From ANA-screening to antigen-specificity: an EASI-survey on the daily practice in European countries

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Abstract

Objective
One of the main goals of the European Autoimmunity Standardisation Initiative (EASI) is the harmonisation of test-algorithms for autoantibodies related to systemic autoimmune rheumatic diseases (SARD).

Methods
A questionnaire was used to gather information on methodology, interpretation, and the algorithm for detection of anti-nuclear antibodies (ANA) in relation to their antigen-specificity. The questionnaire was sent to 1200 laboratories in 12 European countries.

Results
The response rate was 47.2%. The results reveal not only apparent differences between countries, but also within countries.

Conclusion
Awareness of these differences may as such already stimulate harmonisation, but the observed differences may also direct recommendations that may further contribute to achieving the EASI goal of harmonisation of autoimmune diagnostics for SARD.

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Introduction

Testing for anti-nuclear antibodies (ANA), in relation with tests for antibodies to dsDNA and extractable nuclear antigens (ENA), is an important tool in the diagnosis of systemic autoimmune rheumatic diseases (SARD). ANA are traditionally detected by indirect immunofluorescence (IIF) and can be considered either as part of classification criteria, like systemic lupus erythematosus (1, 2), or as screening-test for samples that require further testing for antigen-specificity (3). Testing for ANA is also often used to exclude presence of SARD, rather than select cases that are worthy of further investigation. Recently, the IIF has been reinforced by the American College of Rheumatology (ACR) as the gold-standard for ANA detection (4). However, many new methodologies for autoantibody detection, in particular for ANA, but also for anti-dsDNA and anti-ENA antibodies, are increasingly applied in clinical laboratories. This further increases the need for standardisation of autoantibody testing in SARD.

In 2002 the European Autoantibody Standardisation Initiative (EASI) was founded in order to improve diagnostics in SARD. The goals of EASI are threefold:

1. improvement of the communication between clinicians and laboratory specialists
2. standardisation of methodology, tests, and interpretation of results
3. harmonisation of test algorithms (5).

To achieve these goals, an international EASI-group operates in close collaboration with national EASI-teams (5, 6). The Dutch EASI-team developed a questionnaire in order to gather information on the methodology, interpretation, and algorithm for detection of ANA, anti-dsDNA, and anti-ENA antibodies (7). The next step was to translate this questionnaire into English, which was then used by national EASI-teams of 11 other European countries. The current study summarises the outcome of these questionnaires and enables the identification of national differences in testing for ANA and related autoantibodies.

Methods

The questionnaire with respective answer-options was developed by Liesbeth Bakker-Jonges (Medical Laboratories, Reinier de Graaf Gasthuis, Delft, The Netherlands) and Jan Damoiseaux (Central Diagnostic Laboratories, Maastricht University Medical Center, Maastricht, The Netherlands). The questionnaire was commented on by the Dutch EASI-team, consisting of 3 clinicians and 6 laboratory specialists (6). After revision the questionnaire was translated in English and distributed to the other national EASI-teams. In some cases, the English version of the questionnaire was translated into the language of the respective country with the explicit request not to change or leave out any questions and answer-options. In total the questionnaire consisted of 57 questions in 5 categories: laboratory organisation (n=4), ANA-testing (n=14), anti-dsDNA antibody testing (n=8), anti-ENA antibody testing (n=15), and the algorithm in use (n=16). The complete questionnaire can be found on www.easi-network.com. Questionnaires were distributed to laboratories considered to be involved in ANA-testing, primarily because of participation in national external quality assessment programmes. Data were gathered by the national EASI-teams, summarised in a standard Excel-file, and sent to the coordinator of the study (JD).

The results are reported as absolute numbers, *i.e.* number of laboratories, and percentages. As indicated, the percentages refer to the total number of responding laboratories of the total study or the respective country, or to the subgroup of analysis, *i.e.* the number of laboratories that perform ANA, anti-dsDNA, or anti-ENA antibodies, respectively.

