

ADVANCING AUTOANTIBODY DETECTION: A JOURNEY FROM CONVENTIONAL TO STATE OF THE ART METHODS

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Abstract. Autoimmune diseases constitute a diverse array of disorders characterized by immune dysregulation leading to abnormal B cell and T cell responses against the body's own components. Among the hallmarks of these conditions is the common presence of autoantibodies. These autoantibodies often target various intracellular or extracellular components such as nucleic acids, structural proteins, enzymes, RNA binding proteins, receptors, and cytokines, underscoring the complexity of the immune responses involved. The detection of autoantibodies plays a pivotal role in diagnosing and classifying individuals with autoimmune diseases. Moreover, research indicates that certain autoantibodies can emerge years prior to the clinical onset of autoimmune diseases. Traditionally, methods such as enzyme-linked immunosorbent assay (ELISA), immunofluorescence, and fixed tissue or cell-based assays have been commonly employed for autoantibody detection. However, these technologies exhibit limitations in terms of sensitivity, specificity, and sample throughput. Consequently, ongoing efforts focus on developing novel experimental techniques to uncover new autoantigens associated with autoimmune diseases. Despite technological advancements, challenges in the detection of autoantibodies persist. Issues such as sensitivity and specificity, temporal variability in autoantibody levels, technical constraints, subjective interpretation, and the absence of standardized quality controls pose hurdles. It is crucial to recognize that autoantibody tests alone are diagnostic tools and should be complemented by clinical evaluation for accurate diagnosis. In this chapter, we will explore the technologies utilized in the detection of both classic and emerging autoantibodies. We will delve into the assessment of clinical relevance, accuracy, validation processes, and potential regulatory requirements necessary for establishing these diagnostic tests as approved tools for routine clinical practice.

Key words: autoimmune diseases, autoantibodies, antigen arrays, immunoassay.

INTRODUCTION

Autoimmune diseases are defined as a state, in which an aberrant immune response directed to a normal body constituent causes inflammation, cell damage, or functional injury with clinical manifestations. The exact causes of autoimmune diseases are not fully understood, but they are believed to result from a combination of genetic, epigenetic, immunological, and environmental factors.

Autoimmune diseases can be classified into two categories: systemic and organ-specific. Systemic autoimmune diseases are characterized by immune responses affecting multiple organs or body systems, including conditions like systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and systemic sclerosis (SSc). On the other hand, organ-specific autoimmune diseases are characterized by immune responses primarily targeting specific organs or tissues, such as type 1 diabetes (T1D), multiple sclerosis (MS), Hashimoto's thyroiditis, and celiac disease [1].

While autoimmune diseases are often considered relatively rare, their impact on mortality and

morbidity is substantial. The estimated prevalence of autoimmunity in the general population is around 3-5%. There is an increased frequency of autoimmune diseases in women, with a female-to-male ratio ranging from 10:1 to 1:1. The incidence and prevalence of autoimmune diseases differ between geographical regions [2]. For example, the incidence of MS is 0.8-8.7 per 100,000 person-years in Europe, 2.7-7.5 per 100,000 person-years in North America, and 0.7-3.6 per 100,000 person-years in Asia and the Middle East [3]. The incidence of T1D is 5-10, 10-20, and < 1 per 100,000 person-years in the populations of Europe, USA, and China, respectively. The global prevalence of RA was estimated at 0.24% in 2010 [4].

Among these diseases, the most prominent immunological manifestation is the production of autoantibodies caused by abnormal reactivity of B and T cells towards normal components of the body. Autoantibodies contribute to the pathogenesis through various mechanisms: (a) direct recognition of the autoantigen by the Fab fraction or activation of com-

plement by binding to Fc receptors through the crystallizable fraction; (b) formation of immune complexes that deposit within tissues, activate complement, and promote the recruitment of neutrophils, myeloid cells, and lymphoid cells; (c) inhibition of cell–cell interactions; (d) binding to soluble molecules, such as cytokines, that regulate the immune response and hinder host regulatory interactions [5].

Autoimmune diseases exhibit various patterns of seropositivity throughout the disease progression. Autoantibodies targeting extracellular domains, such as membrane receptors or secreted molecules, are pathogenic as they disrupt the normal function of proteins or signaling pathways. Diagnostic biomarkers frequently involve autoantibodies directed against intracellular proteins, including structural proteins, enzymes, splicing machinery, RNA-binding proteins, and RNA polymerases. These autoantibodies can be detected several years before the diagnosis, indicating that autoimmune mechanisms remain active and persistent over time [6].

Autoimmune disorders pose challenges in diagnosis, often presenting multiple overlapping sub-phenotypes with shared clinical signs and symptoms. Currently, autoantibodies serve as important biomarkers for the diagnosis, classification, and disease activity [7], thanks to advantages such as:

- 1) detection of autoantibodies can aid in the early diagnosis of autoimmune diseases, even before clinical symptoms appear;

- 2) highly specific to certain autoimmune diseases, autoantibodies provide crucial information to differentiate among different disorders and achieve an accurate diagnosis;

- 3) autoantibodies can be used to assess the activity and progression of an autoimmune disease; changes in their levels or patterns over time can indicate treatment response or disease exacerbation;

- 4) some autoantibodies have been associated with specific clinical outcomes or prognoses, providing insights into the likely course of the disease and guiding treatment decisions;

- 5) the presence of autoantibodies can help tailor treatment to specific therapies targeted at the underlying mechanisms of the disease.

