedimento técnico, à padronização e à interpretação dos resultados da pesquisa de autoanticorpos em células HEp-2.
Métodos: Participaram do evento 23 pesquisadores e especialistas de Universidades e laboratórios brasileiros. Foram abordados diferentes tópicos, discutidos amplamente a fim de se estabelecer recomendações específicas.
Resultados e conclusão: O IV Consenso integrou à árvore de decisão o padrão citoplasmático em Anéis e Bastões, o padrão nuclear pontilhado *Quasi*-homogêneo (QH) e o padrão misto CENP-F. Discutiu-se ainda a necessidade de atenção para a classificação do padrão misto relacionado à presença de anticorpos anti-DNA topoisomerase I (Scl-70), compreendendo os componentes nuclear pontilhado fino, nucleolar homogêneo, NOR na placa metafásica e citoplasmático pontilhado fino. Foram sugeridas diretrizes para o controle de qualidade do teste, diluição de triagem e diluição de esgotamento, e foi emitido alerta quanto à necessidade de atenção em relação à heterogeneidade de substratos disponíveis no mercado e a utilização de metodologias automatizadas para detecção de autoanticorpos.

Introduction

Over the last decade, the evaluation of autoantibodies against cellular antigens by indirect immunofluorescence (IIF) on HEp-2 cells underwent elaborate process of national standardization. These actions began in 2000, bringing several repercussions in Brazilian territory for the completion and interpretation of the test,[1-4] stimulating even similar international initiatives.[5-8]

The first Consensuses were aimed at establishing of actions in order to standardizing criteria for reading and interpreting the different patterns of autoantibodies on HEp-2 cells. Classification algorithms, based on morphological criteria and establishing five main groups of patterns (nuclear, nucleolar, cytoplasmic, mitotic apparatus and mixed) were prepared. Each algorithm was presented with orientation manuals, and the main clinical relevance of the different findings have also been addressed.[1,2]

The III Consensus, conducted in 2007, aimed to upgrade the clinical relevance of the test, to suggest effective measures for quality control, and to evaluate the difficulties in implementing the standardization norms.[3,4] The definition of still controversial aspects of the definition of nucleolus positivity was also discussed in III Consensus, and it was suggested the incorporation, to the algorithms, of two new

fluorescence patterns that remained reserved, so that further studies were performed to obtain scientific evidence for its recognition – Cytoplasmic Rods/Rings Pattern and Speckled Quasi-homogeneous Pattern.[3,4] In view of continuing education and of the need to keep up with scientific developments , the IV Brazilian Consensus for Autoantibodies in HEp - 2 cells was held. The event was held in Vitória - ES, and gathered 23 experts on the subject from different regions of Brazil, and again the difficulties and advances in standardization of the test were discussed. The IV Consensus integrated, to the decision tree, the rings/rods pattern, the nuclear quasi-homogeneous speckled pattern (QH), and the mixed pattern observed with the presence of anti-CENP-F antibodies. Also was discussed In the Consensus the need for attention to the classification of mixed nuclear pattern associated with the presence of anti-DNA topoisomerase antibodies.[9] Guidelines for quality control for the test and for the screening and exhaustion dilutions were suggested. Finally, warnings were issued about the heterogeneity of substrates available on the market and about the use of automated methodologies for detection of autoantibodies.

The results of the IV Consensus allowed further progress in improving the criteria that enable the satisfactory control and utilization of the potentiality of this auxiliary method of diagnosis.

Work methodology

During the XXIX Brazilian Congress of Rheumatology (BCR), 23 researchers and experts from universities and private laboratories in different regions of Brazil took part in the IV Consensus in Vitória (ES), on September 18, 2012. In this event, recommendations related to technical procedures, standardization in the implementation and interpretation of test results were discussed and approved. Commercial representatives of different manufacturing companies attended the meeting as listeners, without commenting, voting, or presentation rights. Different topics related to the description of new patterns, the technical procedure of the test, the dilution and titration of sera, the reproducibility of different brands of substrates, the use of automated methods for identifying autoantibodies, and presentation of reports were discussed. Each of the topics was presented to members of the assembly by relators and discussed widely, in order to establish recommendations. Each relator was based on data from the literature and from the presentation of personal studies.

