PRATIQUE QUOTIDIENNE

Pratique actuelle des laboratoires d'analyses médicales pour le dépistage des anticorps anti-nucléaires en Tunisie

Current laboratory practice for anti-nuclear antibody testing in Tunisia

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Résumé

Introduction : Les résultats de la recherche des anticorps anti-nucléaires (AAN) sont cruciaux pour le diagnostic, la classification, la surveillance et le pronostic des maladies auto-immunes. Cependant, il existe encore peu de standardisation pour cette pratique. Ainsi, des enquêtes de terrain permettraient de mieux comprendre les pratiques de routine et de mettre en évidence les différences entre les laboratoires. La présente enquête a été menée pour évaluer les pratiques actuelles des laboratoires en matière de dépistage des AAN en Tunisie et de proposer des recommandations.

Méthodes : Une enquête de 26 questions a été envoyée à 250 biologistes des secteurs privé et public en Tunisie. Elle a été réalisée par l'application Google Forms sur une durée de 5 mois (septembre 2021-février 2022).

Résultats : Le taux de participation était de 45 % correspondant à 113 biologistes. L'immunofluorescence indirecte (IFI) sur HEp-2 était la principale méthode de dépistage des AAN pratiquée (93,3 %). La dilution de dépistage était de 1:80 dans 51,2 % des laboratoires. Près de la moitié (48,8 %) d'entre eux utilisaient une dilution de dépistage distincte chez les enfants. En cas de positivité, tous les laboratoires rapportaient un titre et un aspect de fluorescence. Nous avons remarqué une hétérogénéité dans le rendu du résultat notamment entre les laboratoires du secteur privé et public. En ce qui concerne le contrôle qualité (CQ), seuls 32,6 % des participants effectuaient régulièrement un contrôle interne et 17,8 % participaient à un programme externe de qualité.

Conclusion : Les résultats de cette enquête ont présenté un aperçu des pratiques de laboratoire actuelles de la recherche des AAN en Tunisie. A notre connaissance, il s'agit de la première étude africaine. Globalement, malgré le nombre limité de laboratoires effectuant cette analyse, la plupart d'entre eux semblent suivre les recommandations internationales concernant la technique utilisée, le titrage et l'interprétation des fluorescences. Néanmoins, des progrès restent à faire, notamment en ce qui concerne la dilution du dépistage et le contrôle qualité.

Mots clés : Anticorps anti-nucléaires, antigènes nucléaires solubles, Anticorps anti-DNA, Enquête, standardisation