The identification of sensitive and specific laboratory methods for detecting and measuring autoantibodies has been considered a milestone in clinical immunology over the last century. These methods have evolved from conventional immunoassays that detect individual autoantibodies to multiplexed platforms capable of quantifying multiple autoantigens simultaneously. Immunofluorescence (IIF) and

enzyme-linked immunosorbent assay (ELISA) are commonly used for measuring autoantibodies, despite limitations in specificity, sensitivity, and sample throughput. The main challenges in adopting an evidence-based approach to autoantibody testing are the discrepancies seen in biomarker and disease follow-up studies. Various studies have demonstrated differing levels of agreement between assays used for measuring autoantibodies [8]. Consequently, the initial step towards addressing these challenges involves studying both conventional and emerging technologies and their limitations. This comprehensive evaluation is essential to overcome the existing challenges and improve the accuracy and reliability of autoantibody testing in clinical practice.

CONVENTIONAL TECHNIQUES

Clinical laboratories often rely on conventional immunoassays for measuring autoantibodies in order to diagnose autoimmune conditions. However, these strategies may have limitations when it comes to identifying new autoantigens. While conventional immunoassays are valuable for detecting known autoantibodies and established autoantigens, they may not capture the full range of potential autoantibodies that could be associated with emerging or less well-characterized autoimmune disorders.

The identification of new autoantigens provides crucial information about the immunological processes and mechanisms that trigger the autoimmune response. New autoantigens can serve as diagnostic markers to aid in the more precise classification of autoimmune diseases. These autoantigens can be targeted for the development of therapeutic drugs that selectively modulate the autoimmune response, thereby improving efficacy and reducing side effects of current treatments. Additionally, new autoantigens can be used to assess disease activity, predict treatment response, and monitor therapeutic effectiveness over time. In summary, the identification of new autoantigens is essential for a better understanding, diagnosis, classification, treatment, and prognosis of autoimmune diseases.

The presence of autoantibodies alone is not sufficient for a definitive diagnosis, and a comprehensive evaluation of the patient's clinical picture is crucial. Relying solely on autoantibody testing for the diagnosis of autoimmune diseases has several limitations and challenges, such as:

- 1) Limited specificity: Autoantibodies can be present in multiple autoimmune diseases, including in healthy individuals.

2) Sensitivity issues: Not all individuals with autoimmune diseases produce detectable levels of autoantibodies, especially in the early stages of the disease.

3) Variability in antibody profiles: Autoantibody profiles can vary among individuals and even within the same individual over time.

4) Lack of standardized testing methods and reference ranges for autoantibody testing.

While autoantibody testing plays a valuable role in the diagnosis of autoimmune diseases, it should not be solely relied upon. A comprehensive approach that combines clinical evaluation, medical history, and other diagnostic tests is necessary for accurate diagnosis and optimal patient care.

Indirect immunofluorescence (IIF)

IIF is a technique that involves immobilizing specific antibodies from biological fluids by reacting them with antigens present on various substrates. The resulting reaction is then detected using secondary labeling with fluorochromes. The substrates used for capturing and immobilizing antibodies can include cryosections of animal and human tissues such as the liver, kidney, stomach, esophagus, pituitary, pancreas, bladder, or nervous tissue. Additionally, isolated cells of human carcinoma, *Crithidia luciliae*, granulocytes, neutrophils, fibroblasts, intestinal cells, VSM 47 cells, or human embryonic kidney 293 (HEK-293) cells can also be used as substrates [9].

The IIF assay has been widely used for over 50 years as the gold standard for detecting anti-nuclear antibodies (ANA) and anti-neutrophil cytoplasmic antibodies (ANCA). It is also employed as a confirmatory test for detecting anti-dsDNA antibodies. Furthermore, this technique allows the measurement of other autoantibodies, including anti-mitochondrial, anti-smooth muscle, anti-adrenal cortex, anti-pituitary, and anti-skin antibodies. One advantage of the IIF method is that it provides a semi-quantitative result and staining patterns that offer insights into potential specific autoantibodies [10].

IIF assays using human epithelial cell type 2 (HEp-2) are the gold standard for the measurement of ANA [11]. The 2019 EULAR/ACR classification criteria for SLE maintain the presence of ANA in HEp-2 cells, within the criteria [12]. The HEp-2 IIF assay demonstrates high sensitivity, although it also exhibits high interobserver variability and low specificity, these autoantibodies can be found in infectious diseases, inflammatory conditions, and even in healthy individuals [13]. The interpretation of fluorescence patterns can vary among different observers, even

among experienced professionals. This is because patterns can be subtle or difficult to distinguish, which can lead to discrepancies in the results. There can also be subjectivity in the assessment of signal intensity, and discrepancies in assigning categories such as “positive” or “negative” contribute to result variability. Interpretation of results can be subjective and largely depends on the experience and skills of the observer. This can lead to variations in results and a lack of reproducibility among different laboratories or health professionals.