Results

Response on questionnaire
and type of participating laboratories
The data on response and type of participating laboratories per country are summarised in Table I. In total, the questionnaire was distributed among 1200 laboratories in 12 European countries. The number of laboratories addressed strongly varied per country

Table I. Response on questionnaire and type of participating laboratory per country.

	Austria	Belgium	Finland	France	Israel	Italy	Netherlands	Norway	Portugal	Sweden	Switzerland	Ukraine
Laboratories[1]	51	130	7	290	18	400	76	12	160	23	29	5
Participation[2]	36 (70.6%)	127 (97.7%)	6 (85.7%)	45 (15.5%)	15 (83.3%)	149 (37.3%)	66 (86.8%)	6 (50.0%)	67 (41.9%)	18 (78.3%)	26 (89.7%)	5 (100%)
University[3]	7 (19.4%)	8 (6.3%)	4 (66.7%)	13 (28.9%)	8 (53.5%)	10 (6.7%)	7 (10.6%)	3 (50.0%)	7 (10.4%)	8 (44.4%)	6 (23.1%)	3 (60.0%)
Non-university[3,4]	13 (36.1%)	80 (63.0%)	-	13 (28.9%)	1 (6.7%)	110 (73.8%)	50 (75.8%)	3 (50.0%)	23 (34.3%)	8 (44.4%)	8 (30.8%)	-
Private[3,5]	13 (36.1%)	39 (30.7%)	2 (33.3%)	19 (42.2%)	6 (40.0%)	15 (10.1%)	3 (4.5%)	-	32 (47.8%)	2 (11.2%)	12 (46.2%)	2 (40.0)
Other[3]	3 (8.3%)	-	-	-	-	14 (9.4%)	6 (9.1%)	-	5 (7.5%)	-	-	-
Accreditation[3]	24 (66.7%)	24 (18.9%)	6 (100%)	13 (28.9%)	13 (86.7%)	104 (69.8%)	59 (89.4%)	2 (33.3%)	35 (52.2%)	18 (100%)	20 (76.9%)	5 (100%)

[1] Number of laboratories that were addressed.
[2] Number of laboratories that responded (percentage of laboratories that were addressed).
[3] Number of laboratories (percentage of laboratories that responded).
[4] Non-university refers to laboratories within hospitals (public or private) not directly associated with a medical university.
[5] Private refers to laboratories not directly associated with a hospital.

with a range of 5 (Ukraine) to 400 (Italy). In total 566 laboratories responded, revealing an overall response rate of 47.2% with a range of 15.5% (France) to 100% (Ukraine). Because the results presented are those as obtained from the responding laboratories and participation was not 100%, the country data given below refer to the participating laboratories and not the country as a whole.

The participating laboratories were either associated with university hospitals (n=84; 14.8%) or laboratories within hospitals (public or private) not directly associated with a medical university (n=309; 54.6%), or were private laboratories not directly associated with a hospital (n=145; 25.6%). The remaining laboratories were categorised as "other" (n=28; 4.9%). The distribution differed between countries. University laboratories were less prevalent in Belgium (n=8; 6.3%), Italy (n=10; 6.7%), the Netherlands (n=7; 10.6%), and Portugal (n=7; 10.4%). Non-university hospital laboratories were absent in Finland and Ukraine; in Israel only 1 laboratory (6.7%) belonged to this category. Private laboratories were less prevalent in Italy (n=15; 10.1%), the Netherlands (n=3; 4.5%), Norway (n=0; 0%), and Sweden (n=2; 11.2%). An accreditation status was available in 323 laboratories (57.1%), ranging from 18.9% (Belgium) to 100% (Finland, Sweden, and Ukraine).