General recommendations

I- Cytoplasmic rods/rings pattern

The rods/rings pattern was presented during the III Consensus, although at that time without defined immunological identity and only with preliminary scientific evidence. Therefore, this pattern was not incorporated into the decision tree at that occasion, with the recommendation of additional studies. After these studies, inosine monophosphate dehydrogenase 2 (IMPDH2) and cytidine triphosphate synthase 1 (CTPS1) were recognized as antigenic targets.[10] These are essential enzymes in the biosynthesis of cytidine triphosphate and guanosine triphosphate, respectively. CTP is involved in the biosynthesis of nucleic acids (DNA, RNA) and phospholipids, with an important role in cell proliferation.[11] IMPDH2 catalyzes the NAD-dependent oxidation of inosine monophosphate to xanthosine monophosphate, an essential process in the biosynthesis of guanosine monophosphate – therefore, an activity also closely related to the cell proliferation mechanism.[12] From the pharmacological inhibition of CTPS1 (6-diazo-5-oxo-L-norleucine [Acivicin]) and IMPDH2 (Ribavirin), a dose-dependent induction of cytoplasmic rods/rings structures on substrates of neoplastic cells, including HEp-2 cells, was evidenced.[10]

The Keppeke et al. (2012) study confirmed the close association between cytoplasmic rods/rings pattern and HCV infection. In a sample of 597 subjects with several clinical conditions, antibodies associated with the rods/rings pattern occurred exclusively in patients with HCV. Among 342 patients with HCV, the autoantibody occurred in 38% of those treated with ribavirin and interferon alpha, but in none of the other patients, including those receiving one of these drugs as monotherapy. Demographic parameters, time since diagnosis, response to treatment, virus genotype or viral load were not correlated with the pattern.[13] In the study by Covini et al. (2012), the production of autoantibodies against cytoplasmic structures after treatment with Ribavirin/IFN was observed in 15 HCV-positive participants.[14]

The IV Consensus integrated the rods/rings pattern to the decision tree (Fig. 1); this pattern was classified as an independent Cytoplasmic Pattern, not entailed to other cytoplasmic patterns. A relevant information on the characterization of this pattern was related to the fact that the rods/rings structures are not expressed in all commercial substrates.[15] Therefore, the IV Consensus suggested that, in the report, be informed that the recognition of this pattern is dependent on substrate.

II - Quasi-homogeneous speckled pattern (QH)

The IV Consensus integrated the nuclear quasi-homogeneous speckled (QH) pattern into the nuclear pattern decision-making tree. This pattern fits into the interpretation guide as optional (Fig. 1) and is characterized by extremely thin specked nuclear fluorescence, approaching the homogeneous texture, with the metaphase plate similarly stained. This is a pattern distinct of the nuclear homogeneous and of the nuclear dense fine speckled patterns, where a single antigenic specificity is not noted, but miscellaneous antigenic targets are recognized. França et al. showed that the quasi-homogeneous fine speckled pattern exhibits a autoantibody profile intermediate between that of the dense fine speckled pattern and the homogeneous pattern. Also, the clinical profile associated with quasi-homogeneous fine speckled pattern lies in a gray area between the dense fine speckled pattern and the homogeneous pattern.[16] Therefore, the identification of this pattern suggests further investigation of the clinical diagnosis, because it can be related to systemic autoimmune rheumatic diseases.[17]