Abstract

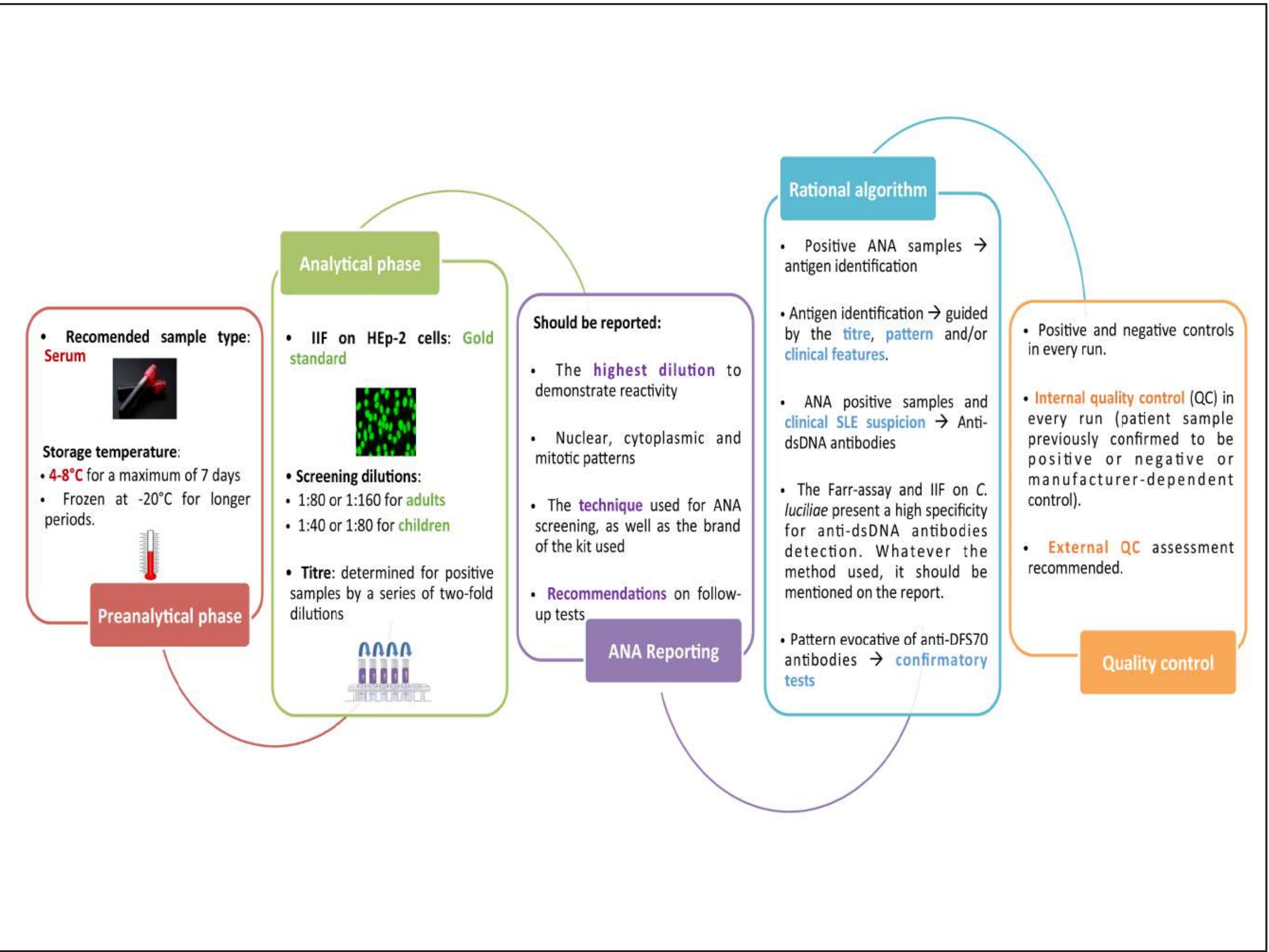
Background: Antinuclear antibodies (ANA) testing results are crucial to autoimmune disease diagnostic,classification, monitoring, and prognosis. However, there is still little standardization for AAN testing and reporting. To achieve this, surveys help gaining insight into routine practices and point out differences between laboratories. The present survey was conducted to evaluate current laboratory practices in ANA testing in Tunisia and propose recommendations.

Design and Methods: A survey of 26 questions was sent to 250 biologists of both private and public sectors involved in autoimmunity diagnostics in Tunisia. Carried out by the Google forms application for 5 months (September 2021-February 2022), the survey aimed to gather insights into prevailling practices.

Results: The participation rate reached 45% corresponding to 113 biologists. Indirect immunofluorescence (IFI) on HEp-2 emerged as the predominant performed ANA screening method (93.3%). The screening dilution was 1:80 in 51.2% of laboratories. Almost half (48.8%) of them used a distinct screening dilution in children. In case of positivity, all laboratories reported a titer and a fluorescence pattern. We noticed heterogeneity in reporting the different ANA patterns as well as in the diagnostic algorithm especially between private and public sector laboratories. Regarding quality control (QC), only 32.6% of participants regularly performed an internal QC and 17.8% participated to an external QC program.

Conclusion: This survey sheds light on the current laboratory practices in ANA testing in Tunisia. To the best of our knowledge, this is the first African study looking to the practice of AAN screening. Overall, despite the limited number of laboratories performing this analysis, most of them seem to follow international recommendations regarding the technique used, interpretation of fluorescence patterns and titration. Nevertheless, progress remains to be made, particularly regarding the screening dilution and quality control measures.

Key words : Anti-nuclear antibodies, soluble nuclear antigens, Anti-DNA antibodies, survey, standardization


Preanalytical phase
Recomended sample type: Serum
Storage temperature:
4-8°C for a maximum of 7 days
Frozen at -20°C for longer periods.
Analytical phase
IIF on HEp-2 cells: Gold standard
Screening dilutions:
1:80 or 1:160 for adults
1:40 or 1:80 for children
Titre: determined for positive samples by a series of two-fold dilutions
ANA Reporting
Should be reported:
The highest dilution to demonstrate reactivity
Nuclear, cytoplasmic and mitotic patterns
The technique used for ANA screening, as well as the brand of the kit used
Recommendations on follow-up tests
Rational algorithm
Positive ANA samples → antigen identification
Antigen identification → guided by the titre, pattern and/or clinical features.
ANA positive samples and clinical SLE suspicion → Anti-dsDNA antibodies
The Farr-assay and IIF on C. luciliae present a high specificity for anti-dsDNA antibodies detection. Whatever the method used, it should be mentioned on the report.
Pattern evocative of anti-DFS70 antibodies → confirmatory tests
Quality control
Positive and negative controls in every run.
Internal quality control (QC) in every run (patient sample previously confirmed to be positive or negative or manufacturer-dependent control).
External QC assessment recommended.