ANA testing
Data on ANA testing per country are summarised in Table II. In total 494 participating laboratories (87.3%) perform ANA testing by the IIF technique. However, in some countries, the number of participating laboratories that do perform ANA testing by IIF is limited: in particular, in the Netherlands (n=43; 65.2%), Norway (n=3; 50%), Portugal (n=43; 64.2%), and Ukraine (n=2; 40%). In most of the laboratories reading of the ANA slides is performed by 2 observers, but in 176 laboratories (35.6%) primarily by 1 observer. Reading by one observer was common in Portugal and Sweden (above 60%), but was not reported in Finland and Ukraine at all.
The screening titer for ANA testing varies considerably between participating laboratories that perform ANA by IIF.

Overall, the 1/80 dilution is used most often (n=299; 60.5%). The 1/40 dilution is used in 77 laboratories (15.6%) and this is relatively most prevalent in the participating laboratories of Israel (n=4; 28.6%), the Netherlands (n=17; 39.5%), Norway (n=1; 33.3%), and Switzerland (n=8; 32.0%). On the other hand, the 1/160 dilution is used in 74 laboratories (15.0%) and this is relatively most prevalent in the participating laboratories of Israel (n=5; 35.7%), Norway (n=1; 33.3%), and Portugal (n=22; 51.2%). Most laboratories that use IIF for ANA detection perform a titration on samples with a positive ANA (n=424; 85.8%). This is less frequently performed in the participating laboratories of the Netherlands (n=15; 34.9%). Interestingly, 484 out of the participating 494 laboratories (98.0%) that determine ANA by IIF also discriminate distinct fluorescence patterns. The most apparent patterns, like homogenous, speckled, centromere, and nucleolar are distinguished in these laboratories. Depending on the source of the slides (HEp-2000), also an SSA-pattern or atypical speckled pattern is distinguished. While in most countries the

Table II. ANA testing.

	Austria	Belgium	Finland	France	Israel	Italy	Netherlands	Norway	Portugal	Sweden	Switzerland	Ukraine
Laboratories[1]	36	127	6	45	15	149	66	6	67	18	26	5
ANA by IIF[2]	34 (94.4%)	124 (97.6%)	6 (100%)	45 (100%)	14 (93.3%)	137 (91.9%)	43 (65.2%)	3 (50.0%)	43 (64.2%)	18 (100%)	25 (96.2%)	2 (40.0%)
1 observer only[3]	13 (38.2%)	48 (38.7%)	0 (0%)	10 (22.5%)	5 (35.7%)	54 (39.4%)	2 (4.7%)	1 (33.3%)	27 (62.8%)	11 (61.1%)	5 (20.0%)	0 (0%)
1/40 screening[3]	6 (17.6%)	16 (12.9%)	0 (0%)	1 (2.2%)	4 (28.6%)	20 (14.6%)	17 (39.5%)	1 (33.3%)	3 (7.0%)	1 (5.6%)	8 (32.0%)	0 (0%)
1/80 screening[3]	13 (38.2%)	83 (66.9%)	3 (50.0%)	34 (75.6%)	4 (28.6%)	103 (75.2%)	23 (53.5%)	0 (0%)	14 (32.6%)	5 (27.8%)	15 (60.0%)	2 (100%)
1/160 screening[3]	9 (26.5%)	16 (12.9%)	0 (0%)	9 (20.0%)	5 (35.7%)	9 (6.6%)	0 (0%)	1 (33.3%)	22 (51.2%)	1 (5.6%)	2 (8.0%)	0 (0%)
Titration[3]	32 (94.1%)	117 (94.4%)	6 (100%)	45 (100%)	10 (71.4%)	124 (90.5%)	15 (34.9%)	3 (100%)	34 (79.1%)	12 (66.7%)	24 (96.0%)	2 (100%)
Pattern typing[3]	32 (94.1%)	123 (99.2%)	6 (100%)	45 (100%)	12 (85.7%)	137 (100%)	43 (100%)	2 (66.7%)	39 (90.7%)	18 (100%)	25 (100%)	2 (100%)

[1] Number of laboratories that responded.
[2] Number of laboratories (percentage of responding laboratories) that perform ANA testing by indirect immunofluorescence.
[3] Number of laboratories (percentage of laboratories that perform ANA testing by indirect immunofluorescence.

