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Damoiseaux, J., Heijnen, I., Van Campenhout, C. et al. (11 more authors) (2018) An international survey on anti-neutrophil cytoplasmic antibodies (ANCA) testing in daily clinical practice. Clinical Chemistry and Laboratory Medicine, 56 (10). pp. 1759-1770. ISSN 1434-6621

https://doi.org/10.1515/cclm-2017-0306

An international survey on ANCA testing in daily clinical practice

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Short title: An EASI-survey on ANCA testing

Key words: ANCA, myeloperoxidase, proteinase-3, vasculitis, testing algorithm

Word count: 3242

Tables: 2

Figures: 3

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Abstract

Detection of anti-neutrophil cytoplasmic antibodies (ANCA) is important for the diagnosis of the ANCA-associated vasculitides (AAV). For AAV, especially ANCA directed against myeloperoxidase (MPO)and proteinase 3 (PR3) are most relevant. ANCA with less well-defined specificities may, however, also be detected in other inflammatory and non-inflammatory conditions.

A questionnaire, initiated by the European Autoimmunity Standardisation Initiative (EASI), was used to gather information on methods and testing algorithms used for ANCA in clinical laboratories of 12 European countries (EASI-survey).

Four hundred and twenty-nine responses were included in the EASI-survey analysis which revealed differences within countries and between countries. Laboratories overall were poor in adherence to international consensus on ANCA testing. Substantial variation was observed with respect to the use of ANCA indirect immunofluorescence (IIF) in the algorithm, application of distinct methods for MPO- and PR3-ANCA, the daily availability of new ANCA results, and interpretation of test results.

Awareness of these differences may stimulate further harmonization and standardization of ANCA testing. This may be promoted by an update of the international ANCA consensus and the introduction of international standards.

Introduction

Laboratory tests for anti-neutrophil cytoplasmic antibodies (ANCA) are used to diagnose and monitor inflammatory activity of the primary systemic small vessel vasculitides, further referred to as ANCA-associated vasculitis (AAV) [1-3]. According to the international consensus statement on testing and reporting of ANCA "ANCA is best demonstrated in AAV by using a combination of indirect immunofluorescence (IIF) on ethanol-fixed neutrophils and enzyme-linked immunosorbent assays (ELISAs) that detect ANCA specific for proteinase 3 (PR3) or myeloperoxidase (MPO)" [4]. This consensus advocates that serum samples from all new patients with an ANCA request should initially be tested by IIF. Positivity of IIF (*i.e.* a C-ANCA or P-ANCA fluorescence pattern) in combination with a positive test for PR3- or MPO-ANCA, respectively, is highly specific for AAV [5].

However, ANCA detected by IIF that do not react with PR3 or MPO have been described in many inflammatory and non-inflammatory conditions, such as autoimmune diseases of the gastro-intestinal tract [6,7] and liver [8], as well as autoimmune rheumatic diseases, infectious diseases, and adverse drug reaction [9]. Although the clinical relevance of ANCA detection in these non-AAV conditions is limited, several approaches have been evaluated to increase this relevance. These include the use of alternative IIF fixatives, such as formalin and methanol, and immunoassays for other target antigens, such as elastase and lactoferrin. Altogether, the autoantigens recognized by ANCA in non-AAV remain ill-defined and there is no international consensus on their place in diagnostic algorithms. According to the addendum to the international consensus [10], detection of ANCA against such antigens is not recommended in non-AAV, and – in addition – the use of IIF fixatives other than ethanol is not advocated for routine ANCA testing in case of these clinical conditions.

Since the publication of the international consensus on ANCA testing in 1999 many new detection technologies have become available. These include second (capture technology) and third (anchor technology) generation ELISAs [11-15], but also alternative antigen-specific assays, like addressable laser bead immune-assays (ALBIA) [16-18], chemiluminescent immune-assays (CLIA) [19], fluorescent-enzyme immune-assays (FEIA) [20-21], dot and line immuno-assays (DIA/LIA) [22], and even IIF [23,24]. On the one hand, the place of these new techniques in the international consensus testing algorithm is not established and may require revision [25,26], while on the other hand, the diversification of ANCA test methods may be an additional hurdle for standardization of these assays.