INTRODUCTION

Antinuclear antibodies (ANA) are a heterogeneous group of non-organ-specific autoantibodies. Despite their label, ANA refer to autoantibodies recognizing material within various cellular compartments including not only the nucleus, but also the cytoplasm, the membranes, and the mitotic apparatus (1,2).

ANA are a defining feature of systemic autoimmune rheumatic diseases (SARD). Therefore, their detection and quantification are fundamental not only for the diagnosis and classification of such diseases, but also for monitoring their evolution and treatment response (3).

ANA testing is a labor-intensive process that starts with a screening test followed by serial dilutions of the positive sera and identification tests for antigen specificity determination. Indirect immunofluorescence (IIF) using HEp-2 cells is considered the gold standard technique for ANA screening (1,4,5). HEp-2 IIF requires considerable technical expertise as the visual microscope determination of the staining pattern and the endpoint titer makes it dependent on highly skilled operator. Therefore, as the demand for ANA testing increased, other assay methods have become available over the past decades, mainly enzyme-linked immuno-sorbent assays (ELISA), chemiluminescence immunoassay (CLIA) and fluorometric-enzyme immunoassay (FEIA) (4, 5), but also automated computer-assisted HEp-2 IIF assay systems (6). Laboratories may use the alternative assay techniques to supplement HEp-2 IIF testing or, less often, to replace it.

Diversity of ANA detection methods, the differences in their sensitivity and specificity and the lack of standardization of their results lead to discrepancies in the interpretation and reporting of ANA findings between laboratories. Inconsistencies in ANA testing practices could lead to ANA repetitions, a source of anxiety for the patient and unnecessary expenses for the health system. They can also cause delays in diagnosis and consequently impact the prognosis. Therefore, international groups of experts have regularly tried establishing recommendations and creating an accurate pattern nomenclature to harmonize ANA testing in the last years (1,7).

Several published surveys aimed to do a confrontation of daily practices of ANA testing to international recommendations. They concerned mainly European and North American laboratories and their results revealed differences not only between countries, but also within them (8, 9). However, little is known about laboratory practices in Asian and African countries.

An overview of routine laboratory practices was carried out in Tunisia through a survey addressed to health professionals involved in the performance and interpretation of ANA testing. Our aim was to propose locally adapted recommendations as a step forward to standardizing ANA testing results at the national level.

1. Methods

Our team at the Laboratory of Clinical Immunology at Pasteur institute of Tunis developed a survey of 26 questions covering five main items: pre-analytical phase, analytical phase, anti-dsDNA and anti-ENA antibodies testing, result reporting and quality control (QC) (Table 1).

The survey was conducted using the Google Forms application (Google LLC, Mountain View, CA). The link for the survey was sent by email on September25th 2021 and was closed on February 28th, 2022. The survey form was sent to medical biology laboratories involved in ANA-testing both in the public and the private practice in Tunisia. The laboratories coordinates were obtained through a collaborative effort with the Tunisian Society of Immunology (STI), the Tunisian Society of Clinical Biology (STBC) and the syndicate of independent biologists in Tunisia.

Although, the Pasteur Institute of Tunis Biomedical Ethics Committee has confirmed that no ethical approval is required for this study, the questionnaire was conducted with strict respect for the anonymity of the participants.

RESULTS

1.1. Participating laboratories

Among the 250 biologists who received the questionnaire, 113 took part in the survey, i.e.an overall participation rate of 45.2% with a dominance of private practice laboratories (n=94; 83.2%) over university and regional hospitals laboratories (n=19; 16.8%).

While all biologists from public laboratories declared performing ANA testing, only 27.7% (n=26/94) of the private laboratories did, making a total of 45 biologists (39.8%) performing ANA testing. The rest of the participants (n=68/113) outsourced this test to other laboratories.

1.2. Pre-analytical phase

All biologists declared performing ANA testing in the serum. Samples are mainly conserved at a temperature of +4°C (n=34; 75.7%) or -20°C (n=9; 20%) until analyses. Two private sector biologists keep the serum at room temperature and run the ANA testing on the same day of sampling. Only 28 laboratories (62.2%) declared conserving aliquots of the specimen.

