EDITORIAL

Evaluation strategy of anti-mitochondrial antibodies M2-negative: the role of multiplex rodent tissues and related clinical implications

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Abstract

Indirect immunofluorescence on HEp-2 cell line (HEp-2-IIF) remains "gold standard" method for the detection of antinuclear antibodies (ANA). ANA is an operative definition, showing the possibility of autoantibodies (Aab) to bind nuclear, and cytoplasmic antigens. One of the major examples is represented by anti-mitochondrial antibodies (AMAs), which target proteins of the inner and outer mitochondrial membranes, located into the cytoplasm. The standard IIF on rat kidney/stomach/liver tissue sections, with the combined use of other commercial assays, may all be used in ordinary lab life to validate the AC-21 pattern on Hep-2 cells.

The routine lab experience teaches that commercial kits cannot always be detected and define specific AMAs.

In these cases the literature proposes the use of other homemade assays to detect AMAs as immunoprecipitation (IP) and Western blot (IP-WB). However, using IP or IP-WB is difficult to apply in a routine laboratory, because of numerous cases to process and the related troubles.

Where find confirmation of the AC-21 pattern if line-immunoblot and other routine methods (ELISA, CLIA/FEIA assays) fail? We review AC-21 AMA-like sera from our patients (year 2022) and propose a revised diagnostic algorithm based on the combined use of IIF on Hep-2 cells, line immunoblot and IIF on rodent tissue as a third line method. We demonstrated that, particularly in cases where the second level test was unsuccessful, the application of IFI on rodent tissues became indispensable to verify the existence of AMAs.

Introduction

Indirect immunofluorescence on HEp-2 cell line (HEp-2-IIF) remains the "gold standard" method for the detection of antinuclear antibodies (ANA) (1). ANA is an operative definition, showing the possibility of autoantibodies (Aab) to bind nucleus and cytoplasmic antigen.

One of the major examples is represented by anti-mitochondrial antibodies (AMAs), which target proteins of the inner and outer mitochondrial membranes, located in the cytoplasm.

Serum AMAs is directed against several components of the mitochondrial membrane and represents the hallmark of Primary Biliary Cholangitis (PBC) (2).

AMA pattern was reported by the International Consensus on Antinuclear Antibody Patterns (ICAP; www.autoab.org) as a "*coarse granular filamentous staining extending throughout the cytoplasm by IIF on cellular substrates such as HEp-2 cells (AC-21)*" (ICAP; www.autoab.org) (3). AMA-related AC-21 pattern may be identified by IIF before the PBC clinical suspect, or during routine ANA screening tests.

Conventionally, AMAs is classified into 9 distinct categories, referred to as M1 through M9 subtypes. Most

AMAs specific bind to a trypsin-sensitive antigen of the inner mitochondrial membrane named M2 (2), mainly targeting a core structure component of the pyruvate dehydrogenase complex (PDC), named E2, but the E1 and E3 subunits are also recognized (AMA-M2, E3). Another subunit, named E1α of the pyruvate dehydrogenase complex (PDC-E1α) can be also detected (2).

Over the years other proteins have been discovered. M4 (sulphite oxidase) and M9 (glycogen phosphorylase) are added as PBC-specific Aab (4, 5).

In routine lab life the AC-21 pattern and the presence of related AMAs can be confirmed by commercial line-immunoblotting, ELISA, CLIA/FEIA assays, and the standard IIF on rodent kidney/stomach/liver tissue sections (2).

The routine lab experience also teaches that commercial kits cannot always be detected and define specific AMA.