Standardization of the IIF technique presents challenges due to variations among manufacturers of the substrate, the fixation process, and the characteristics of the secondary antibody [14]. Interlaboratory differences in microscopy apparatus and, particularly, the subjective interpretation of results also contribute to the difficulty of standardization. Furthermore, technical obstacles arise in the processing and reading of IIF slides, including manual reading, real-time interpretation, the requirement of a dark room, and the transcription of handwritten results. These factors collectively contribute to the complexity and potential variability associated with the IIF assay.

Genetic technologies have emerged as a means to enhance the sensitivity and specificity of ANA testing. One such advancement is the use of the HEp2000 substrate, which is a knock-in cell line transfected with Ro60 cDNA [15]. This modification improves the detection of antibodies targeting Ro60 antigen. Additionally, a knock-out substrate has been developed more recently to specifically detect antibodies against dense fine speckled (DFS)-70 antigen in the ANA IIF screening test [16]. These genetic-based approaches enhance accuracy and specificity in ANA testing, facilitating the identification of specific autoantibodies associated with certain autoimmune conditions.

To address the subjectivity associated with the diagnostic platform, automated digital reading systems have been developed, along with solid-phase immunoassays utilizing well-characterized antigens. Automated ANA pattern recognition systems utilize hardware and software to identify, store, and analyze IIF patterns obtained from HEp-2 cell substrates [17]. These automated systems typically consist of a microscope, a digital camera, a LED light source, and software for image acquisition. The systems measure fluorescent light intensity to categorize samples as positive or negative and perform pattern analysis. Some automated platforms can even report ANA titers based on fluorescence

intensity without the need for serial dilutions [18]. Although these systems are highly reliable in distinguishing positive and negative results, the precision of patterns is not comparable to human interpretation.

Enzyme-Linked Immuno Sorbent Assay (ELISA)

ELISA is a plate-based assay technique used for the detection and quantification of autoantibodies. In ELISA, the autoantigen of interest is immobilized on a solid surface, such as the wells of a microtiter plate. When the patient's sample, containing the autoantibodies, is added to the plate, the autoantibodies bind to the immobilized antigen, forming an antigen-antibody complex. This complex is then detected using an enzyme-conjugated secondary antibody that recognizes and binds to the patient's autoantibodies. The enzyme-substrate reaction produces a measurable signal, typically a color change, which is proportional to the number of autoantibodies present in the patient's sample. ELISA assays are performed in 96-well plates, which allow for the simultaneous measurement of multiple samples in a single experiment.

During the 1980s, indirect ELISA emerged as a widely utilized technique and has since remained a fundamental method for the diagnosis of autoimmune diseases. ELISA panels are available to measure antibodies against various specific autoantigens such as SSA/Ro, SSB/La, Smith (Sm), Sm/ribonucleoprotein (RNP), Scl-70/topoisomerase I, Jo-1, centromere, and cardiolipin. Unlike the indirect IIF method, which reports results in terms of serum dilutions, ELISA provides quantitative measurements of antibody levels in specific units. This quantitative approach allows for a more precise assessment of antibody concentrations, aiding in the diagnosis and monitoring of autoimmune diseases. The ability of ELISA to quantify autoantibodies against specific antigens has contributed to its continued prominence in clinical laboratories.

ELISAs designed for the detection of ANAs utilize a combination of autoantigens obtained from native sources and/or produced using recombinant technologies [19]. These immunoassays typically include a panel of specific autoantigens, such as SS-A/Ro60, SS-B/La, Scl-70/topoisomerase I, CENP-B, Jo-1, U1-RNP, Sm, dsDNA, PM/Scl, or P ribosomal proteins [20]. Individual autoantigens can exhibit distinct biochemical properties when it comes to their binding to the solid matrix used in ELISA assays. The use of mixtures of antigens in ELISAs can

potentially lead to masking effects and a reduction in available epitopes for binding by human autoantibodies. This occurs because the autoantibodies may bind to other antigens present in the mixture, thereby interfering with the specific recognition of their intended target.

While ELISAs designed for detecting individual autoantibodies generally demonstrate improved performance compared to assays utilizing antigen mixtures, they also have limitations in terms of autoantibody binding to exposed epitopes on autoantigens. The accessibility of specific epitopes can vary, and some autoantibodies may fail to recognize certain epitopes, leading to potential false-negative results. Therefore, the selection of appropriate antigen combinations or individual antigens in ELISAs requires careful consideration to ensure optimal sensitivity and specificity. The recognition of epitopes by autoantibodies can be influenced by several factors. The conformational structure of the autoantigen may play a role in exposing or concealing certain epitopes, affecting the binding of autoantibodies. Additionally, variations in post-translational modifications of autoantigens can also affect the recognition of epitopes by autoantibodies. Furthermore, the production of autoantibodies can be dynamic, with changes in antibody levels and specificity over time. This technique presents other limitations that contribute to the occurrence of false negative results. Autoantibody levels are variable in early stages of the disease or during periods of remission, and they may be too low to be detected by the ELISA test, resulting in a false negative result. Additionally, if the relevant epitopes are not present in the sample or if the conformation of the epitopes is altered, the autoantibodies may not properly bind during the test, leading to a false negative result. It is important to address these limitations and consider the specific requirements of the autoimmune disease being investigated when designing and interpreting ELISA assays for autoantibody detection [21].

