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THE QUALITY ASSURANCE AND ORGANIZATION OF AUTOANTIBODY LABORATORY

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10.1 Introduction

There has been an enormous effort engineered in the past 20 years to find an easy, cost-effective and as-fast-as-possible way to detect autoantibodies (autoAb) especially against intracellular antigens. For years laboratory professionals have been seeking the best screen at the shortest time possible in the most cost-effective way. But, unfortunately, nobody has yet found the best solution to meet all the needs of laboratory personnel and expectations of medical specialists and insurance companies

Autoantibody laboratory (AAL) usually services specific medical speciality:

- Systemic autoimmune
- Haematological
- Gastrointestinal
- Renal
- Liver
- Endocrine
- Cardiovascular
- Neurological
- Skin

Or more specific diseases:

- Antiphospholipid Syndrome
- Anti-GBM disease
- Anti-Synthetase Syndrome
- Churg-Strauss Syndrome
- Dermatomyositis
- Primary biliary cirrhosis
- Rumatoid arthritis
- Scleroderma (diffuse, limited)
- Scleroderma, limited
- Scleroderma /Myositis overlap

- Drug induced lupus
- Inflammatory bowel disease
- Microscopic polyangiitis
- Mixed connective tissue disease
- Polymyositis
- Sjögren Syndrome (primary, secondary)
- Subacute cutanous lupus erythematosus
- Systemic lupus erythematosus
- Vasculitis, systemic
- Wegener granulomatosis

When sending samples to an AAL autoAbs testing medical specialists should always be aware of the following:

- Reliability and overall quality assurance of AAL
- Repertoire of autoAb tests and methods used
- Reproducibility, comparability of test results in time (continuous care of rectification/adjustment of any changes in kits/reagents with relevant internal standards, method(s)' adaptation/ modification)
- Skilfulness in reporting test results

AutoAb tests should not be performed at primary level laboratories at Public Health departments or doctor's private practises due to:

- Complexity of organisation and management of an AAL
- Quality assurance
- Possibility of long-effective consequences of laboratory findings

10.2 Setting-up/organizing an autoantibody laboratory

In order to start and/or organize an AAL several very important premises and prerequisites should be considered:

10.2.1 Competence

Defining a doctrine of an AAL - a set of guidelines for the dynamics of autoAb testing: screening tests, specific tests for single autoAb: hierarchy of necessary tests to follow each other, what to report to a clinician. In order to implement these basic premises following questions should be answered first

- Where: hospital/institution (institutes, universities, university hospitals), independent
- How much: number of tests/year influenced by a number of medical specialists and/or hospital departments sending samples for autoAb testing. An estimation of approximate number of people potentially needing its services would be very useful.
- What: routine, research, both

- Who: number of laboratory professionals: immuno-chemist (biochemist, biologist, etc), specially trained lab technicians
- How: techniques, methodology: in-house, kits
- To whom: rheumatologists, nephrologists, neurologists, other internal medicine specialists

10.2.2 Quality control

All aspects (internal, external): traceability to metrological etalons (where possible) or internationally acceptable control materials and interlaboratory comparability (national or international level, national quality assurance schemes)

10.2.3 Education

It is prerequisite to employ adequately educated (type, degree, previous training on the field) personnel with the assurance of continuous education and close relations with medical doctors in order to exchange knowledge on both parts, suggestions, information in order to make necessary changes and/or adaptations. Unfortunately, autoAb tests are not always carried out by specially trained technicians. Replacing in-house methods with kits and introducing more manufacturer and thus more kits on the market even stimulates and encourages employing cheap and thus incompetent personnel.

10.2.4 "PR (public/professional relations)"

Collaboration with similar/complementary institutions/hospitals, single/group of medical specialist(s), patient(s), other related professionals, laypersons etc.