The diversity of the available ANCA IIF substrates, antigen-specific assays and technologies, and variety of test algorithms may result in highly diverse ANCA testing procedures in clinical laboratories which, eventually, may cause variation in outcomes for patients. Therefore, although consensus guidelines have been available for over 15 years, we here aim to evaluate adherence to existing guidance in diagnostic laboratories internationally, in order to identify and address any issues with harmonization. As such, this European Autoimmunity Standardisation Initiative (EASI) study addresses the EASI aspirations for “standardization of methodology, tests, and interpretation of results” and “harmonization of test algorithms” [27].

Methods

A questionnaire on ANCA testing was first developed for Dutch clinical laboratories by Renate van der Molen (Radboud University Medical Center, Nijmegen), Caroline Roozendaal (University Medical Center Groningen), and Jan Damoiseaux (Maastricht University Medical

Center). This questionnaire was distributed to all Dutch laboratories participating in the external quality assessment for ANCA [28]. Next, the questionnaire was translated to English and distributed by national EASI-teams in 11 other European countries. In the United Kingdom (UK) and Ireland the questionnaire was distributed in collaboration with UK NEQAS. In most other countries (Austria, Belgium, Finland, France, Ireland, The Netherlands, Spain, UK) the questionnaire was distributed only to laboratories that were involved in ANCA testing, while in some other countries either all laboratories were contacted without previous knowledge or certainty if ANCA-tests were being performed (Portugal, Sweden, Switzerland), or laboratory specialists known to be potentially involved in ANCA testing were selected (Italy).

In total, the questionnaire consisted of 54 questions in 5 categories: laboratory organization (n=5), ANCA IIF testing (n=16), ANCA specificity testing (n=11), the algorithm for ANCA testing (n=16), and ANCA testing with short turn-around-time (STAT; n=6). STAT testing was defined as having results available within 24 hours. Data of the participating countries, further referred to as EASI-survey, were collected by the national EASI-teams or UK NEQAS. These data were summarized in a standard Excel-file and sent to the coordinator of the study (JD). Since the results of the UK and Ireland were compiled in a single dataset, the results were also combined in the analyses.

The results are reported as absolute numbers, *i.e.* the number of laboratories, and percentages of either A) the total number of responding laboratories that perform ANCA testing, or B) to a subgroup of analysis, as indicated in the text. Non-responders were excluded from the total denominator for each question. Ethical approval: the conducted research is not related to either human or animals use.

Results

Response on questionnaire and ANCA workload of participating laboratories

The data on response and type of participating laboratories per country are summarized in Table I. In total, the questionnaire was distributed among 628 laboratories in 11 European countries (Italy excluded). In total, 328 laboratories (52.2%) responded. In Sweden (n=3) and Switzerland (n=8) some laboratories responded that they do not perform ANCA tests; these responses were excluded from further analyses. In Italy, the questionnaire was only distributed to known laboratory specialists (n=300). In total, 145 Italian laboratory specialists (48.3%) responded. Thirty-three Italian responses were not included because some laboratory specialists answered only a few questions, some reported contrasting results, and some responded twice. Altogether, 429 responses were included in the analyses (Fig. 1).

Workload of laboratories was very variable. In Finland a relatively large weekly number of ANCA requests are reported by the large university laboratories. Many of the participating laboratories in the UK/Ireland (n=39; 67.2%) also received high numbers (>50) of weekly ANCA requests (Table I). In Belgium (n=31; 43.1%), Italy (n=58; 43.2%), the Netherlands (n=17; 39.6%), and Portugal (n=15; 44.1%) many participating laboratories reported a relatively low number (≤15) of weekly ANCA requests.