Line immunoassay (LIA)

LIA is a diagnostic technique used for the detection of autoantibodies. It involves the preparation of a nitrocellulose membrane that has specific antigens coated in lines. These antigens can be derived from various autoantigens associated with different autoimmune diseases.

When a patient's serum or sample is applied to the LIA, any autoantibody present in the sample will bind specifically to their corresponding antigen on the nitrocellulose membrane. To visualize the bound

autoantibodies, a secondary antibody conjugated to an enzyme, typically horseradish peroxidase (HRP), is added. This secondary antibody recognizes and binds to the patient's autoantibodies that are already bound to the antigens on the membrane. Next, a specific substrate is added to initiate an enzymatic reaction with the HRP enzyme. This reaction produces a detectable signal, often a color change, at the location of the corresponding antigen–antibody binding on the membrane. The presence and intensity of these signals can be interpreted as positive results for specific autoantibodies, indicating the presence of particular autoantigens associated with autoimmune diseases.

LIA offers a high degree of specificity and allows the simultaneous detection of multiple autoantibodies in a single test. It is commonly used as a confirmatory assay in the diagnosis of autoimmune diseases and provides valuable information about the autoantibody profiles of patients.

There is a diverse range of commercially available LIAs, primarily utilized for the identification of ANAs. LIAs have been employed as screening tests to detect disease-specific autoantibodies associated with conditions such as SLE, SSc, primary biliary cirrhosis, idiopathic inflammatory myopathies, and paraneoplastic syndromes [22]. However, it is important to note that LIAs may have limitations in terms of sensitivity and specificity for certain autoantibodies. Some autoantibodies may not be effectively captured or detected by the antigens used in a particular LIA, leading to false-negative or false-positive results [23]. Therefore, it is crucial to consider the specific clinical context, and to complement LIAs with additional diagnostic tests to ensure accurate and comprehensive evaluation of autoantibody profiles in patients.

Chemiluminescent immunoassay (CLIA)

CLIA is a technique used for the detection of autoantibodies. In CLIA, a solid phase is used, which are magnetic particles coated with specific autoantigens. These autoantigens can include dsDNA, RNP/Sm, SSA/Ro, SSB/La, Scl-70, Jo-1, CENP-B, and mitochondria [24]. The assay involves the binding of autoantibodies present in the serum or plasma sample to the immobilized antigens. A secondary antibody, conjugated to a chemiluminescent molecule, is then added. When the reaction occurs, the chemiluminescent molecule emits light as it goes from an excited state to a ground state. The emitted light is measured by a photomultiplier, and the intensity of the light signal is proportional to the concentration

of autoantibodies in the sample. The results are typically reported in relative light units [25].

Various immunoassays utilizing chemiluminescent technology have been developed to measure specific autoantibodies in different autoimmune conditions. For example, in SSc, immunoassays based on chemiluminescence have been developed to detect anti-Th/To antibodies [26]. Similarly, in immune-mediated necrotizing myopathies, CLIA have been employed to measure anti-3-hydroxy-3-methylglutaryl-coenzyme A reductase antibodies [27]. In the context of SLE, CLIA has been employed to measure various autoantibodies that are associated with the disease such as anti-dsDNA, anti-Sm, anti-RNP, anti-SSA/Ro, and anti-SSB/La antibodies [28]. In RA, CLIA has been particularly valuable in measuring autoantibodies such as rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibodies. CLIA has also been utilized to measure other autoantibodies that can be found in RA, including anti-mutated citrullinated vimentin antibodies and anti-RA33 antibodies [29].

Chemiluminescent analytical methods offer several key advantages in the field of autoantibody detection. One of the main advantages is their wide dynamic range, allowing for the accurate measurement of autoantibodies across a broad concentration range. Additionally, these methods provide high signal intensity, resulting in strong and reliable analytical signals. Another advantage is the absence of interfering emissions, which ensures greater specificity and reduces the potential for background noise. This is particularly important in achieving accurate and reliable results. Furthermore, CLIA offer rapid acquisition of the analytical signal, allowing for efficient and timely data acquisition. One limitation is the potential for limited antigen detection. Another consideration is that CLIA often operate within closed analytical systems. The availability of test panels may be limited in CLIA, which could restrict the range of autoantibodies that can be measured simultaneously [30].

EMERGING TECHNOLOGIES

In recent years, autoantibody measurement techniques have evolved significantly, driven by the continuous discovery of new autoantigens. This progress has facilitated improved classification of clinical phenotypes and enhanced our understanding of the underlying pathophysiology of autoimmune diseases. To expand our understanding of autoantigens and improve diagnostic capabilities, it is im-

portant to complement conventional immunoassays with emerging technologies and approaches (Figure 1). These can include fluid-phase immunoassays, and high-throughput screening methods that enable the simultaneous measurement of multiple autoantibodies against a broader array of autoantigens. By embracing new technologies, clinical laboratories can enhance their ability to identify novel autoantigens, improve diagnostic accuracy, and gain deeper insights into the complex landscape of autoimmune diseases.