10.2.5 Financing

Insurance companies, founds (private, institutional, governmental), research grants

10.2.6 Logistics/functioning/information system

AAL may be an advanced expert laboratory, which excellency is closely interrelated with good infrastructure and expertise of specialized medical support or, it may be estranged and melted into some large laboratory service apart from medical departments and clinicians (as an integrated part of Clinical and Biochemical Lab) and thus cut from continuous flow of knowledge and information on both sides. Adequate laboratory software for all levels of AAL functioning/activity is essential to assure an uninterrupted functioning of AAL (promptness with optimal quality, reliability, accurateness etc). To avoid any confusion defined rules should be set for the selection of big and small AAL equipment, reagents/kits, and suppliers. A transparency of all in- and out-coming people and materials (patients' sera and test results, reagents, chemicals, kits, other laboratory material, trash, dangerous waste) is obligatory and must be tracked down to the very source/beginning. Together with good software it contributes to minimizing the costs.

All the above can be more easily accomplished and facilitated by locating AAL in close vicinity of the most important customer/medical department (i.e. a division of internal medicine or even single departments, like rheumatology). It results in a very prosperous and successful collaboration in every aspect of a routine and/or research engagement. The Dept. of Rheumatology, Univ. Med. Center, Ljubljana, Slovenia with its Immunology laboratory (IL), is a good example of such successful constellation. Nevertheless, this is a very rare situation and therefore needs to be further elucidated. Autoantibody tests are much more commonly performed in a routine clinical chemistry laboratory. Most of the times, laboratory professionals (immunochemists, or immunobiochemists, or immunobiologists ...) and clinicians sit on two benches separated by a wide river without seeing/understanding each other - many times there is no established collaboration between them or at least an articulation of a need to do something in this direction. A single telephone call now and than does not count: a verbal exchange of information should always take place in the form of a consultancy.

10.3 Functioning of AAL

Good laboratory practice and quality work very much depend on same issues as any other type of medical laboratory (adequately educated and well trained personnel, etc according to ISO15189). So far we have enquired about effectiveness (good services), however, functioning of an AAL crucially depends on its financing which makes it essential to take account of the efficiency of the AAL, i.e. the performance related to the expenditure. Regardless of the source of financing, ordering autoAb tests is always restrictive in approval of new and continuation of already, good established tests, due to huge and constant raise in medical costs at all levels:

Governmental/institutional laboratories: Each country/state has (not) established its own way of setting-up and organizing such type of laboratories due to current/legal health care strategy which does or does not influence insurance companies and their policy regarding payments for autoAb tests (i.e.: completely different situations in USA, EU, Eastern Europe, undeveloped world. Or inside EU: England, Germany, France, Denmark, Slovenia etc).

Private (almost always smaller) laboratories usually run very selective, single routine tests avoiding to follow guidelines proposed by the professional experts on autoAb. They tend and actually do reduce their costs on almost everything: kits are selectively approved (many times depending on "more results for less money"), constantly cutting down the costs for materials and human resources. In many countries they often hire inadequately educated and thus cheaper labour to run the tests, which often result in a very poor laboratory performance regularly neglecting quality controls on all levels. Results are unreliable, inaccurate, irreproducible, incomparable in time and inadequately interpreted. They are unable to make any quality discussion with relevant medical specialists, often ignoring and disregarding suggestions for possible further steps in quality assurance of laboratory diagnosis. It goes beyond a simple AAL/clinician communication, which usually ends up in none at all.

From all the above autoAb tests should always be performed by laboratory specialists with a history of extensive training on the field.

10.4 AAL in Rheumatology

Focusing on rheumatology as one of the main users and beneficiaries of an AAL the following autoAb tests against organ non-specific antigens should definitely be included:

a. Screening tests:

- ANA test* (antinuclear antibodies: meaning a differentiation of imunofluorescence (IF) patterns of nuclear membrane, nucleoplasm, nucleoli, spindle apparatus and cytoplasm on Hep-2 cells together with semiquantitative evaluation of a titer).
- Anti-ENA (antibodies against extractable nuclear antigens) **
- ANCA* (antibodies against neutrophyl cytoplasmic antigens)

b. Tests on autoAb against specific antigens:

- ds-DNA (double stranded DNA)
- Sm, U1RNP, Ro, La, Scl-70, Jo-1**
- CL (cardiolipin),
- beta2GPI (beta2glycoprotein I),
- LA (lupus anticoagulant)
- CCP (cyclic citrulinated peptide)
- MPO (myeloperoxydase)
- PR3 (protein 3)