1.3. Analytical phase

IIF is used for ANA screening by 95.6% (n=43/45) of laboratories. Two private sector laboratories reported using ELISA for ANA testing without performing IIF first. For IIF, HEp-2 cells were the main cell substrate used. Only one laboratory in a teaching hospital

Table 1: Summary of the main items of the survey

Main items of the survey
Type of laboratory
Pre-analytical phase
ANA testing outsourcing
Specimen used
Samples conservation
Analytical phase
Screening dilution for adults and children
Substrate used
Reagents used
Immunoglobulin isotype detected
Use of counterstain
Use of positive and/or negative controls
Lens magnification used when reading IIF on HEp-2 cells
Frequency of maintenance of the microscope
Rational algorithm
Confirmatory testing for anti-DFS70 antibodies
Algorithm in case of positive ANA testing
Anti-dsDNA antibodies testing method
Result reporting
Reporting of the technique, substrate and commercial kit used
Fluorescence patterns reported
Titre assessment and reporting
Quality control
Accreditation according to EN/ISO 15189:2012 directives
Frequency of quality control
Type of quality control performed

declared using frozen sections of rat liver. The most used reagent for HEp-2 cell slides for IIF was that of the EUROIMMUN® firm (Germany) (n=38 /42; 90.5%). Other reagents were less commonly used, such as those of BioSystems® (Spain) (n=5), BIORAD® (USA) (n=3) and AESKU® (Germany) (n=2) firms. Five biologists used more than one brand of reagent.

For laboratories using the IIF technique, the initial screening dilution in adults was 1:80 for 51.2% of the laboratories (n=22/43), 1:100 for 37.2% (n=16/43) of the laboratories, 1:160 for 7% of the laboratories (n=3/43) and 1:180 for the rest of them (n=2/43). Regarding ANA screening in children, the 1:40 and 1:80 dilutions were used by the same number of laboratories (n=16/43; 37.2%) while 23.3% (n=10/43) of laboratories used a 1:100 dilution instead. In total, only 21 laboratories (48.8%) used a distinct screening dilution for children. For positive samples, most laboratories (n=29/43; 67.4%) determined the titer by a series of two-fold dilutions while the rest of them declared just reporting an estimated titer.

For the revelation of the antigen-antibody reaction, 90.7% (n=39/43) of the biologists used a secondary fluorescein-labelled antibody detecting IgG class antibodies whereas the other biologists preferred using a triple conjugate recognizing IgG, IgM and IgA isotypes. To

reduce nonspecific fluorescence, a counterstain was used by only 20 biologists (46.5%), 12 of them from the public sector. Most laboratories (n=39 /43; 90.7%) run a positive control in each series of IIF while only 58.1% (n=25 /43) of them run a negative control.
For the microscopic analysis of the immunofluorescence staining, 95.3% (n=41/43) of participants used the 40x magnification while only two private sector biologists declared using the 100x magnification. Concerning the fluorescence microscope, nearly half of the participants (n=21/43; 48.8%) performed a maintenance check only in case of a malfunction. The rest of laboratories did perform a maintenance check on a regular basis, eight of them did it every six months (n=8/43; 18.6%) and 14 of them annually (n=14/43; 32.6%).

1.4. Results reporting

The survey revealed heterogeneity in reporting fluorescence patterns. Some patterns were not systematically mentioned, particularly those concerning the cytoplasm and the mitotic apparatus. The different patterns recognized and reported by the laboratories are presented in Table2. Isolated cytoplasmic patterns were considered ANA negative by 21 participants (48.8%) and ANA positive by 14 laboratories (32.6%). Eight biologists (18.6%) added a note indicating the presence of cytoplasmic staining according to the clinical context.

Table 2 : Fluorescence patterns on HEp-2 cells recognized and reported

Fluorescence patterns	N	Proportion (%)
Homogenous	43	100
Anti-DFS70-like pattern	33	76.7
Speckled	43	100
Nucleolar	40	93
Centromere	38	88.4
Nuclear envelope	39	90.7
Nuclear dots	33	76.7
PCNA– like pattern	31	72.1
Mitotic apparatus	26	60.5
Cytoplasm	39	90.7

In case of a positive immunofluorescence reaction, all laboratories reported a fluorescence pattern and a titer. The titer was expressed by the last positive dilution by 65.1% (28/43) of the biologists while it was expressed by the inverse of the last positive dilution by the rest of them. Seventy percent (n=31/43) of the biologists declared mentioning the technique and the cell substrate used in the analysis report, and 55.8% (n=24/43) of them reported the brand for the reagent used either for IIF or for ELISA.
In case of an evocative fluorescence pattern of anti-DFS-70 (Dense Fine Speckled 70 kD) antibodies,74.4% (n=32/43) of biologists confirmed the presence of anti-DFS70 antibodies by an immunoenzymatic technique whereas 8 biologists, most of them (n=7/8); from the private sector, declared reporting anti-DFS70 antibodies directly after only IIF analyses without any further testing.

