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Harmonization of anti-nuclear antibody testing (ANA) by indirect immunofluorescence assay: Results from ten years of UK NEQAS external quality assessment

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A B S T R A C T

External quality assurance (EQA) programs play a pivotal role in monitoring laboratory practices, allowing each laboratory to evaluate the consistency of results across different methods as well the ability of individual laboratories to compare and improve over time their own performance.

The objective of our study was to analyze the UK NEQAS EQA reports for the "Antibodies to Nuclear and Related Antigens" program from 2013 to 2023, to assess the overall level of harmonization of the responses for anti-nuclear antibody (ANA) testing by indirect immunofluorescence (IIF), in terms of both pattern and titer consensus. As a second aim, we analyzed the impact of the introduction in UK NEQAS EQA reports of the International Consensus on ANA Patterns (ICAP) nomenclature and of digital image recognition on the harmonization of the ANA HEp-2 IIF test.

The percentage of consensus for positive/negative results was significantly higher (90.9 ± 1.4) in 2023 than in 2013 (64.0 ± 7.8, $p < 0.001$). Common to all years in the investigated period, consensus on pattern recognition was significantly lower for the homogenous pattern (70.5 ± 16.0) compared to the centromere (84.9 ± 14.9), the speckled (90.3 ± 12.3), and the negative (84.5 ± 18.6, $p < 0.001$) samples, while it was significantly higher for titers 1:80–1:320 than for titers > 1:320 ($p < 0.001$).

The difference between manual reading and digital reading was not significant (93.8 % *vs*. 92.4 %; $p = 0.078$), but it was significant between the pre- and post-use of the ICAP nomenclature (82.6 % *vs*. 93.8 %; $p < 0.001$).

This study shows that the variability in ANA recognition and reporting is pattern (homogeneous > speckled > centromere) and titer (high titer > low titer) dependent. While we did not find any difference between the use of manual reading compared to digital reading, the adoption of the ICAP nomenclature greatly improved the harmonization of ANA reporting.

1. Introduction

The presence of antinuclear antibodies (ANA) is the hallmark of autoimmune rheumatic diseases, such as systemic lupus erythematosus (SLE), systemic sclerosis, Sjögren's syndrome, mixed connective tissue disease and idiopathic inflammatory myopathies [1,2].

Indirect immunofluorescence (IIF) on human epithelial cells (HEp-2) is considered the reference method for ANA testing [3], but biological and non-biological issues may affect the standardization of the method. In particular, visual evaluation suffers from intra- and inter-laboratory variability due to discrepancies in recognition and description of the ANA patterns, and is highly dependent on reader expertise and on the substrate used [4–6].

ANA HEp-2 IIF testing allows detection of antibodies to specific intracellular antigens, resulting in different staining patterns that are usually categorized based on the recognized cellular components along

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the consecutive cell-cycle phases. In addition, a semi-quantitative assessment of the autoantibody serum concentration is provided by the fluorescence intensity or titer [7,8]. Proper recognition of a well-defined ANA staining pattern is crucial in determining the most likely specific autoantibodies for further cascade testing [8,9].

Guidelines for ANA detection using HEp-2 IIF recommend reporting titer and pattern [7,10]. The higher the antibody titer, the higher the likelihood for disease [11–13]. In addition, the combination of antibody level and antibody pattern can also provide helpful information on the most possible associated diseases [14]. An improper reporting and clinical interpretation of the test can lead to misdiagnosis, inappropriate therapies, and unnecessary costs.

In order to harmonize the recognition and description of distinct HEp-2 IIF patterns, the International Consensus on ANA Patterns (ICAP), a working group of the Autoantibody Standardization Committee (ASC) of the International Union of Immunology Societies (IUIS), proposed a comprehensive nomenclature system, which currently includes 32 distinct ANA patterns, each assigned an AC (anti-cell) code, ranging from AC-0 to AC-31 [15–19]. Over the years, this nomenclature has gained widespread use and is now available in 18 languages.

UK NEQAS, an international provider for external quality assessment (EQA) aims to promote harmonization in testing, interpreting, and reporting of results, and since 2016 has incorporated the use of the ICAP nomenclature system within their EQA program.