* Certain specific fluorescences should be confirmed by tests on specific antigens: AMA like - anti-PDH (pyruvatedehydrogenase), ribosomal like - anti-ribRNP, PCNA like - anti-PCNA (proliferating cell nuclear antigen), different ASMA IF etc; pANCA - MPO, cANCA - PR3)
** Not a screen when tested on single antigens

We believe it is of vital importance that the repertoire of an AAL is consisted of those autoAb tests that cover most diagnostic needs (i.e. rheumatology), a complete service in one place in order to assure minimal quality standards for AAL functioning from employing skilled personnel to internal and external quality controls. Namely, an adequate testing for autoAb should always begin with "ANA" testing on Hep-2 cells which represents the first screening test, although not an ideal and not a complete one. Further testing depends on several facts and assumptions: i.e. positivity/negativity of a tested serum, (presumed) diagnosis, availability of specific test(s) in the same AAL where ANA was performed, money. Besides ANA, some specific autoAb tests should be

included, based on patients' diagnosis thus forming some typical sets of initial autoAb tests. Based on national guidelines or agreement among AAL, rheumatologists and insurance companies, groups of tests can be preset in advance.

a. In an ANA positive serum there are two possibilities for further testing:

- Second screen for anti-ENA with CIE,
- Testing on single selected autoAb depending on (presumed) diagnosis (with the awareness of relevant diagnostic criteria and incidence for particular autoAb)

b. There has been no consensus how to proceed testing (if at all) of a serum declared as ANA negative. However, it is not a clear-cut decision whether a serum is positive or negative; it depends on several things:

- Method of choice (in-house, kit), manufacturer of Hep-2 cells, conjugates
- Starting serum dilution (cut-off point)
- Method accurateness
- Microscopy performances
- Skilfulness of the person reading the IF and differentiating IF patterns

Evaluation of ANA by IF begins with a starting serum dilution, which represents the cut-off. It ranges from 1:40 to 1:160 and is one of the main disagreements and differences among AALs. There are several reasons for this discrepancy and money is the most important one: selecting 1:80 or even 1:160 as the cut-off means more negatives and less further testing for autoAbs. By our opinion, declaring a serum negative at the dilution 1:40 is not the same as is at 1:80 or 1:160. For more than 20 years IL has been using 1:40 dilution as the cut-off dilution for ANA testing on Hep-2 cells (the same manufacturer of Hep-2 cells preparations). Over 17 years CIE has been suggested as the second screen for more specific anti-ENA thus giving clinicians much more information to work with, since there are no CIE false positives.

We believe that using both tests speeds up the diagnosis of those patients who would stay (at least for a long time) undiagnosed especially in cases of poor (or none at all) collaboration between AAL and clinicians (Table 1). Again, it should not be solely AAL to decide about further autoAbs testing but all three parties involved: AAL, rheumatologists, financier (eventually it all ends up in higher costs).

Table 1. *IFANA and CIE/anti-ENA in 5431 patients in 1998/1999*

ANA	**No. of patients**	**anti-ENA pos: anti-Ro, Jo-1, UDA**
negative	1728	52 (3%)
1:40	1320	80 (6%)
1:80	820	90 (11%)
≤1:80	3868	222 (6%)

At this point it should not be overlooked the effort of a group of experts who in order to improve the quality of autoAb testing, constituted the European Consensus Study Group for Autoantibodies (Consensus group) who meet annually at the European Workshop for Rheumatology Research (EWRR). The group was formed in 1988 to examine the sensitivity and reproducibility of different methodologies for the detection of autoAb to intracellular antigens such as immunofluorescence, gel techniques (immunodiffusion (ID) and counterimmunoelectrophoresis (CIE), ELISA, Western blotting, and then to improve the performance of these tests. Therefore, every year 10 different sera samples are sent to 35-40 leading laboratories from 21 different European countries to test for as many autoAb against intracellular antigens as possible, and the results of these exercises show a tremendous improvement in detection rates. A significant step in the life of the EWRR has been reached when laboratory experts in the consensus group agreed to submit their protocols, of which the best were chosen and published in the Manual of Biological Markers of Disease. Participating laboratories often function as national expert laboratories to disseminate the knowledge from the consensus meetings to other laboratories and clinicians. One of the main conclusions of the Consensus group was that different detection techniques could not be directly compared. Immunofluorescence as a good screening method should precede all the more specific ones. Borderline results obtained by ELISA or immunoblotting tests should be further confirmed by one of the gel techniques (ID or CIE).