1.5. Anti-dsDNA and ENA antibodies testing

If ANA testing is positive, 35.6% (n=15/45) of laboratories systematically proceeded to anti-double stranded DNA (dsDNA) and anti-ENA on the positive sera, while 44.4% (n=20/45) of biologists rather recommended further testing for these antibodies (Table 3).

Table 3 : Process in case of positive ANA testing

In case of positive ANA testing	Public sector laboratories N	Private sector laboratories N	Total N (%)
Proceed with anti-dsDNA and ENA testing	14	2	16 (37.2)
Recommend anti-dsDNA and ENA testing	5	14	19 (44.2)
Report positive ANA without any recommendation	0	8	8 (17.8)

Anti-dsDNA assessment was performed by almost all laboratories (n=44/45) using different techniques as presented in the table 4, knowing that 39% of laboratories declared using more than one technique for this test. IIF on *Crithidia luciliae* was the most prevalent technique for anti-dsDNA detection both in the public and the private sectors. While public sector laboratories proceeded to anti-dsDNA testing mainly when facing a positive ANA serum, private sector laboratories rather assessed these antibodies only if requested, independently of ANA-screen test result (Table 4).

Table 4 : Methods and context of anti-dsDNA antibodies testing

Anti-dsDNA detection technique	**Public sector laboratories N**	**Private sector laboratories N**	**Total N (%)**
Farr-assay	0	0	0 (0)
IIF on Crithidia luciliae	17	17	34 (75.6)
ELISA	9	11	20 (44.4)
Immunodot	4	5	9 (20)
Context of anti-dsDNA antibodies testing			
If requested	6	20	26 (57.8)
In case of positive ANA testing	11	7	18 (40)
In case of positive ANA testing with clinical suspicion of SLE	10	3	13 (28.9)

1.6. Quality control

None of the participating laboratories declared being accredited according to EN/ISO 15189 directives. Fourteen biologists (14/43; 32.6%) reported regularly using an internal QC (IQC) for IIF. Six biologists performed an IQC in every IIF run (14%), three of them run an IQC monthly (7%), four biologists run the IQC every six months (9.3%) and one biologist declared running an IQC every six months (2.3%). The internal and external QCs used by the different participants are shown in Table 5.

Table 5 : Quality controls performed

Internal quality controls	**Public sector laboratories N**	**Private sector laboratories N**	**Total N (%)**
HEp-2 kit-related commercial control	7	2	9 (20)
Kit-independent commercial control	2	0	2 (4.4)
‘In-house’ control (single or pooled patient samples)	1	2	3 (6.7)
External quality controls			
Inter-laboratory exchange of patients’ samples	0	0	0
External quality control program	5	3	8 (17.8)

DISCUSSION

The results of this survey allowed comparing practices from the answer of 45 participating laboratories performing ANA testing in Tunisia. Although the demand for ANA testing has increased remarkably, there are still not so many laboratories in Tunisia performing autoimmunity tests. Most private laboratories would rather outsource ANA testing to more qualified laboratories. This is most likely due to the fairly high cost of setting up an immunology diagnostic platform (reagents, fluorescence microscope, etc.), as well as the expertise required for the validation of autoimmunity tests.

Groups of experts, whether rheumatologists or biologists, stated that IIF is the reference method for ANA detection (1, 4). This decision is principally based on the use of positive ANA as an entry criterion in 2019 EULAR/ACR classification criteria for systemic lupus erythematous (SLE) (10), but also for the advantages of IIF. Compared to other techniques, IIF allows the detection a larger panel of autoantibodies whether to well-defined or to still unknown target antigens whatever their cellular location is. Solid-phase assays rely on either purified antigens that purification steps may lead to denaturation of the native protein, or recombinants that may lack conformational epitopes, both processes leading to loss of antigenicity (11). Our data showed that almost all laboratories in Tunisia followed the international recommendations by using IIF for ANA screening.