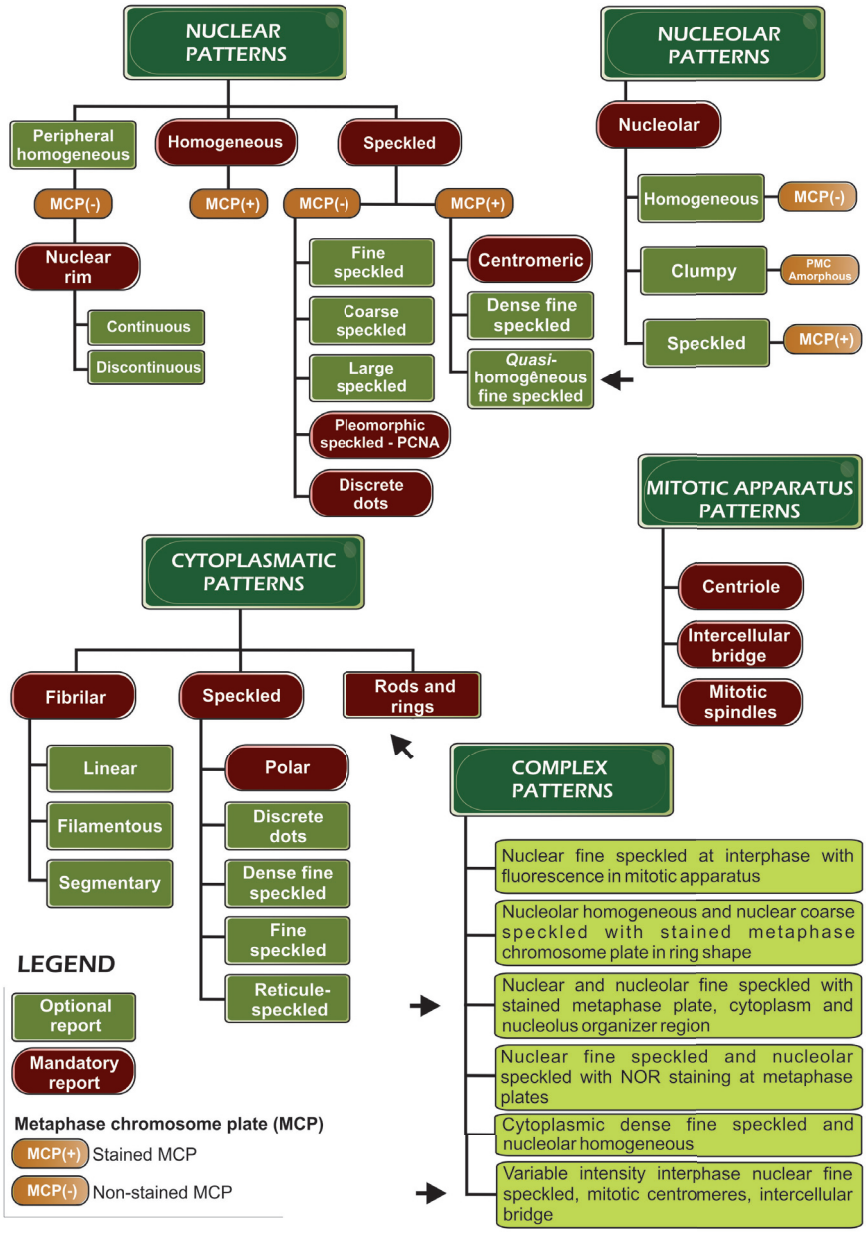

Fig. 1 – Classification trees for nuclear, nucleolar, cytoplasmic, of the mitotic apparatus and mixed patterns. The arrows indicate the additions of new recognized patterns.

III – Mixed pattern of CENP-F type

The IV Consensus integrated the CENP-F pattern into the mixed pattern decision tree. This pattern is characterized by thin specked fluorescence of variable intensity in the nuclear matrix in interphase cells and with nucleoli usually negative. In this pattern a delicate lacy decoration of kinetochores is noted, being predominantly visible in prophase and metaphase. The mitotic apparatus still exhibits occasional punctual markings in the central region of the intercellular bridge, in telophase cells. Finally, the figures in prophase exhibit delicate coloration of the nuclear envelope.[18]

This is a complex pattern, caused by antibodies against a 350 kDa protein, known as CENP-F or mitosin. This protein plays an important role in the organization of cytoplasmic microtubules, methylation of histone H3, regulation of some transcription factors and cell cycle progression to mitosis.[19,20] Rattner et al. identified the serum pattern from a patient with lung cancer[18] and later in breast cancer.[21,22] Cassiano et al. reported positivity for the pattern in different neoplastic diseases, chronic liver diseases, chronic renal allograft rejection and Crohn's disease.[23] The presence of the CENP-F pattern in a patient with colorectal carcinoma has been reported.[24] As a whole, the literature points to the suspicion of neoplastic disease in patients with this pattern.

IV - Mixed pattern of anti-DNA topoisomerase type

The IV Consensus drew attention to the compound pattern related to the presence of anti-DNA topoisomerase I (Scl-70).