ANCA testing by indirect immunofluorescence

Three hundred and thirty four of the 429 responding laboratories (77.9%) performed ANCA IIF on ethanol-fixed neutrophils (Table II). This is not the case in >20% of the participating

laboratories in Austria (n=8; 50.0%), Italy (n=27; 25.0%), the Netherlands (n=9; 20.9%), Portugal (n=16; 47.1%), and Sweden (n=7; 63.6%). In total 221 laboratories (66.2% of laboratories that use ANCA IIF) also perform ANCA on formalin-fixed neutrophils. Particularly in Sweden (n=0; 0%) and UK/Ireland (14.9%) formalin-fixed slides are not or hardly used at all. The majority of the laboratories (n=122; 55.2%) that additionally perform ANCA IIF on formalin-fixed slides use this strategy for all ANCA requests. Most of the other laboratories (n=83; 37.6%) restrict this to samples that are positive on ethanol-fixed slides (either all positives or pattern dependent). Of the remaining 17 laboratories few laboratories (n=5) utilize formalin-fixed slides only for requests for gastroenterologic diseases and the other laboratories (n=12) for miscellaneous reasons (not specified in the questionnaires).

As recommended at the First International ANCA Workshop [29], the majority of the participating laboratories used 1:20 serum dilution for ANCA IIF screening. In the Netherlands a 1:16 screening dilution is used by 7 laboratories (20.6%). Only half of the EASI-survey responding laboratories consistently perform titrations (n=165; 50.0%). However, this appeared very heterogeneous in the different European countries, varying from <25% in Spain (n=4; 20%) and UK/Ireland (n=12; 23.5%) to 100% in Austria (n=8) and Finland (n=4).

Distinction of ANCA IIF patterns, on the other hand, is consistently performed by nearly all participating laboratories that perform ANCA IIF testing (n=321; 96.1%). Basically all these laboratories report C-ANCA and P-ANCA patterns, while about 80% and 15% of the laboratories report atypical ANCA and other ANCA patterns, respectively. The definitions used for P-ANCA and atypical ANCA, however, are quite diverse (Table III).

Antigen-specific ANCA testing

According to the international consensus on ANCA testing, specificity for MPO and PR3 should be tested by ELISA [4]. In recent years, many different ELISA methods as well as alternative immuno-assays have become available. The assays used for detection of MPO- and PR3-ANCA in the different countries are summarized in Figure 2. In general, individual laboratories used the same method for MPO- and PR3-ANCA. Capture ELISA's, however, were slightly more prevalent for detection of PR3-ANCA than MPO-ANCA in Belgium, the Netherlands, Spain, and Switzerland. In Portugal some participating laboratories (n=5; 14.7%) reported that they do not perform antigen-specific assays. In general, these laboratories use an external laboratory for antigen-specific ANCA testing.

Some technologies are remarkably linked to specific countries: ALBIA is quite prevalent in French laboratories (n=10; 27.8%), DIA/LIA are particularly used in Belgium (n=16; 22.2%) and France (n=6; 16.7%), while the category 'other' in Italy (n=22; 22.2%) is predominantly represented by CLIA. The majority of the participating laboratories report MPO- and PR3-ANCA in a quantitative way (82.1%). This ranges from 68.8% (n=11; Austria) and 69.6% (n=48; Belgium) to 100% (n=4; Finland).

Besides testing for MPO- and PR3-ANCA, laboratories may also offer the possibility to analyze if autoantibodies to other ANCA specificities (azurocidin, bactericidal/permeability increasing protein, cathepsin G, elastase, lactoferrin, lysozyme, etc.) are present in a patient. These kind of tests are available in a minority of the laboratories participating in the EASI-survey (n=50; 13.0%).