IL has been a part of the consensus group since the very beginning, contributing to the CIE protocol for the detection of autoAb against different intra-cellular antigens (anti-ENA). In the last 15 years, over 205.000 sera have been routinely tested for different autoAb; among them 100.000 have been screened for more than 10 different autoAb specificities by our unchanged version of CIE (5). Almost exclusively in-house methods have been used for autoAb with most satisfactory results at the lowest possible cost. The same version of the CIE method has been used in IL as the second screening test for different anti-ENAs since 1987, therefore we like to expose certain aspects of the method:

Since immune complexes formed in CIE gel precipitate as distinct lines, all the reactions between autoAb and relevant antigens are actually seen. This includes also those with unknown specificities. Therefore, many rare, but sometimes diagnostically important autoAb (PCNA, SL, Ku, PM/Scl, etc.) were discovered by simple routine testing. Considering the results, CIE is neither expensive nor complicated: it can be performed at very low cost in a very reasonable time, 24 hours for the overall positive/negative result and additional 24 hours to test for specific autoAb. This is, for this type of analytes, more than acceptable. A trained technician can test over 100 sera in just 2 hours needed for screening for positivity with two antigen substrates and to further characterisation of all positives using relevant standard antisera. The negative CIE test means that the tested serum is negative for all autoAb against most common antigens (Ro, La, Sm, U1RNP, Jo-l, Sd-70) and also against some rare, but very important ones (Table 2) PCNA, PM/Scl, Mi-2, PL-4, PL-7, PL-12, Ku, SL), as well as against many unknown antigens. Therefore, there is no need to run separate tests for each autoantibody separately as is the case with ELISA technique.

Table 2. *Autoantibodies against some rare antigens (4)*

Antigen	**Disease (aprox. %)**
Ku	SSc (2,3%)*, PM, DM, SLE (?)
Mi-2	PM (5%)
PCNA/cyclin	SLE (3%)
PM/Scl	PM, DM (8%), SSc (3,6%)*
SL	SS (3%), SLE (3-8%)
PL-4	SLE (3%)
PL-7, PL-12	PM, DM

SLE-systemic lupus erythematosus, SS-Sjoegren's syndrome, SSc-Systemic clerosis, PM-polymyositis, DM-dermatomyositis (* An on-going multicentre study leading by Dept. of Rheumatology, Ljubljana: anti-ENA/CIE on over 600 SSc patients)

Based on over 17 years of experience, we have summarised major practical advantages and disadvantages of the two-step CIE procedure and its relationship to ELISA.

10.4.1 Advantages of CIE

1. Positive/negative testing for all known and unknown autoAb precipitating in CIE gel can be performed in a single CIE run.
2. CIE methodology can easily be adapted to run many sera in a single run (from one to 50 or more).
3. During the first CIE step defining anti-ENA positivity/negativity of samples no standard antisera are required.
4. The sensitivity of the CIE for routine purposes is excellent (10-20 µg/mL of antigen).
5. There are no false positive results: if the test is positive, it is positive for one or more of autoAb against more than 15 known and many unknown antigens.
6. CIE is easy to perform: an average technician needs only 2-3-weeks training in a specialist laboratory.
7. Since CIE is always an "in-house technique, all the test procedures (including antigen extraction) are transparent and can be readily controlled and adapted for specific purposes.
8. An individual laboratory can establish its own pool of secondary standards according to primary CDC or WHO standards.
9. CIE detects autoAb mainly against native antigens
10. By introducing different substrates and some minor modifications of the CIE procedure, we can determine antibodies against other important antigens (i.e. antibodies against pyruvate dehydrogenase complex).