In this study, we reviewed the UK NEQAS EQA reports for the "Antibodies to Nuclear and Related Antigens" program submitted by participating laboratories worldwide from 2013 to 2023, to assess the overall level of response harmonization in terms of both positive/negative results, pattern and titer consensus. As our second aim, we analyzed the impact of introducing digital image recognition (from 2015 onward) and the ICAP nomenclature (from 2016 onward) on the level of harmonization of the ANA HEp-2 IIF test.

2. Methods

Data were extracted from the UK NEQAS EQA program participant summary reports. In accordance with UK NEQAS guidelines, participants are instructed to treat the specimens as routine clinical samples and to report results along with details on pattern and titer. We reviewed all reports from 2013 to 2023, including 60 distributions and 120 individual samples, for a) positive/negative consensus; b) pattern consensus; c) titer consensus (endpoint dilution); d) manual *vs*. digital reading; and e) pre-ICAP *vs*. post-ICAP consensus.

According to UK NEQAS, the expected or designated response is based on consensus of the results submitted by the participants. In addition, although UK NEQAS separately reports data entered by laboratories that performed manual or digital readings, we cannot be certain that there was no operator intervention in the reading of digital data.

3. Statistical analysis

Data were summarized using mean and standard deviation. The groups (target titers and pre-ICAP *vs.* post-ICAP) were compared using the *t*-test for independent samples. The comparison between dependent groups (manual *vs*. automated reading) was performed using the *t*-test for paired samples. Different patterns and average consensus recorded over different years were compared using the ANOVA test with Bonferroni post-tests and t-tests for independent samples. The analyses were conducted with SPSS statistical software (IBM Armonk, New York, USA, v28). A value of $p < 0.05$ was considered statistically significant.

4. Results

Over the time period reviewed, the number of participating laboratories submitting results for ANA of the EQA scheme increased from 567 in 2013 to 597 in 2023.

4.1. Positive/Negative consensus

The percentage of responses complying with the expected negative or positive target were analyzed for the investigated period. The percentage of consensus responses was significantly higher (90.9 ± 1.4) in 2023 (n = 537) than in 2013 (n = 441) (64.0 ± 7.8, $p < 0.001$). In particular, a major increase was observed starting from 2022 ($p < 0.001$ when compared to the period ranging from 2013 to 2021) (Fig. 1).

4.2. Pattern consensus

On average (from 2013 to 2023), 346 participating laboratories submitted a response for any given HEp-2 IIF pattern of the EQA program. The percentage of laboratories reporting the consensus response differed depending on the ANA pattern type, being significantly lower for the homogenous pattern (AC-1) (70.5 ± 16.0) compared to the centromere pattern (AC-3) (84.9 ± 14.9), to the speckled (AC-4,5) (90.3 ± 12.3), and to samples that were scored as negative (AC-0) (84.5 ± 18.6) (Table 1). The improvement in positive/negative consensus percentage (described in Fig. 1) observed in the years 2022 and 2023, it is likely due to the higher prevalence of samples with speckled pattern (70 %) than with homogeneous pattern (8 %) distributed in those years compared to the years < 2022 (47 % speckled and 20 % homogeneous).

4.3. Titer consensus

On average (from 2013 to 2023), 311 laboratories submitted a response for the HEp-2 IIF titer (endpoint dilution). The percentage of consensus response was significantly higher for titers within the range of 1:80–1:320 than for titers > 1:320 ($p < 0.001$) (Table 2).

4.4. Manual vs. Digital reading

Since 2015, UK NEQAS has allowed for laboratories to report ANA results based on automated digitally-acquired data alongside data acquired from traditional manual reading of HEp-2 IIF slides. On average (from 2015 to 2023), 386 participating laboratories submitted a response using manual interpretation compared to an average of 88 participating laboratories submitting a response using automated computer-aided diagnosis (CAD) interpretation. Of the 28 samples (4 negative, 3 centromere, 2 homogeneous, 19 speckled patterns) which were distributed between 2015 and 2023 (from sample coded 215–1 to