Despite the great development of alternative techniques, they are not considered by experts to be appropriate for ANA screening (1, 4). They are based on restricted mixtures of well-defined nuclear antigens that differ in number, nature (purified native antigens, recombinant proteins, or synthetic peptides) and method of synthesis or purification (5). So many variables that contribute to the possible discrepancies in results obtained for the same serum with different techniques or reagents used (4, 12, 13).This survey showed that two private laboratories used ELISA for ANA detecting, probably to compensate for non-experienced workforce in IIF interpreting. ACR ANA Task Force reported that many large private laboratories as well as some small hospital laboratories have been testing for ANA by ELISA or coated beads instead of IIF in the USA, causing a significant increase in false negative tests (4).This can lead to a delay in the diagnosis process for patients presenting autoantibodies detectable by IIF and not by solid-phase substrates.

The IIF technique on rodent tissue sections as a substrate had been considered as the reference method for ANA testing for a long time until HEp-2 cells were discovered mid-1970s (5). Our survey revealed there was one laboratory still using rat liver sections as IIF substrate. Since HEp-2 cells are derived from laryngeal carcinoma, they have bigger nuclei, allowing easier visualization of its structures, and they express antigens in various stages of the cell cycle which is useful for the identification of peculiar autoantibodies such as anti-mitotic spindle apparatus, anti-PCNA or anti-centromere antibodies (5). Nowadays, there are numerous brands of commercially available HEp-2 cell slides. Although the production of this type of cells in large number is easier than cryopreserved sections of rodent tissue, there is a lack of standardization of cell culture, permeabilization and fixation methods for HEp-2 cells. These parameters influence the cell distribution of autoantigens and the preservation of epitopes (14). Subsequently, there is a lack of reproducibility between HEp-2 kits that seems more frequent in weakly reactive samples (14). Therefore, biologists as well as rheumatologists must pay attention when comparing results. This reinforces the importance of mentioning not only the technique, but also the brand of the kit used for ANA testing on the analyses report. Our results revealed that more than half of the laboratories did mention the brand of the kit used.

Many parameters interfere with IIF performance. Beside the brand of the HEp-2 kit used, the screening dilution, the choice of the anti-globulin, the use of controls or counterstain are just as important factors that need to be considered when harmonizing ANA screening.

Expert groups of EASI (European Autoimmunity Standardization Initiative) and IUIS (International Union of Immunological Societies) standardization committees established international recommendations for ANA testing. Of these, the titer corresponding to the 95th percentile of the local healthy control population was set as the optimal screening dilution (1). This titer was shown to correspond to a 1:160 dilution by the study of Tan et al. on healthy controls from different countries (USA, Europe, Japan, Canada, Australia) (15). No data on the local healthy population is available in Tunisia, but only 7% of the laboratories used the 1:160 as the initial dilution. More than half of them rather used the 1:80 dilution. Indeed, while the international recommendations advocate for a screening dilution defined locally, the 2019 ACR/EULAR classification criteria call for a positive ANA at a titer of at least 1:80 (10). Although at this starting dilution 13.3% of healthy individuals are ANA positive, the sensitivity for SARD diagnosis is higher than that with the 1:160 dilution. A recent study demonstrated through ROC analysis that the ideal screening dilution for distinction between healthy and SARD populations was1:80 (16). Moreover, an ICAP (International Consensus on ANA Patterns) survey revealed the 1:80 dilution was the most used in laboratories around the world (17).

Our results showed more disparities regarding screening dilutions in children. The 1:40 and 1:80 dilutions were the most commonly applied. There is no clear consensus for screening dilutions in pediatric samples. Nevertheless, numerous publications report a screening dilution of 1:80 in children (18, 19). In a large series of healthy children aged 6 months to 15 years, Wananukul et al. reported an ANA prevalence of 9% at 1:40 dilution and only 3% at 1:80 (19). The starting dilution 1:80 may then seem appropriate at first sight. But the same study reveals that in SLE patients, ANA were positive in 91% at a serum dilution of 1:40 against 78% at 1:80. The high sensitivity and specificity as well as the negative predictive value of 97% may justify a screening dilution of 1:40 in children (19).