Cell based Assay (CBA)

CBA is a relatively new technology that enables the detection of antibodies against self-antigens in their three-dimensional conformation. Pathogenic autoantibodies tend to recognize antigens in their native conformation more effectively than in denatured or linear configurations. CBA utilizes eukaryotic cell lines, such as HEK-293 and Chinese hamster ovary cell (CHO), which have been transfected with plasmids containing the gene encoding the protein of interest. These transfected cells express high levels of the target protein in its native folded state on the cell membrane. This approach is particularly valuable for validating new antigen targets for autoantibodies [31]. The use of eukaryotic expression systems allows the simulation of the natural state of the protein, resulting in the presentation of a three-dimensional structure that is readily recognizable by autoantibodies *in vitro*. Fluorescein-labeled antibodies are then applied, which can specifically bind to autoantibodies present in serum or cerebrospinal fluid samples.

CBA offers several advantages, including the ability to detect autoantibodies that recognize conformational epitopes, mimicking the *in vivo* situation. This technique allows for a more accurate representation of the autoantibody profile and contributes to the identification of novel autoantigens. By using fluorescein-labeled antibodies, the binding of autoantibodies can be visualized and quantified using fluorescence microscopy or flow cytometry [32].

CBA has significantly improved the serological detection of antibodies in myasthenia gravis [33, 34]. By employing fluorescein-labeled antibodies, CBA enables the binding of autoantibodies present in serum or cerebrospinal fluid samples. This technology has been successfully applied in routine screening tests targeting proteins expressed on the surface of neurons and in glia. Examples of these proteins include the N-methyl-D-aspartate receptor, contactin-associated protein 2, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor,

γ-aminobutyric acid B, glycine receptor, aquaporin-4, and myelin oligodendrocyte glycoprotein [33].

While CBAs have found extensive application in the diagnosis of neuroimmune diseases, the assessment of their sensitivity and specificity lacks defined criteria. Nonetheless, CBA offers several advantages, including the use of native target antigens, high signal intensity, reconstruction of visible three-dimensional images, and localization of the target antigen. These features contribute to the enhanced detection and characterization of autoantibodies in the context of neuroimmune disorders.

Fluid-phase radio binding assay (RBA)

RBA is a technique where the autoantigen is labeled with a radioactive isotope and mixed with the patient's sample. If antibodies are present, antigen-antibody complexes form and can be collected by centrifugation. The amount of antibody is determined by measuring the radioactivity in the pellet using gamma counting. A disadvantage of this technology is that it uses low levels of radiation with ^{125}I , ^{35}S -methionine, and ^3H -leucine.

RBA assays are widely used for the detection of linear and conformational autoantibodies in various autoimmune diseases, such as T1D, celiac disease, and thyroid disease [35]. In these assays, the autoantigen is evaluated in solution rather than being immobilized on a solid surface. RBA employs radioactively labeled proteins, which are typically produced through *in vitro* transcription and translation methods. One advantage of RBA is the ability to generate any protein, including large proteins and those that are not readily available commercially.

RBA has been successfully applied for the specific detection of antibodies against glutamic acid decarboxylase (GAD65) and islet tyrosine phosphatase 2 (IA2) in 90% of patients with T1D [36]. Fluid-phase RBA has demonstrated higher sensitivity compared to ELISA for the measurement of anti-GAD65 antibodies [36]. This could be attributed to the fact that in ELISA, GAD65 is bound to the plastic surface, potentially altering the conformation of the epitopes, whereas, fluid-phase RBA allows for high-affinity antigen binding. Furthermore, RBA has been employed for the quantification of specific antibodies against proinsulin and insulin [37] as well as antibodies targeting Znt8, another important autoantigen in T1D [38].

Moreover, the bactericidal/permeability-increasing fold-containing B1 (BPIFB1), a recently identified autoantibody associated with interstitial lung disease, has been quantified by RBA. The seropositiv-

ity of BPIFB1 autoantibodies has been reported to be 15% in patients with interstitial lung disease and 12% in patients with other idiopathic lung disorders. RBA enables the measurement of BPIFB1 autoantibodies in a quantitative manner, providing valuable insights into their prevalence and potential association with lung diseases [39].

Luciferase Immunoprecipitation Systems (LIPS)

LIPS is an immunoglobulin capture assay that utilizes genetically encoded recombinant luciferase fusion proteins. This method uses immunoglobulin-binding protein A/G beads and after washing, the immunocomplex is analyzed through a luminometer. The luciferase gene derived from *Renilla reniformis* is employed as the reporter in LIPS because of its linear output range that spans over seven orders of magnitude. This technology uses various protein targets, including full-length proteins, protein variants, fragments, short peptides, and other recombinant proteins. LIPS provides a versatile and quantitative approach for studying antibody responses and antigen-antibody interactions [40].

LIPS has been utilized for the detection of autoantibodies in various autoimmune conditions such as autoimmune encephalitis [41], SLE [42], Sjogren's syndrome [43], biliary cirrhosis [44], and SSc [45]. On the other hand, LIPS assays have been employed to identify autoantibodies against IA2, IA2-beta, and GAD65 in T1D [40]. LIPS technology is particularly useful in research settings for the discovery of novel autoantigens. Recently, it has been used to identify anti-insulin autoantibodies in patients with T1D [46].

LIPS offers a distinct advantage in discriminating between seropositive and seronegative signals. Its broad dynamic range of detection and low background noise make it well-suited for monitoring changes in antibody levels over time in autoimmune diseases. This technology has proven to be essential in assessing autoantibodies against plasma membrane receptors, proteins with extracellular domains, and proteins released as cytokines [47]. The wide dynamic range of LIPS allows for precise quantification of antibody levels, even in samples with low antibody titers or fluctuating levels.