10.4.2 Disadvantages of CIE

1. Some rare autoAbs, i.e. ribRNP, do not precipitate in agarose CIE gel.
2. Recombinant antigens ("single epitope" antigens) are too small to precipitate in CIE gel; therefore autoAb against such antigens cannot be detected by CIE.

10.4.3 CIE versus ELISA

The point, which is many times ignored, is that ELISA can never replace CIE: each of them has their role in autoantibody testing. Results obtained by CIE and ELISA methods cannot be compared directly: they are useful for different purposes and give different answers:

1. With CIE, all major autoAb can be detected in two CIE runs.
2. ELISA tests apply only to selected antigens, giving one answer at a time, while CIE uses cell extracts (multiple antigen substrates), each time giving multiple answers.
3. Classic ELISA, or its automated versions, can never be good screening techniques as results obtained with regard to five or more specific antigens do not mean that a particular serum is also negative for other autoAb not included into same kind of repertoire.
4. ELISA gives too many false positive results sometimes leading to inadequate medical treatment. Setting-up cut-off values is even more problematic than in IF ANA testing.
5. Some ELISA kits give unreliable results due to unacceptable variations in batch-to-batch analysis.
6. It is not the analytical sensitivity but the analytical specificity, which remains one of the major problems with different ELISAs.

Many laboratories throughout Europe introduce only ELISA tests for autoantibody detection into their routine practice, which, we think, is inadequate and may be dangerous. We evaluated anti-ENA by CIE in sera from over 5.000 patients previously tested by IF ANA on Hep-2 cells. In patients with negative and low positive ANA (titre ≤1:80) there was about 6% of those with positive anti-ENA on anti-Ro, Jo-1 and UDA (Table 2.).

Considering recommendations of the consensus group, each laboratory analysing autoAb to intracellular antigens should be able to perform several basic techniques:

- Immunofluorescence test on Hep-2 cells as the first screening.
- If routine tests for specific autoAb are performed by ELISAs and a particular result is problematic, it should be confirmed/retested by a second technique such as immunoblotting, or by one of the gel techniques (RID, CIE).
- The results in such cases should always be interpreted for clinicians and not just reported.

We also believe that autoantibody testing should be performed with an utmost care. Therefore, it should be in the hands of highly professional personnel in expert laboratories covering all the necessary techniques for autoantibody testing.

10.5 Conclusions

Due to the dramatic shortage of resources in health system on one side and the fast extension of laboratory tests on the other, services of an AAL is continuously pressurised to increase its efficiency. To meet the needs of all patients and clinical

personnel responsible for human healthcare, minimal requirements should be set up. ISO 15189 provides particular requirements for the quality and competence of medical laboratories. For its implementation into AAL adequately educated and trained laboratory professionals is obligatory.

10.6 Take-home messages

In order to organize an AAL the following premises should be considered: competence (where, how much, what, who, to whom, how), quality control, education, financing, and logistics/informational system.

1. AutoAb tests should always be performed by laboratory specialists with a history of extensive training on the field, who is able to evaluate and interpret analytical results into laboratory (interpreted) findings.
2. The first screening test for autoAb in rheumatology is "ANA" testing on Hep-2 cells (by indirect immunofuorescence).
3. All sera (ANA positive or negative) should be screened for anti-ENA with CIE in those patients where connective tissue disease is suspected or tested on single autoAb(s), being aware of all limitations of single autoAb testing.
4. Counterimmunoelectrophoresis is fast, easy and cost-effective method for the detection of autoAb to intracellular antigens and dramatically lowers the overall costs for autoAb search.
5. If routine tests for specific autoAb are performed by ELISAs and a particular result is problematic, it should be retested by a second technique such as immunoblotting, or by one of the gel techniques (immunodiffusion, CIE).
6. AutoAb test should be performed in a laboratory, covering all the necessary techniques for autoantibody testing - autoAb tests should not be performed at primary level laboratories at Public Health departments or doctor's private practises
7. ISO 15189 provides particular requirements for the quality and competence of medical laboratories. For its implementation into AAL adequately educated and trained laboratory professionals is obligatory

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