Literature proposes the use of other homemade methods to detect AMAs. In our a very recent work Ceribelli and colleagues, using IFI and homemade methods such as immunprecipitation (IP) and Western blot (IP-WB), demonstrated the presence of antibodies against the PDC components in rheumatic patients with PBC or without liver dysfunction. (6). However, the application of IP or IP-WB in a routine laboratory is very difficult, because of troubles. Related to these methods, where find confirmation of the AC-21 pattern
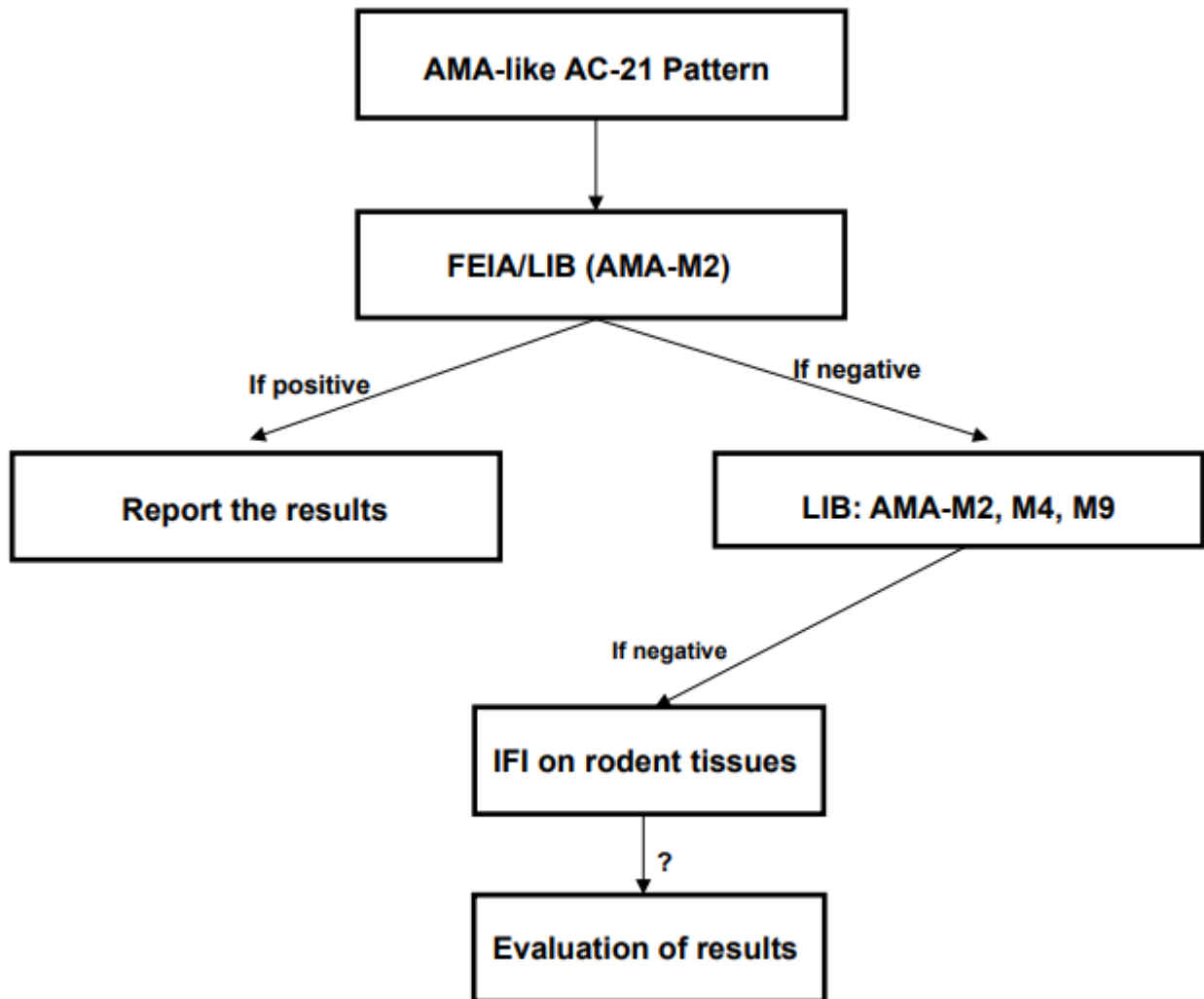
Figure 1. Diagnostic flow chart applied on tested sera. FEIA: Fluorescence immunoassay; LIB: line immunoblot

if line-immunoblot and other routinely methods (ELISA, CLIA/FEIA assays) fail? For these reasons, we review AC-21 AMA-like sera from 3598 tested ANA (year 2022). Sera from patients with AC-21 IFI pattern were tested as shown in Figure 1 (flow chart), described here. AMA-M2 antibodies were searched with FEIA (Phadia, Termofisher) and line immunoblot (EUROLINE, Euroimmun).