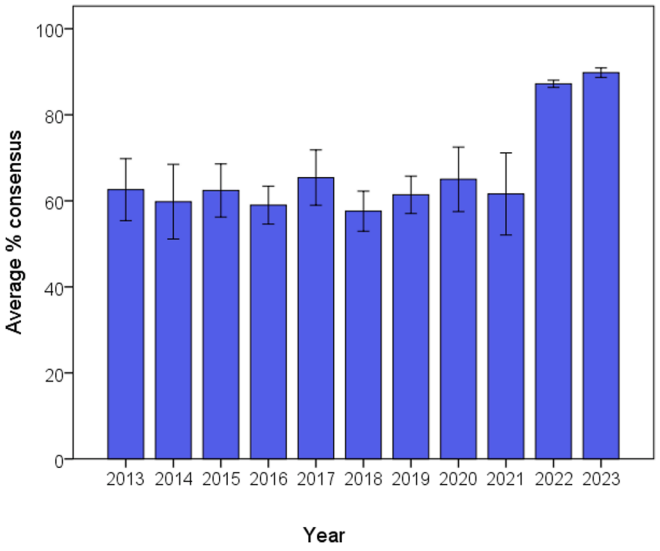

Fig. 1. Average percentage of consensus in different years. Bars represent ± 1 standard deviation from the average value.

Table 1
Consensus (%) of participating laboratories in reporting four selected HEp-2 ANA IIF patterns from 2013 to 2023.

	Speckled (AC-4,5)	Centromere (AC-3)	Homogenous (AC-1)	Negative (AC-0)	*p*-value
Number of samples deemed as specific pattern	60	13	22	16	
Number of laboratory responses	22,428	4937	8481	3902	
Correct consensus response (mean % ±SD)	90.3 ± 12.3^	84.9 ± 14.9	70.5 ± 16.0*	84.5 ± 18.6^	<0.001

*Significantly different from the centromere pattern.
^Significantly different from the homogenous patter.

Table 2
Consensus (%) response (target titer considered the one which received most responses).

	Target titer 1:80–1:320	Target titer > 1:320	*p*-value
Number of samples	53	58	
Number of laboratory responses	18,157	21,135	
Correct consensus response (mean %±SD)	56.8 ± 11.6	34.1 ± 3.3	<0.001

sample coded 236–2), we found a manual reading consensus for positive/negative response of 93.8 %±10.3, while the digital reading consensus was 92.4 %±12.5 ($p = 0.078$) (Table 3).

4.5. Pre-ICAP vs. post-ICAP

From the end of 2016, UK NEQAS included the ability to report ANA patterns based on the ICAP nomenclature (from sample 166–1 onwards). On average (from 2016 to 2023), 81 % of laboratories submitted an ANA response using the ICAP nomenclature. A significant difference in consensus was observed between pre- and post-introduction of the ICAP nomenclature for ANA testing (62.7 % ±7.7 *vs.* 73.7 % ±14.3, respectively; $p < 0.001$) (Table 4).

5. Discussion

The search for ANA is the first step in the serological diagnosis of patients with suspected autoimmune rheumatic diseases and the entry criterion for SLE classification. IIF on HEp-2 cells is still the reference method to detect the presence of ANA, providing information about the antigenic target of the autoantibodies and their clinical association. However, the process of reporting ANA patterns is prone to inconsistency due to the intrinsic subjectivity in image interpretation and variability of methods used and reader expertise in pattern recognition of

Table 3
Consensus (%) obtained with manual *vs.* automated CAD reading of the HEp-2 ANA IIF test.

	Manual reading	Automated reading	*p*-value
Number of samples	28	28	0.078
Number of laboratory responses	10,806	2458	
Correct pos/neg consensus response (mean % ±SD)	93.8 ± 10.3	92.4 ± 12.5	

Table 4
Consensus obtained in the pre-ICAP *vs.* post-ICAP eras.

	Pre-ICAP	Post-ICAP	*p*-value
Number of samples analysed	41	70	
Total number of laboratory responses	21,135	26,786	
Correct consensus response (mean %±SD)	62.7 ± 7.7	73.7 ± 14.3	<0.001

HEp-2 cells. This scenario poses considerable challenge in the harmonization of ANA HEp-2 IIF even among highly qualified personnel.

EQA providers are key stakeholders for achieving harmonization, given their role in monitoring the performance of individual laboratories as well the performance of different methodologies across different testing sites. In addition, EQA provides the evidence for identifying critical issues and highlights where further actions for improvement are required.

Over the years, autoimmunity laboratories have gained increasing awareness that participation in EQA programs is a valuable tool to improve testing outcome. Moreover, EQA can be used as a measure of the effectiveness of efforts made by international committees, like ICAP, in promoting the harmonization process. In the present study, we show that significant improvements have been made along the past 10 years in terms of harmonization of the ANA HEp-2 IIF test, in defining positive *vs.* negative results.