LIPS offers several key features that make it a highly promising tool for the study of autoantibodies. The non-radioactive nature, stable antigen storage, high signal-to-noise ratio, and wide dynamic range make LIPS a powerful tool for the detection, quantification, and characterization of autoantibodies in autoimmune diseases.

Antigen Microarray Technologies

Antigen microarray technologies have revolutionized the field of autoantibody detection and profiling by enabling the simultaneous analysis of antibody reactivity against a large number of antigens. These technologies offer a high-throughput and multiplexed approach for studying autoantibodies in various autoimmune diseases. Antigen microarray technologies offer several advantages, including high sensitivity, multiplexing capability, and the ability to analyze large numbers of samples simultaneously. They provide a comprehensive view of autoantibody profiles, facilitate the discovery of novel autoantibodies, and can potentially aid in disease diagnosis, prognosis, and monitoring of treatment responses in autoimmune diseases. Antigen array technologies and platforms are described below:

Planar arrays

Planar antigen arrays are a type of antigen microarray technology that involves immobilizing a large number of antigens on a solid support, such as a slide or nitrocellulose membrane. The process of creating planar arrays is similar to DNA microarrays, utilizing printing equipment and procedures adapted for protein immobilization [48]. The antigens are loaded onto the solid support in nanoliter volumes at specific locations, forming microdots. The spatial density of antigens can be quite high, reaching up to 2000 per cm² or more. The capture reagents, such as antibodies or other proteins are immobilized on the solid support using the printing equipment. To detect autoantibodies, fluorescently labeled IgG or IgM antibodies are used. If any autoantibody in the sample bind to the immobilized antigens, they can be detected through the fluorescence emitted by the labeled antibodies. Fluorescence-compatible laser scanners, commonly used in DNA microarrays, can be employed to measure the signals from the planar arrays. These scanners provide excitation at specific wavelengths, such as 532 nm and 633 nm, allowing the detection of fluorescent dyes like cyanine, Alexa Fluor, and R-phycoerythrin.

Alternatively, some approaches utilize the enzyme alkaline phosphatase, which produces a color reaction when incubated with a substrate. This enzymatic reaction is used as a signal for the presence of autoantibodies bound to the antigens on the planar array.

Planar antigen arrays offer the advantage of high-density immobilization of antigens, enabling the analysis of a large number of antigens simultaneously. They utilize established equipment and pro-

cedures from DNA microarray technology, making it easier to adapt and apply this technology to the study of the complete proteome. The use of fluorescent detection or enzyme-based color reactions allows for sensitive and specific detection of autoantibodies on the planar arrays. Planar antigen arrays offer the advantage of testing small amounts of body fluids to assess the diversity of the autoimmune repertoire. However, a limitation of planar antigen arrays is the reliance on commercially available antigens associated with autoimmunity. To overcome this limitation, research studies have proposed an alternative approach using internally produced matrices containing large collections of antigens obtained from the Human Protein Atlas [49]. Other limitation is the inability to identify autoantibodies against proteins with post-translational modifications. To detect conformation-sensitive autoantibodies, protein arrays must contain full-length proteins. This is particularly important for the identification of autoantibodies such as anti-myelin oligodendrocyte folded glycoprotein autoantibodies. Oligodendrocyte-specific autoantibodies can only recognize the denatured form of the protein and not its folded conformation [50].

In SLE research, planar antigen arrays have been utilized to identify and characterize various autoantibodies, including those targeting nuclear antigens such as dsDNA, nucleosomes, histones, and other nuclear proteins [51]. A miniaturized and multiplexed array platform consisting of 48 recombinant plexes has shown high performance and applicability in detecting autoantibodies and searching for serum biomarkers in patients with lupus nephritis. In RA research, planar antigen arrays have been used to identify and analyze various autoantibodies, including those targeting citrullinated peptides, RF, and other RA-associated autoantigens [52]. This demonstrates the potential of planar antigen arrays in autoimmune diseases research and diagnosis [53].

Bead-based arrays

Bead-based arrays are a versatile and widely used technology for the detection and quantification of various analytes, including autoantibodies. These arrays involve the immobilization of specific capture reagents onto distinct sets of microspheres, which serve as solid supports. The capture reagents include antibodies, antigens, or other molecules that specifically bind to the target analytes. The microspheres used in bead-based arrays are functionalized with carboxyl or thiol groups, which allow for the covalent attachment of the capture reagents [54]. For instance, capture reagents containing biotin can be

immobilized onto microspheres through the use of avidin-coupled microspheres. This system involves the use of fluorochrome-labeled detection antibodies or other reporter molecules that specifically bind to the target analytes. The fluorescent signals emitted by the microspheres are then detected and analyzed by the flow cytometer, allowing for the identification and quantification of the captured targets [55].

Bead-based arrays can be used to detect various autoantibodies associated with SLE, including anti-dsDNA, ANA, anti-Sm, anti-RNP antibodies, among others [56]. This technology has been used to classify and stratify SLE patients based on their autoantibody profiles. By analyzing the presence or absence of specific autoantibodies, researchers can identify distinct subgroups of SLE patients with different clinical features, disease severity, or treatment responses [57]. In addition, bead-based arrays have been employed in biomarker discovery studies in SLE [58].