When negative results are present, we tested sera with a second commercial line-immunoblot (EUROLINE, Euroimmun) coated with AMA-M2, M2-E3, M4, and M9

antigens. The line-immunoblot test kit provides qualitative in vitro determination of human autoantibodies of the immunoglobulin classes IgG and IgM to 4 different antigens AMA-M2 (pyruvate-dehydrogenase complex), M2-3E (BPO, fusion protein of the E2 subunits of the alpha-2-oxoacid dehydrogenases of the inner mitochondrial membrane), M4 (sulphite oxidase) and M9 (glycogen phosphorylase) in serum to support the diagnosis of PCB. Antibodies against M2 are always present in sera harboring antibodies against M4. If other negative results were also present, sera were tested with
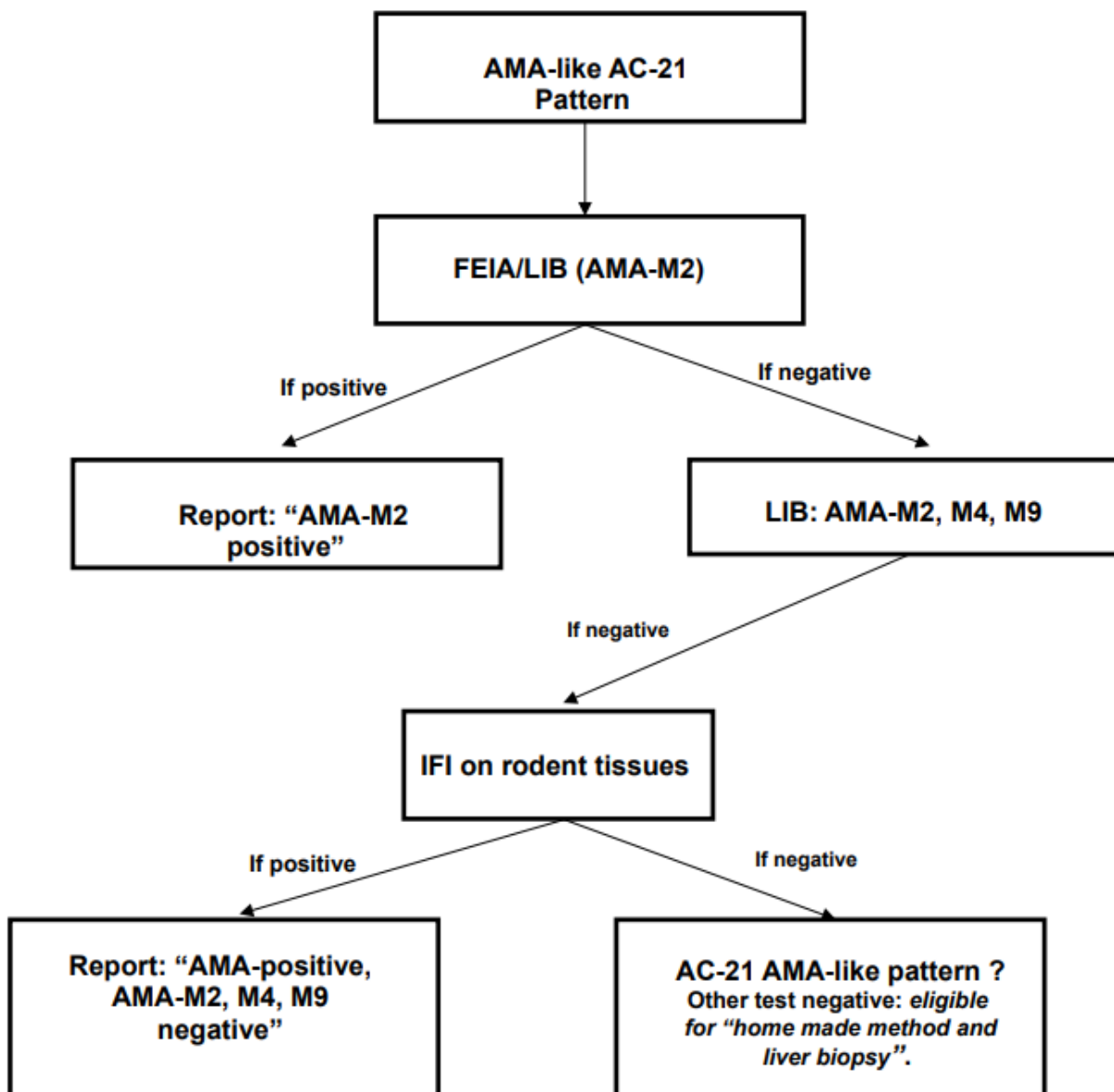

Figure 2. New diagnostic flow chart. FEIA: Fluorescence immunoassay; LIB: line immunoblot

IFI on rodent tissues substrate (Figure 1). IIF on a commercial rat liver, kidney, and stomach tissue was considered positive if a characteristic staining pattern was present: granular diffuse cytoplasmic staining of the Kupffer cells and hepatocytes of the renal tubules (strongest staining is noted in distal which is mitochondria-rich) and parietal gastric cells.