ANCA testing algorithm

Analysis of the responses about the ANCA testing algorithm is provided in Figure 3A. Overall, about half of the laboratories (n=202; 53.2%), follow the minimal requirements of the international consensus, *i.e.*, screening by IIF and if positive antigen-specific immuno-assays for both MPO- and PR3-ANCA [4]. A minority of laboratories (n=22; 5.8%) only test for either MPO- or PR3-ANCA, based on the staining pattern observed in IIF. The optimal consensus algorithm [4], both IIF and antigen-specific immuno-assays on all samples, is executed in only 16.8% of the laboratories (n=64). As noted above, about 20% of the participating laboratories do not use IIF in their algorithm. A minority of laboratories (n=17; 4.5%) have reversed the sequence of testing compared to the international consensus: they screen by antigen-specific immuno-assays and perform IIF only on the positive samples. Significant heterogeneity in testing algorithms within and between European countries is evident in Figure 3A. Only in Finland (n=4) the testing algorithm is completely harmonized in the participating laboratories, *i.e.*, they all use the optimal consensus algorithm.

Since AAV patients benefit from an early diagnosis, we surveyed rapid reporting (STAT) of ANCA testing, defined as having results available within 24 hours of requesting. The majority of laboratories recognized both pulmonary alveolar hemorrhage and rapidly progressive glomerulonephritis as the most relevant clinical manifestation warranting STAT ANCA testing. However, one third (n=125; 30.7%) of the laboratories participating in the EASI-survey do not offer such STAT ANCA testing (Figure 3B). This is most apparent in Austria (n=8; 50.0%), Belgium (n=32; 44.4%), Italy (n=46; 56.1%), and Portugal (n=22; 64.7%). In addition, many laboratories (n=158; 38.8%) do not offer this STAT service during the weekend. This is explicitly the case in Finland (n=3; 75.0%), France (n=32; 88.9%), and Spain (n=15; 75.0%). Only thirteen percent (n=53) of EASI-survey respondents offered rapid testing

including the weekend. All laboratories that offer STAT testing use antigen-specific immuno-assays for both MPO- and PR3-ANCA. In the laboratories participating in the EASI-survey about half of the laboratories additionally test by IIF (n=122; 57.8%) and for anti-glomerular basement membrane (GBM) antibodies (n=115; 54.5%).

If a patient is diagnosed as suffering from AAV, follow-up testing is to be performed with the ANCA specificity that was originally positive. The assay(s) used for follow-up of MPO- and PR3-ANCA patients is illustrated in figure 4. Only very few participating laboratories (<5%) do not perform antigen-specific immunoassays for follow-up, but use IIF testing instead. About 2/3 of the laboratories always use IIF next to the ANCA specificity initially identified. Also, about 1/2 of the laboratories simultaneously test for the reciprocal antibody specificity, *i.e.*, MPO-ANCA in PR3-ANCA patients and *vice versa*. The most striking difference in follow-up between MPO-ANCA and PR3-ANCA patients is related to the way results are presented: qualitative or quantitative. In case of MPO-ANCA 27,7% of the participating laboratories report only qualitative results, while this is 10.1% in case of PR3-ANCA.

ANCA testing is clinically relevant for AAV, but is also used as an adjunct to diagnosis of other disorders, such as gastrointestinal autoimmune diseases. For the latter, performing antigen-specific immuno-assays does not appear to be of added value. Therefore, we surveyed if laboratories are able to discriminate ANCA requests for AAV or for gastro-intestinal autoimmune diseases, and if yes, whether this affected the testing algorithm. Less than half of the participating laboratories (n=167; 43.4%) are able to determine the clinical background of the ANCA request, *i.e.* AAV *versus* gastroenterology (Figure 3C), and only 26.9% (n=45) of these laboratories consequently use an alternative algorithm. Large differences are observed between the participating countries.

Discussion

In the current study we have presented the results of a questionnaire on testing for ANCA in 12 European countries (EASI-survey) as compared to the international consensus on ANCA testing [4,10]. The results reveal major differences between and within countries. In particular with respect to the use of ANCA IIF testing the laboratories participating in the EASI-survey often seem to deviate from the international consensus.