According to the recommendations from the ICAP group, a report on a positive ANA result should include, along with the endpoint-titer, immunofluorescence patterns with their AC nomenclature, possible autoantibody associations, and remarks on follow-up testing (17). To date, there have been 6 ICAP workshops that aimed to harmonize the HEp-2 pattern nomenclature (7). A total of 29 different patterns have been categorized including nuclear, cytoplasmic, and mitotic patterns (7). Recognition of the different patterns is subjective and varies according to the reader. Therefore, HEp-2 IIF reading and interpreting can differ not only between laboratories but also within the same team. Consequently, it is crucial that two experienced examiners read HEp-2 slides and determine ANA results in agreement. Even the use of an automated computer-assisted diagnostic system does not exempt from double checking ANA patterns as it can only replace one of two necessary readers (20).

The ICAP working group suggests replacing the outdated term for ANA IIF testing with anti-cell antibodies testing (17). Indeed, HEp-2 IIF allows the identification of a wide array of autoantibodies' patterns, including those targeting cytoplasmic and mitotic antigens. These uncommon patterns, qualified by "rare", have been described in organ-specific and non-organ-specific autoimmune diseases (AIDs) (21). Even though no disease-associated specificities have been demonstrated, these patterns should be communicated to the clinician as they could lead to the suspicion of an AID (21). Therefore, experts group stated that, besides nuclear patterns, cytoplasmic and mitotic apparatus patterns should also be reported (1).

Almost half respondents in our survey considered isolated cytoplasmic staining to be ANA negative, versus 33% of laboratories reported in a recent international survey (17).According to ICAP nomenclature, there are 9 cytoplasmic patterns (2). They can be related to several clinically relevant antibodies, such as anti-ribosomal P protein or anti-tRNA synthetase antibodies for fine speckled staining, anti-mitochondrial antibodies for reticular staining or anti-cytoskeletal antibodies for fibrillar staining (2). Disregarding cytoplasmic patterns can mislead the clinician or delay the diagnosis of AIDs autoimmune cholangitis or inflammatory myositis (21).

ANA endpoint titer and pattern provide crucial information to the clinician. Indeed, whatever the pattern, the higher the ANA titers, the higher likelihood of ANA-associated AIDs (22). International recommendations for ANA testing designed the highest dilution to demonstrate reactivity to be reported (1). However, although ANA are hallmarks of AIDs, no ANA signs a diagnosis by its mere presence. In the same way, the absence of ANA positivity or specificity does not discard the diagnosis of AID, especially if the clinical context is highly evocative. The interpretation of ANA testing must always consider the associated clinical and biological features. Therefore, the request for ANA testing must always be accompanied by the patient's clinical features. Depending on ANA screening results and the clinical features, the biologist will decide whether to perform follow-up tests for ANA specificity identification. In case of positive ANA, it is recommended to identify the target antigen (1). The choice of the technique and antibodies panel tested is guided by the pattern, the titer and/or clinical setting (1). Nowadays, multiparametric assays detecting multiple autoantibodies simultaneously are commercially available. These tests are presented in different panels of autoantigens that are disease oriented, hence the importance of clinical information. When correctly identified, ANA pattern can also guide the biologist for the choice of the panel as some patterns can point to a group of target autoantigens or, sometimes, to one or more diseases (23).

According to international recommendations, positive ANA should also be followed by anti-dsDNA antibodies testing when there is clinical suspicion of SLE (1). More than half of the participants in the survey revealed testing for anti-dsDNA antibodies only if requested by the clinician. The technique mostly used was the IIF on *Crithidia luciliae*. Although, the Farr-assay is the gold standard for anti-dsDNA antibodies (24). This technique requires dedicated facilities and safe handling and disposal of radioactive isotopes, thus explaining the preferred use of alternative methods in the diagnostic routine. Among the different techniques available, both the Farr-assay and IIF using the kinetoplast DNA of the hemoflagellate *C. luciliae* as the autoantigen offer high clinical specificity and are recommended by the EASI and IUIS working groups for SLE diagnosis (1). For monitoring disease activity, anti-dsDNA titration is better achieved by quantitative assays like the Farr-assay or ELISA (1).Whatever technique adopted, it must be the same used for SLE activity monitoring, hence the importance of always mentioning the technique used on the analysis result report (1).