Both, bead-based arrays and planar arrays have advantages and applications in autoantibody detection. Bead-based arrays have a higher multiplexing capability compared to planar arrays. With bead-based arrays, different capture reagents can be attached to distinct sets of microspheres, allowing for the simultaneous detection of multiple targets in a single assay. Planar arrays typically have a lower density of immobilized antigens and are less amenable to high levels of multiplexing. Bead-based arrays generally offer higher sensitivity compared to planar arrays. The three-dimensional nature of the microspheres in bead-based arrays allows for efficient capture and detection of target analytes, resulting in enhanced sensitivity. Planar arrays offer more flexibility in terms of antigen selection and customization. The selection between these technologies depends on the specific research or diagnostic needs and resources available.

Addressable laser bead immunoassay (ALBIA)

ALBIA method is a novel technique that incorporates multiplexing technology to detect simultaneously multiple autoantibodies using small sample volumes. In this method, specific antigens immobilized on magnetic beads capture the autoantibodies. The presence of the captured autoantibodies is then determined using a fluorescently-labeled anti-human secondary antibody. ALBIA offers several advantages in terms of its ability to measure multiple autoantibodies in a single assay, thereby saving time and reducing sample volume requirements.

The instrument software analyzes the fluorescence intensity of each magnetic bead and automatically converts it into units, providing quantitative results for each autoantibody.

ALBIA utilizes the Luminex platform (Luminex Corp., Austin, TX) in clinical diagnostic laboratories for autoantibody measurement. Various automated platforms enable rapid analysis of samples to measure anti-dsDNA and extractable nuclear antigen (ENA) antibodies [59]. With ALBIA, autoantibodies such as ANCA, celiac disease-related antibodies, and ANA can be detected with improved accuracy [60].

The high sensitivity and specificity of ALBIA make it a valuable tool in autoimmune diagnostics and research. Its ability to analyze multiple autoantibodies in a single test improves efficiency and provides a more comprehensive assessment of autoimmune diseases, contributing to better patient care and management.

Cytobead

Cytobead is a technique that integrates the detection of autoantibodies and confirmatory tests by automating the interpretation of IIF and enabling quantitative multiplexing of autoantibodies using addressable microspheres. This method involves dividing the wells on conventional glass slides into compartments using teflon barriers to facilitate the combination of these systems. Cytobead enables the evaluation of bead reactions under routine microscopes by utilizing green fluorescence for signal detection. The differentiation of antigen-coated red fluorescent beads based on size is achieved using reference beads.

The CytoBead technique utilizes a multiplexing strategy for the detection of ANA. It involves the detection of ANA in HEP-2 cells and testing of ENAs using antigen-coated microspheres in a reaction environment [61]. This approach is also employed for the detection of ANCA in the diagnosis of ANCA-associated vasculitis. The international consensus recommends initial testing by IIF on ethanol-fixed human neutrophils, followed by confirmation through ELISA. The prevalence of positive ANCA results obtained through traditional tests showed good agreement with results obtained using the CytoBead technique [62]. Based on the same principle, CytoBead enables the simultaneous measurement of IgA antibodies against tissue transglutaminase, endomysium, and deamidated gliadin, as well as total IgA levels, for the diagnosis of celiac disease [63].

Rapid Extracellular Antigen Profiling (REAP)

REAP is a technique for comprehensive and high-throughput discovery of exoproteome-target-

ing autoantibodies, which can have significant implications in the field of autoimmunity research. By profiling the exoproteome and identifying autoantibodies targeting extracellular antigens, REAP may provide valuable insights into the pathogenesis of autoimmune diseases and potentially uncover novel diagnostic markers or therapeutic targets. The high-throughput nature of REAP screens a large number of antigens simultaneously, enabling a comprehensive analysis of the autoantibody repertoire.

In REAP, patient samples are exposed to a genetically-barcoded library that consists of 2,688 distinct members of the human exoproteome displayed on the surface of yeast cells. When samples from patients are applied to the library, autoantibodies present in the samples have the potential to bind to specific antigens displayed on the yeast cells. The antibody-coated cells are then isolated using magnetic selection, which allows for the enrichment of cells with antigen-bound autoantibodies. Following the isolation step, the barcodes associated with the antibody-coated cells are subjected to deep sequencing. By analyzing the sequencing data, researchers can identify the specific barcodes and their corresponding antigens that were recognized by the patient's autoantibodies.

The application of REAP in the study of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) patients has demonstrated its sensitivity and specificity in detecting autoantibody reactivities. It has revealed the presence of autoantibodies targeting various antigens, including type I interferons, IL-17, IL-22, gastric intrinsic factor, and glycoprotein hormone GPHB5. In addition, REAP has revealed novel autoantibody reactivities against a wide range of antigens in the context of SLE. These antigens encompass growth factors, extracellular matrix components, cytokines, and immunomodulatory proteins that play significant roles in the severity and clinical presentation of SLE [64].