The position of the IIF test in the testing algorithm for AAV has been disputed for many years [30-32]. However, assays have changed substantially over the years and recently the results of a multi-center ANCA study on AAV have suggested that screening for ANCA by IIF is not of added value when using a high-quality antigen-specific immuno-assay [26]. Not all tests are the same, however, and performance of each type of assay would need to be validated or verified in laboratories accredited to ISO 15189 and performance continually monitored in External Quality Assessment schemes. While performance of MPO- and PR3-ANCA immunoassays in AAV diagnosis has been acknowledged, it may not be true for other disorders, such as autoimmune liver diseases and inflammatory bowel disease. It should be kept in mind, though, that some assays for PR3-ANCA commonly reveal low-positive results in ulcerative colitis [33]. Nevertheless, alternative algorithms for use of antigen-specific immunoassays and IIF have been proposed for AAV and non-AAV, respectively [25,28]. In order to triage samples for alternative testing algorithms sufficient clinical details and laboratory expertise is required, but the results of our study suggest that this is not the case in a substantial number of laboratories.

The clinical relevance of ANCA testing in non-AAV conditions is controversial. Diagnostic criteria for autoimmune hepatitis do not include ANCA in the diagnostic score, as it was not considered helpful [34]. Similarly, the European evidence-based consensus on the diagnosis and management of ulcerative colitis and Crohn's disease stated that the sensitivity of ANCA, in particular P-ANCA, is far from high enough to justify the use of ANCA testing in routine diagnosis [35,36], and even that the use of ANCA in combination with other serologic markers such as anti-saccharomyces cerevisiae antibodies (ASCA) is ineffective at differentiating colonic Crohn's disease from ulcerative colitis [36].

The use of alternative IIF fixatives, *i.e.*, formalin and/or methanol, has been proposed to better differentiate between ANCA related to AAV *versus* other (non-)inflammatory conditions, but there is again no consensus on this issue. The results of the recent multi-center ANCA study have revealed that, as compared to only ethanol-fixed slides, the combination of ethanol- and formalin-fixed slides has a significantly better performance for the diagnosis of AAV [37]. However, in this study ANCA requests from gastroenterology were excluded. The use of formalin- (and methanol)-fixed ANCA slides, however, also has contributed to the many different definitions used for ANCA patterns, in particular for P-ANCA and atypical ANCA (data not shown). Therefore, if the use of alternative IIF fixatives is going to be supported, the terminology used for describing the distinct ANCA patterns should be re-addressed in terms of harmonization.

Rapidly progressive glomerulonephritis and pulmonary alveolar hemorrhage are well recognized as clinical manifestations that require early diagnosis and appropriate treatment. This may be facilitated by STAT ANCA testing. For this purpose special test devices are available in multiple assay formats [19, 22, 38-40]. Nevertheless, this option is not offered to

the full extent, *i.e.*, 7 days a week, by the majority of laboratories participating in the EASI-survey. The respective clinical manifestations harbor a relatively high pre-test probability of AAV, but the pulmonary-renal syndrome is also associated with anti-GBM disease, also known as Goodpasture's disease [41]. As such, it is important that, in case of STAT ANCA testing, anti-GBM antibodies are analyzed simultaneously, because presence of these antibodies would have impact on the treatment protocol. According to our questionnaire results, inclusion of anti-GBM antibodies is not a standard protocol in about half of the participating laboratories (data not shown).

This study provides an important insight into self-reported practice in ANCA diagnostics internationally and adherence to ANCA consensus guidelines. However, the study has some limitations. The timeframe that was used for distributing the questionnaires in the participating countries was different. While most questionnaires were distributed in 2014 and 2015, this was significantly earlier in the Netherlands (2010). Obviously, the ANCA procedures and assays may have changed over time. The external quality control program in the Netherlands has observed that more laboratories have abandoned ANCA IIF over time: in 2014 this had increased from 20% to 40% [28]. This is less of an issue in the UK NEQAS programs (W. Egner, personal communication), so caution is required in generalizing, and there are many external influencers for methodological changes. Furthermore, existing methods have been altered by manufacturers in the same period, *e.g.* FEIA MPO- and PR3-ANCA were changed from a first generation to a third generation test [25]. Identifying all relevant laboratories to survey has been addressed differently in different countries by necessity. This may not have been equally comprehensive. As always in surveys, some laboratories did not answer all questions. Although this was not a major problem, for