When ANA pattern is evocative for anti-DFS70 antibodies, confirmatory assays should be performed (2,25). When no other ENA is recognized, anti-DFS70 antibodies are considered to be a negative predictor for the development of SARD within 10 years of follow-up (26). Confirming the DFS70 reactivity is thus important as it makes ANA-related SARD diagnosis less likely. More than half of the participating laboratories in this survey proceed to second line-tests to confirm anti-DFS70antibodies. The AC-2 pattern, defined by a dense fine speckled fluorescence, may be caused by autoanti-

bodies to other antigens than DFS70 (27), hence the importance of confirming the reactivity directed to DFS70. But for that, the DFS pattern should first be accurately recognized, which can be challenging (27). As it can be mistaken for a homogenous pattern, failure to identify and report the DFS pattern can not only mislead the diagnosis but also lead to unnecessary clinic visits and follow-up testing (26).
Unlike other laboratory analyses, standardization of autoimmunity diagnostic tests is a complex task. Even the EN/ISO 15189:2012 requirements are subject to interpretation for autoimmunity laboratories, making accreditation in the field of immunology a real challenge (20). In Tunisia, no immunology laboratories have yet received accreditation.
Considering QC, our survey showed that a limited number of laboratories in Tunisia were using internal QC or participating to external quality assessment (EQA) programs. Along with the use of manufacturer-provided positive and negative controls, the use of manufacturer-independent control samples is highly recommended (25). Internal QC can also be in-house determined, by choosing a patient sample that had been confirmed positive in order to ensure repeatability of pattern and titer interpretation as well as to detect lot-to-lot variations (25). The choose of a cut-off control using a borderline positive patient sample or a commercial low positive control enables uniformity in test sensitivity and prevents false negative results (25,28). Regarding IQC frequency, only 14% of the laboratories declared performing an IQC in every run, which is recommended by the Croatian society of medical biochemistry and laboratory medicine (25).
Unfortunately, no national EQA program is yet available in Tunisia. This is the case in of many other countries like Croatia whose EQA programs do not cover autoimmunity diagnostics (29). This is certainly related to the complexity of standardizing autoantibody testing owing to the wide spectrum of antigen-antibody reactivity in terms of avidity, affinity, epitope specificity (30). Only eight laboratories reported participating in EQC program in our survey. To ensure reproducibility, the exchange of patients' samples between laboratories can be a cost-effective alternative.

CONCLUSION

Altogether, the results of this survey presented an insight of current laboratory practices in ANA testing in Tunisia. To the best of our knowledge, this is the first African study looking to the practice of AAN screening. Overall, despite the limited number of laboratories performing this analysis, most of them seem to follow international recommendations regarding the technique used, interpretation of fluorescence patterns and titration. Nevertheless, progress remains to be made, particularly regarding the screening dilution and quality control. There is no national consensus for ANA testing In Tunisia. The results of such surveys allow comparison with other laboratories locally but also learn more about the practices in other countries. This survey constitutes a first step aiming to standardize practices in ANA testing and reporting through the development of recommendations. Main recommendations that can be drawn from this study are summarized in Table 6.

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Author contributions: IZ and SB conceived and designed the study. IZ, SB and YG collected and analyzed the data. SB, FK and IZ wrote the manuscript. IZ and MBA revised the manuscript. All authors read and approved the final manuscript.

Table 6: Recommendations for ANA testing and reporting

Preanalytical phase	• Serum is the recommended sample type for the detection of autoantibodies. • Serum should be stored at 4-8°C for a maximum of 7 days or frozen at -20°C for longer periods.
Analytical phase	• The IIF on HEp-2 cells is the reference method for ANA screening. • Screening dilutions of 1:80 or 1:160 are appropriate for ANA detection in adults. • Screening dilutions of 1:40 or 1:80 are appropriate for ANA detection in children. • The titer should be determined for positive samples by a series of two-fold dilutions.
ANA Reporting	• The highest dilution to demonstrate reactivity should be reported • Nuclear cytoplasmic and mitotic patterns should be reported • The technique used for ANA screening and follow-up tests should be reported, as well as the brand of the kit used • Recommendations on follow-up tests should be mentioned on the result report
Rational algorithm	• Positive ANA samples should be tested for antigen identification • Antigen identification should be guided by the titer, pattern and/or clinical features. • Anti-dsDNA antibodies should be tested in ANA positive samples in case of clinical SLE suspicion • The Farr-assay and IIF on *C. luciliae* present a high specificity for anti-dsDNA antibodies detection. Whatever the method used, it should be mentioned on the report. • When the pattern is evocative for anti-DFS70 antibodies, confirmatory tests should be performed, or at least recommended to the clinician.
Quality control (QC)	• Positive and negative controls should be included in every run. • Internal quality control (QC) should be performed in every run. Internal QC can be a patient sample previously confirmed to be positive or negative or the manufacturer-dependent control. • Participating in an external QC assessment program is recommended.

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