In the literature, the classic description of the pattern associated with anti-DNA topoisomerase I antibodies is restricted to the nucleus and nucleolus, with no specificity in this finding. It was recently shown that anti-DNA topoisomerase I antibodies cause an extremely specific pattern, characterized by decoration of five cellular domains, namely, nucleus, nucleolus, cytoplasm, nucleolus organizer region and metaphase plate chromosomes.[9]

V - Titration of the conjugate and quality control of the assay

The IV Consensus stressed again the need for a rigorous quality control of the assay in order to restrict false-positive reactions in non- autoimmune individuals, but with request for the test and with the purpose of minimizing the differences of results between different laboratories. The continued need for titration of the conjugate for equalization of Brazilian laboratories' systems and the use of adjacent negative and positive controls was recommended. This orientation, previously established and detailed in III Consensus,[4] underscores the need for the Brazilian laboratories ensure the quality of the test. In this sense, it must be emphasized the need for specific training and qualification of technical staff, in addition to considering the heterogeneity of commercial kits and of optical equipment between different services. The need of performing the titration of the conjugate for each new batch of commercial kit was reinforced, based on the use of commercial reference sera, or of sera coming from other services.[4]

Considering that the investigation of autoantibodies on HEp-2 cells depends technically on multiple factors (microscope power lamp ranging from 20, 50 or 100 W; concentration and protein/fluorescein rate of the conjugate; minimal reactivity of control sera in dilution 1/80; qualification and inherent subjectivity of the observer), it appears that the titration of the conjugate is a fundamental parameter and subject to adjustment, in order to ensure the recognition of the nominal title of control sera. This measure is considered vital, in order to achieve objectivity and accuracy for the method.[4]

VI – Screening dilution and titration of sera

The IV Consensus urged the Brazilian laboratories the use of a screening dilution of 1 /80. This recommendation is based on the fact that some autoimmune patients may have titles of 1/80, although most of them present moderate (1/160 and 1/320) to high (≥ 1/640) autoantibody titles on HEp - 2 cells, while healthy subjects tend to have low titers (1/40 and 1/80).[17,25] Another aspect which reinforced the need for this recommendation was the fact that the test continues to be requested by a variety of specialists that attend patients from various types in services, where the autoimmune rheumatic diseases are less prevalent. The IV Consensus emphasizes that the test should be requested in the presence of a compelling clinical suspicion of autoimmune disease, preventing that the request of the test in an inappropriate clinical context (low pretest probability) result in confusion in the clinical reasoning.[4]

Another orientation was related to depletion of serum up to 1/640: the positive samples can be released as ≥ 640, for it has been shown that up to this title there is substantial gain in terms of positive predictive value for the diagnosis of autoimmune rheumatic diseases.[17,26] In some circumstances, the continuity of dilution for discriminating one (or more than one) concurrent pattern may be desirable.

VII-Reproducibility of different patterns

The IV Consensus warned about the reproducibility of different patterns among different commercial brands. There is a degree of variation among different commercial substrates available in the Brazilian market, and this variability can affect differently the definition of different patterns. The variations may be related to the batches, being inherent to the manufacturing process of the kits.

In a recent study, Dellavance et al. (2013) analyzed 17 patterns of recognized diagnostic relevance in eight substrates. The processing of reactions and the reading were done blindly and independently by three diagnostic centers. Generally a good reproducibility of the 17 tested patterns was evidenced.[27] However, some patterns showed significant variability of recognition in some commercial substrates, such as the CENP-F pattern, the cytoplasmic fine speckled (associated with anti-Jo-1) pattern, and the nuclear speckled pattern type PCNA pleomorphic. Such patterns have been recognized in only two of the eight tested substrates.[27] This study showed that the most part of the patterns was adequately recognized in most antigenic substrates analyzed. Possibly one of the aspects that subsidized this high rate of reproducibility was the fact that the samples used in the study were immunologically and morphologically well characterized. These results cannot be extrapolated to the situation of samples with fluorescence patterns less well characterized.

Considering that autoimmune patients do not always present monospecific sera, IV Consensus warned to the need to use a panel of control samples for validation of batches and commercial brands of HEp-2 cells used in laboratories, because this measure will ensure greater reliability and safety in the application of the results by clinicians. Furthermore, the use of more than one commercial brand of substrate for specific cases is recommended, and that, for each new batch or slide brand, the reference sera, representing the different cellular regions and patterns, be tested.