calculation of percentages the total number of answers provided was taken as 100%. Finally, at the time the questionnaire was developed, *i.e.*, 2009-2010, some newer technologies were not yet widely available and therefore were not included in the answer options. This holds for instance for the CLIA, which appeared to be quite prevalent in Italy. However, the consensus guidelines also predate most of the newer technologies and none of these factors would affect ability to adhere to the guidance as long as ELISA is taken to include other antigen-specific immuno-assay variants. Finally, some differences between countries might be explained by national reimbursement policies and national guidelines. For instance, in Belgium testing for antigen-specific immunoassays is only reimbursed as reflex-test following a positive result in the IIF. The prevalence of individual technologies is likely to be affected by country-specific commercial factors as well.

In conclusion, there are apparent differences in ANCA diagnostics within and between countries which affect adherence to international guidelines. Technological changes may have made updating of the guidance necessary to reflect changes since publication.
The major differences observed are about the use of ANCA IIF in the algorithm, the definitions used for P-ANCA and atypical ANCA IIF pattern, the widespread use of newer technologies supplanting ELISA, the lack of availability of STAT testing, and ability to differentiate between requests in the perspective of AAV *versus* non-AAV, including gastroenterologic diseases. Harmonization of these issues may improve by two ongoing initiatives: (1) the establishment of a new international consensus on ANCA testing, and (2) the introduction of international standards for both MPO- and PR3-ANCA [42].

Acknowledgements

Dr. Caroline Roozendaal (University Medical Center Groningen, The Netherlands) is greatly acknowledged for her help in constructing the original questionnaire. Also, all the members of the national EASI-teams are acknowledged for their collaboration. In Italy EASI is integrated into the Italian Forum on Autoimmune Disease Research (FIRMA). In Switzerland the EASI activities are covered by the Commission of Laboratory Diagnostics of the Swiss Society for Allergology and Immunology (SSAI). In Spain EASI activities are coordinated by the Spanish Group of Autoimmunity (GEAI). Finally, we also thank all the laboratory specialists who returned the completed questionnaire.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: None declared.

Employment of leadership: None declared.

Honorarium: None declared.

Competing interests: None declared.

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Legends to the figures

Figure 1. Schematic overview of questionnaire distribution, response, inclusion and analysis. [1]Initially, Italy was excluded from the data of the EASI-survey, because [2]Italy addressed laboratory specialists instead of laboratories. Finally, the results were combined for analysis in the total EASI-survey. [3]Exclusion is based on ANCA testing not being performed in the respective laboratories, [4]or based on incomplete, contradicting, or duplicate responses from Italian laboratory specialists.

Figure 2. Methods used for the detection of MPO-ANCA (A) and PR3-ANCA (B) as reported by the participating laboratories in different European countries. Note that laboratories may use more than one method for antigen-specific ANCA detection; therefore total percentage may exceed 100%.

Figure 3. ANCA testing procedures as reported by the participating laboratories in different European countries. The ANCA testing algorithms (A) include: #1 screening by IIF and if positive testing for both MPO- and PR3-ANCA (* minimal consensus requirement), #2 screening by IIF and if positive, depending on the pattern, testing for either MPO- or PR3-ANCA, #3 testing all samples by IIF as well as MPO- and PR3-ANCA (** optimal consensus requirement), #4 MPO- and PR3-ANCA without IIF, #5 MPO- and PR3-ANCA and if positive also IIF, #6 other. Panel B represents the availability of STAT ANCA testing; panel C illustrates the possibility to discriminate ANCA requests from the gastroenterology department and to what extend this has consequences for the ANCA testing strategy.

Figure 4. Methods used for follow-up of AAV patients with MPO-ANCA (A) or PR3-ANCA (B) as reported by the participating laboratories in different European countries. Note that

laboratories may use more than one method for follow-up; therefore total percentage may exceed 100%.