When considering the overall HEp-2 IIF pattern consensus, good performance was achieved, although some differences emerged. Indeed, a lower consensus was found for the homogeneous pattern when compared to the speckled, centromere or negative ones. This result is in line with an international survey [20] showing that readers, most of them with more than 10 years of experience, had a higher concordance in correctly classifying the centromere (AC-3) (95.2 %) or speckled (AC-4,5) (92.8 %) pattern than the homogeneous (AC-1) pattern (80 %). Although in our study the data regarding the level of experience of participants are not considered, it is noteworthy that the homogeneous, as well as the centromere and the speckled patterns are all classified at the competent (i.e., not expert) level by the ICAP committee. Notably, negative samples were recognized with significantly higher accuracy compared to positive samples, providing useful information considering that false positive results could lead to incorrect or over diagnosis, prescriptions of inappropriate drugs and unnecessary concern and anxiety in patients and physicians [21].

Since guidelines for ANA detection recommend reporting both pattern and titer, the percentage of consensus with regard to the IIF end-point titer was also analysed [11].

Our results showed higher concordance for the samples with low IIF titer (below 1:320) compared to samples with higher titers. Although serial sample dilution is considered the conventional quantification method for autoantibody titer assessment, it is recognized that the higher the dilution, the higher the error rate. This is a potential reason why automated approaches based on fluorescence intensity using single sample dilution are emerging [22,23]. However, these results should be taken with caution, as many laboratories using CAD systems report the estimated titer based on fluorescence intensity and not in the real titration, increasing antibody titer imprecision. Moreover, although UK NEQAS asks for reporting the end-titer dilution, we cannot be sure that this indication is really followed by all the participants. In addition, the number of participants not providing an ANA titer (this is not mandatory within the EQA program) makes the overview of the consensus titer in our databank incomplete.

Recently, notable progress in the direction of harmonization has been achieved thanks to the establishment of a new standardized nomenclature and categorization of the ANA-IIF patterns made by ICAP through a comprehensive taxonomy classifying 32 distinct ANA patterns. We show here that since UK NEQAS introduced the ICAP nomenclature in its report formats there has been marked improvement in the description of the ANA pattern, with an increase in consensus of

11 % (from 62.7 % to 73.7 %). Although the ICAP algorithm has first gone online on mid-2015, probably it took some years to catch up and also the fact that UK NEQAS incorporated the possibility that labs use the ICAP nomenclature may have contributed to the use of the ICAP system and fostered the harmonization rate.

It is important to point out that this high level of correct answers from participants apply to the few patterns that were assessed speculating that the percentage of agreement may drop for some other patterns, especially those that are not so common or so easy to identify.

Introducing to clinical laboratories artificial intelligence for ANA pattern recognition and the advent of CAD systems for digitization of HEp-2 IIF images has also played a significant role. An increasing number of laboratories are using CAD systems to the analysis of HEp-2 IIF patterns [24]. Though these systems are not completely optimised as several ICAP patterns as well as mixed patterns are not recognized by CAD systems, some studies have demonstrated improved agreement using digital images both in fluorescence intensity and staining pattern evaluation [25,26]. We did not observe an improvement in recognition of positive or negative samples by digital systems compared to the traditional manual method, but the data is still relevant because it confirms that digital reading is at least as accurate as manual reading. Results from this study conducted in collaboration with the UK NEQAS indicate that even if significant progress has been made in recent years, achieving harmonization in reporting ANA HEp-2 IIF pattern and titer remains one of the biggest challenges for an immunology laboratory and requires further collaborative efforts.

CRediT authorship contribution statement

Maria Infantino: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Conceptualization. **Teresa Carbone:** Writing – review & editing, Writing – original draft, Supervision. **Dina Patel:** Writing – review & editing, Supervision, Data curation. **Ravishankar Sargur:** Writing – review & editing, Visualization, Data curation. **Carol Stanley:** Writing – review & editing, Visualization, Data curation. **Amina Bhayat-Cammack:** Writing – review & editing, Supervision, Formal analysis. **Emirena Garrafa:** Writing – review & editing, Data curation. **Silvia Pancani:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Mariangela Manfredi:** Writing – review & editing, Supervision, Conceptualization. **Luis E.C. Andrade:** Writing – review & editing, Visualization, Supervision, Conceptualization. **Nicola Bizzaro:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Data curation.

Data availability

Data will be made available on request.

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