Table III. Anti-dsDNA antibody testing.

	Austria	Belgium	Finland	France	Israel	Italy	Netherlands	Norway	Portugal	Sweden	Switzerland	Ukraine
Laboratories[1]	36	127	6	45	15	149	66	6	67	18	26	5
Anti-dsDNA ab[2]	31 (86.1%)	113 (89.0%)	5 (83.3%)	43 (95.6%)	13 (87.7%)	138 (92.6%)	63 (95.5%)	6 (100%)	62 (92.5%)	18 (100%)	23 (88.5%)	5 (100%)
Technique:												
CLIFT[3]	12 (38.7%)	30 (26.5%)	4 (80.0%)	8 (18.6%)	4 (30.8%)	112 (81.2%)	17 (27.0%)	4 (66.7%)	22 (35.5%)	17 (94.4%)	9 (39.1%)	0 (0%)
Farr-assay[3]	0 (0%)	2 (1.8%)	0 (0%)	5 (11.6%)	2 (15.4%)	2 (1.4%)	8 (12.7%)	0 (0%)	2 (3.2%)	0 (0%)	0 (0%)	0 (0%)
FEIA[3]	1 (3.2%)	28 (24.8%)	4 (80.0%)	13 (30.2%)	0 (0%)	30 (21.7%)	38 (60.3%)	5 (83.3%)	35 (56.5%)	1 (5.6%)	6 (26.1%)	0 (0%)
ELISA[3]	21 (67.7%)	49 (43.4%)	0 (0%)	22 (51.2%)	6 (46.1%)	53 (38.4%)	5 (7.9%)	1 (16.7%)	17 (27.4%)	2 (11.1%)	12 (52.2%)	4 (80.0%)
Other[3]	2 (6.5%)	26 (23.0%)	0 (0%)	8 (18.6%)	3 (23.1%)	13 (9.4%)	4 (6.3%)	0 (0%)	5 (8.1%)	3 (16.7%)	2 (8.7%)	1 (20.0%)
>1 techniques[4]	5 (16.1%)	22 (19.5%)	3 (60.0%)	13 (30.2%)	2 (15.4%)	89 (64.5%)	9 (14.3%)	4 (66.7%)	19 (30.6%)	5 (27.8%)	6 (26.1%)	0 (0%)
Reporting:												
Qualitative[5]	3 (9.7%)	32 (28.3%)	0 (0%)	3 (7.0%)	0 (0%)	11 (8.0%)	16 (25.4%)	0 (0%)	13 (21.0%)	0 (0%)	3 (13.0%)	0 (0%)
Semi-qualitative[5]	7 (22.6%)	23 (20.4%)	1 (20.0%)	3 (7.0%)	2 (15.4%)	49 (35.5%)	6 (9.5%)	1 (16.7%)	7 (11.3%)	13 (72.2%)	6 (26.1%)	0 (0%)
Quantitative[5]	21 (67.7%)	46 (40.7%)	4 (80.0%)	37 (86.0%)	11 (84.6%)	78 (56.5%)	35 (55.6%)	6 (100%)	49 (79.0%)	5 (27.8%)	14 (60.9%)	5 (100%)

[1] Number of laboratories that responded.
[2] Number of laboratories (percentage of responding laboratories) that perform anti-dsDNA antibody testing.
[3] Number of laboratories that detect anti-dsDNA antibodies by the respective test (percentage of laboratories that perform anti-dsDNA antibodies).
[4] Number of laboratories that perform >1 technique for the detection of anti-dsDNA antibodies (percentage of laboratories that perform anti-dsDNA antibodies).
[5] Number of laboratories that report results qualitative, semi-quantitative, or quantitative, respectively (percentage of laboratories that perform anti-dsDNA antibodies).

participating laboratories also identify cytoplasmic staining (n=418; 84.6%), this is less prevalent in the Netherlands (n=26; 60.5%). Other, miscellaneous patterns are reported by 197 participating laboratories (39.9%). This is quite similar between the participating laboratories with the exception of, on the one hand, Norway (n=0; 0%) and Ukraine (n=0; 0%), and, on the other hand, Sweden (n=17; 94.4%) (data not shown).