VIII - Automated methods for autoantibodies screening

The IV Consensus does not recommend the use of automated assays (EIA and chemiluminescence) in the screening of autoantibodies. There is considerable supply of commercial kits for screening of autoantibodies based in solid phase immunoassays and with antigenically distinct formulations. Despite the considerable progress of the industry in improving these products, its diagnostic performance do not superpose on the traditional indirect immunofluorescence assay with HEp-2 cells. False-negatives in ANA ELISA, for example, can create serious diagnostic problems, with unsuspected consequences.[28,29] In addition, that test allows the preliminary analysis of the likely autoantibodies present in a certain serum, with careful interpretation of the immunofluorescence pattern, while the solid phase immunoassays only provide a numerical result.

IX - Detection of specific antibodies

The IV Consensus alert to the choice of methods for identification of specific autoantibodies such as anti-native DNA antibodies and antibodies against extractable nuclear antigens. The Consensus emphasizes the need to take care for the excessive sensitivity of the immunoenzymatic methods, given that the standardization of the detection of specific autoantibodies and their clinical correlations were originally described based on the method of double immunodiffusion and its counterpart, counterimmunoelectrophoresis.

Automated methods are more sensitive, and its positive predictive value is generally lower, being therefore suitable for carrying screening services in general, but not in specialized laboratories. For the other hand, the double immunodiffusion is a method that ensures excellent clinical correlation, being relevant as a confirmatory test when automated methods are used in a first phase, or as method of choice for supportive laboratories for rheumatology. The use of more sensitive methods tends to produce positive results in clinical contexts different than those in which autoantibodies are expected, and this may impair the diagnostic process. Ultimately, it is possible that the widespread use of ultrasensitive methods compromise the reputation of these autoantibodies as specific biomarkers.

With the use of solid phase immunoassays, it was recommended that the results be confirmed by specific methods (double immunodiffusion, counterimmunoelectrophoresis, *Crithidia luciliae* immunofluorescence, immunoblotting, etc.), to ensure high specificity to the final result. This recommendation is essential in defining the diagnosis, being less relevant in monitoring the patient. Therefore, the choice of method of identification must be deployed with care, based on the profile of patients attending the service.

X- The report

The IV Consensus kept the presentation of the results descriptively, but suggested that the report is presented at the top of the result, making the presentation easier to the Rheumatologist (Fig. 2). It was recommended that the report continues to contemplate the reactivity (fluorescent/non-fluorescent, or reactive/non-reactive) in different cellular compartments (nucleus, nucleolus, cytoplasm, mitotic apparatus), singly or in combination (in the case of mixed patterns).

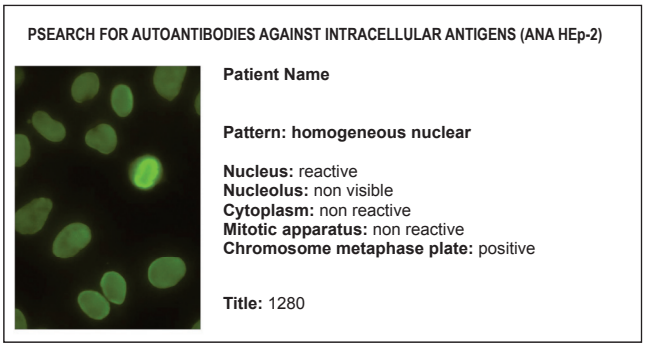

Fig. 2 – Example of a descriptive report based on the recommendations of the IV Consensus, presenting as first information the definition of the pattern.

Acknowledgements

The Authors wish to express their gratitude to Fundação Aroeira and the sponsors of the event, Albert Einstein Medicina Diagnóstica, Alka Tecnologia, Amaral Costa Laboratório, Conselho Federal de Biomedicina, Conselho Regional de Biomedicina - 3ª Região, DASA, Euroimmun Brasil, Grupo Fleury, Hemagen, Hermes Pardini, Medivax, Olimpus, Padrão Laboratório Clínico, Pontifícia Universidade Católica de Goiás - PUC-Goiás, Sociedade Brasileira de Patologia Clínica e Medicina Laboratorial, Sociedade Brasileira de Reumatologia, Thermo Scientific, Wama Diagnóstica and Werfen Group - Werfen Medical.

Conflicts of interest

The authors declare no conflicts of interest.

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