QUALITY AND STANDARDIZATION OF AUTOANTIBODY TESTS

International reference samples play a crucial role in the quality control of autoantibody tests [65]. Organizations such as the Centers for Disease Control and Prevention (CDC) in Atlanta, USA, and the Association of Medical Laboratory Immunologists (AMLI) provide panels of reference sera to standardize ANA IIF tests, establish staining patterns, and assess the performance of various autoantibody immunoassays [66]. However, the number of international reference sera is limited, and not all autoantibodies are available.

The variability in the structures, affinities, and avidities of antibodies produced by each individual patient poses a challenge in standardizing autoantibody-testing methods. To address this issue, one suggested approach is to establish specific likelihood ratios for disease diagnosis based on local validation cohorts. This would assist in the interpretation of test results and enhance comparability between different techniques. By providing standardized measures of likelihood, clinicians can better evaluate the significance of autoantibody test results in the context of a specific disease diagnosis [67].

Indeed, various committees and organizations are actively working towards improving the diagnosis of autoimmune diseases by standardizing autoantibody tests and harmonizing testing algorithms. These initiatives include:

a) Autoantibody Standardization Committee (ASC): ASC aims to establish internationally recognized standards for autoantibody testing. They develop reference materials and protocols to ensure the accuracy and comparability of autoantibody assays.

b) European Autoimmunity Standardization Initiative (EASI): EASI is a collaborative effort among European experts in the field of autoimmunity. Their goal is to promote the standardization of autoantibody testing methods and improve the quality of diagnostic testing across laboratories in Europe.

c) Working Group on Harmonization of Autoantibody Tests (WG-HAT): WG-HAT focuses on harmonizing autoantibody-testing algorithms for specific autoimmune diseases. They develop recommendations for the selection and interpretation of autoantibody tests, aiming to streamline the diagnostic process and improve clinical decision-making.

These committees and organizations play a crucial role in promoting consistency and reliability in autoantibody testing, facilitating accurate diagnoses, and ensuring better patient care in the field of autoimmune diseases.

CONCLUSION

Autoantibody testing plays a crucial role in the serologic diagnosis of autoimmune diseases. As new technologies emerge, they offer both advantages and disadvantages compared to traditional detection methods. Antigen arrays provide the ability to screen a large number of potential autoantibodies or autoantigens, although they may have limitations in detecting conformational epitopes for recombinant proteins. Liquid-phase immunoassays have been described with high sensitivity. RBA, however, has the drawback of utilizing radioactivity, while LIPS offers a radioactivity-free alternative that allows for the quantification of autoantibody titers across a wide dynamic range.

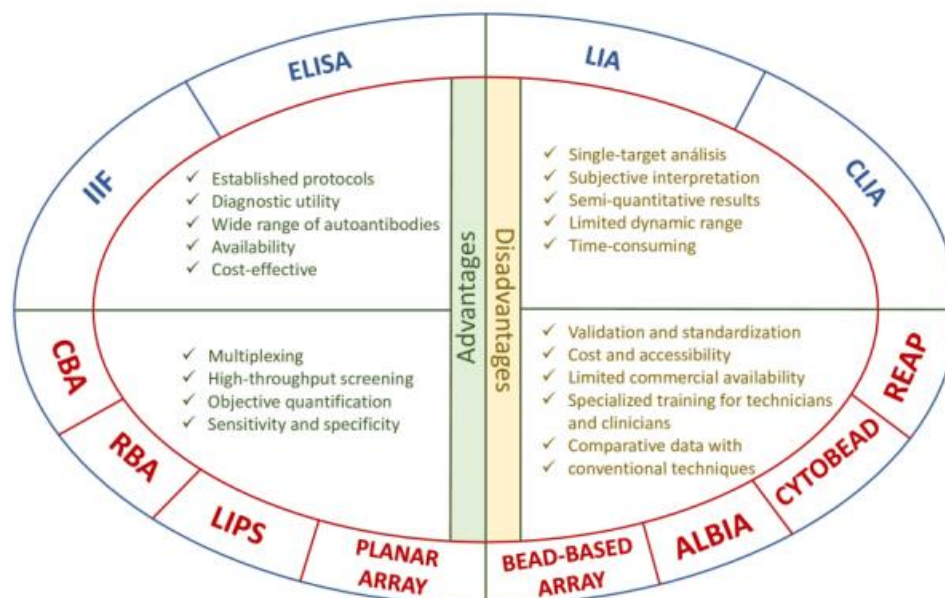


Figure 1. Old and new technologies for autoantibodies testing. A visual representation of the evolving landscape in autoantibody testing, comparing traditional methods with new technologies. The figure illustrates the potential advantages and limitations of various techniques, emphasizing the importance of continuous research and validation studies to enhance diagnostic accuracy and improve clinical outcomes in autoimmune diseases

To assess the reliability, specificity, and sensitivity of new immunoassay methods, cross-validation studies comparing them to conventional techniques are necessary. It is important to evaluate their reproducibility and performance in order to determine their utility in clinical practice.

Research allows the identification of new relevant epitopes associated with autoimmune diseases, leading to the development of more sensitive and specific tests. These new testing methods can detect autoantibodies in early stages of the disease and minimize false-negative or false-positive results. These advancements promote detection that is more accurate, early diagnosis, effective monitoring, and a more personalized approach in the management of autoimmune diseases. As our understanding of these diseases' advances, it is expected that testing methods will continue to evolve and improve, ultimately providing better clinical outcomes for patients.

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