We found 36 (1%, the year 2022) sera with AC-21 IFI pattern. From these patients' sera we able to describe three groups of patients. In Group 1, 25 patients (69%) were present and considered positive for AMA-M2 and/or M2-E3 Aab. Four patients were also positive for gp210 (2 patients 8%), sp100 (one patient 4%) and SSA-Ro52 KDa (one patient, 4%), and anti-centromere B antibodies (CENPB) (4 patients, 16%). Only the AMA-M2 positive patients presented the contemporary presence of other autoimmune diseases. In this contest, complete matches for methods are observed (line immunolbot and FEIA). These patients were characterized by the presence of liver dysfunction in 93% of cases (23/25 patients). Four patients with CENPB were characterized by the contemporary presence of connective tissue disease, with limited - scleroderma features.

Eleven patientswere considered AMA-M3 and M2-E3 negative (11 patients 30.5%). All patients were tested on line-immunoblot (M2-M4, M9), according to the diagnostic flow chart. All patients were d negative. Based on the planned flow chart, we decided to test all patients with IIF on rodent tissues. We showed that eight patients were positive (72.7%, Group 2), with the typical fluoroscopic pattern on rodent tissue. IIF on a commercial rat liver, kidney, and stomach tissue was considered positive if a characteristic staining pattern was present: granular diffuse cytoplasmic staining of the Kupffer cells and hepatocytes of the renal tubules (strongest staining is noted in distal which is mitochondria-rich) and parietal gastric cells. Group 3 is characterized by three patients with AC-21 pattern (27.3%), negative for double line-immunoblot and IIF on rodent tissues. These patients were eligible for IP and WB homemade methods (27.3%) (Figure 2).

AMAs are the undisputed, main hallmark of PBC. In 2018, the ICAP Committee (www.autoab.org) (2) described a reference serum for cytoplasmic reticular IIF pattern (AC-21 in the ICAP nomenclature) with an antigen association with PDCE2/M2, BCOADC-E2, OGDC-E2, E1a subunit of PDC, and E3BP/protein X. An isolated cytoplasmic pattern or other IIF patterns may be linked to an AMA positive on the IFI test in a regularly observed lab setting. Thus, it is necessary to use additional methods to confirm the AMA positivity suspected by IIF using commercial line- immoblotting tests or FEIA/CLIA immunodiagnostic assays. Rarely the clinical pattern need using other laboratory investigations, such as IP and IP-WB for each antigenic component recognized by AMA.

In our very recent work Ceribelli and colleagues, using IFI and homemade methods sdemonstrated the presence of antibodies against the PDC components in rheumatic patients with PBC and others (6). However, due to the large number of cases to handle and the associated analytical issues, using IP or IP-WB in a typical laboratory setting is challenging. Thanks to the improvement of diagnostic assays, nine mitochondrial antigen/antibody patterns —from M1 to M9 — were now described, not only in hepatic disorders, but also in non-hepatic diseases (5). Moreover, only M2, M4, M8, and M9 are specific for PBC; however, anti-M4 and anti-M8 probably represents artifacts of the methods used to detect AMA since they are both targets of AMA-M2 and are predictors of elevated immunological activity of the disease (5).

The routine lab experience also teaches that commercial kits cannot always be detected and define specific AMA.

We recommended the use of IP and IP-WB only for a few and specific patients' sera, for two reasons. First, these methods are very difficult to use out of the research laboratory, because of their long and complicated procedure that are poor compatibility with the time of clinical laboratory. Rapid and precise laboratory testing is essential for the early detection of PBC and the appropriate course of treatment before the development of serious problems from the condition. Second, using these assays as screening methods, if on one hand can identify rare autoantigen, from the common ones without additional testing, are characterized by a lot of steps and high time-cost procedures, that need the great experience of operators.

We strongly encourage clinical laboratories to report AC-21 and another cytoplasmic patterns when analyzing ANA samples (7, 8). Furthermore, recognition of different components of PDC in AMA-positive PBC

patients may define possible clinical association with other clinical subsets, as well as in rheumatic disease patients.

One question arises: where find confirmation of the AC-21 pattern if line-immunoblot and other routine methods (ELISA, CLIA/FEIA assays) fail?

For these reasons, we propose a revised diagnostic algorithm based on the combined use of IIF on Hep-2 cells, line immunoblot, and IIF on rodent tissue as the third line method.

Our data showed that, in the absent of IP and WB, the use of IFI on rodent tissues became decisive to confirm AMAs presence. It provides evidence that the laboratory and its personnel must possess expertise in order to make accurate diagnoses.

Conflict of interests

The authors declare that they have no conflict of interest.

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