Anti-dsDNA antibody testing
The data on anti-dsDNA antibody testing per country are summarised in Table III. In total, 520 of the participating laboratories (91.9%) detect anti-dsDNA antibodies. The percentage of participating laboratories that perform this analysis varies from 83.3% (Finland) to 100% (Norway, Sweden, and Ukraine). There is a wide variation between the participating laboratories on methodologies used for the detection of anti-dsDNA antibodies. IIF on *Crithidia luciliae* (CLIFT) is, except in Ukraine, quite prevalent in use and varies from 18.6% (France) to 94.4% (Sweden). The Farr-assay, however, is not available in the participating laboratories of most countries and has a low prevalence in the other countries, ranging from 1.4% (Italy) to 15.4% (Israel). Fluorescent-enzyme immunoassays (FEIA) are relatively highest represented in Finland (n=4; 80%), the Netherlands (n=38; 60.3%), Norway

(n=5; 83.3%), and Portugal (n=35; 56.5%), while enzyme-linked immunosorbent assays (ELISA) are relatively best represented in Austria (n=21; 67.7%), France (n=22; 51.2%), Switzerland (n=12; 52.2%), and Ukraine (n=4; 80.0%). With respect to other techniques employed, in particular in Belgium 23 laboratories (20.4%) use dot-blots for the detection of anti-dsDNA antibodies. Interestingly, a substantial number of participating laboratories (n=177; 34.0%) has multiple techniques available for the detection of anti-dsDNA antibodies. Whether all techniques are routinely applied to all samples was not questioned.

In most countries quantitative reporting of results is common. Only in Sweden the majority of participating laboratories reports semi-quantitatively (n=13; 72.2%), and this is associated with the relatively high number of laboratories that perform CLIFT (n=17; 94.4%). Qualitative reporting of results for anti-dsDNA antibodies is most prevalent in participating laboratories of Belgium (n=32; 28.3%), the Netherlands (n=16; 25.4%), and Portugal (n=13; 21.0%).

Anti-ENA antibody testing
The data on anti-ENA antibody testing per country are summarised in Figure 1 and Table IV. In total, 517 of the participating laboratories (91.3%) detect anti-ENA antibodies. Except for Ukraine (n=3; 60%), there is little variation in percentage of participating laboratories

performing this test within each country (range: 83.6–100%). Like for anti-dsDNA antibodies, also for anti-ENA antibodies there is a wide variation in methodologies used for determining ENA-specificities (Fig. 1). Anti-ENA antibody typing by ELISA is relatively most prevalent in participating laboratories of Austria (n=20; 62.5%), Italy (n=70; 50.4%), and Ukraine (n=2; 66.6%). In Sweden the line immunoassay (LIA) is relatively most prevalent (n=10; 58.8%), but this method is also commonly used in the participating laboratories of Austria (n=14; 43.8%) and Finland (n=3; 50%). Dot-blots, on the other hand, are primarily used in Belgium (n=58; 50.0%), and to a lesser extend in France (n=15; 34.1%) and Switzerland (n=8; 34.8%). As is the case for anti-dsDNA antibodies, FEIA is the method of choice in the participating laboratories of Finland (n=6; 100%), the Netherlands (n=38; 61.3%), Norway (n=5; 83.3%), and Portugal (n=31; 55.4%). Israel is the only country where addressable laser-bead immuno-assays (ALBIA) predominates (n=5; 62.5%) in the participating laboratories, although this method is also common in France (n=9; 20.5%). Finally, with respect to other methodologies, it is interesting to mention that in Sweden immuno-diffusion techniques are commonly available (n=6; 35.3%), while in Italy chemiluminescent immuno-assays (CLIA) are quite common (n=31; 22.0%. Out of the 517 labora-
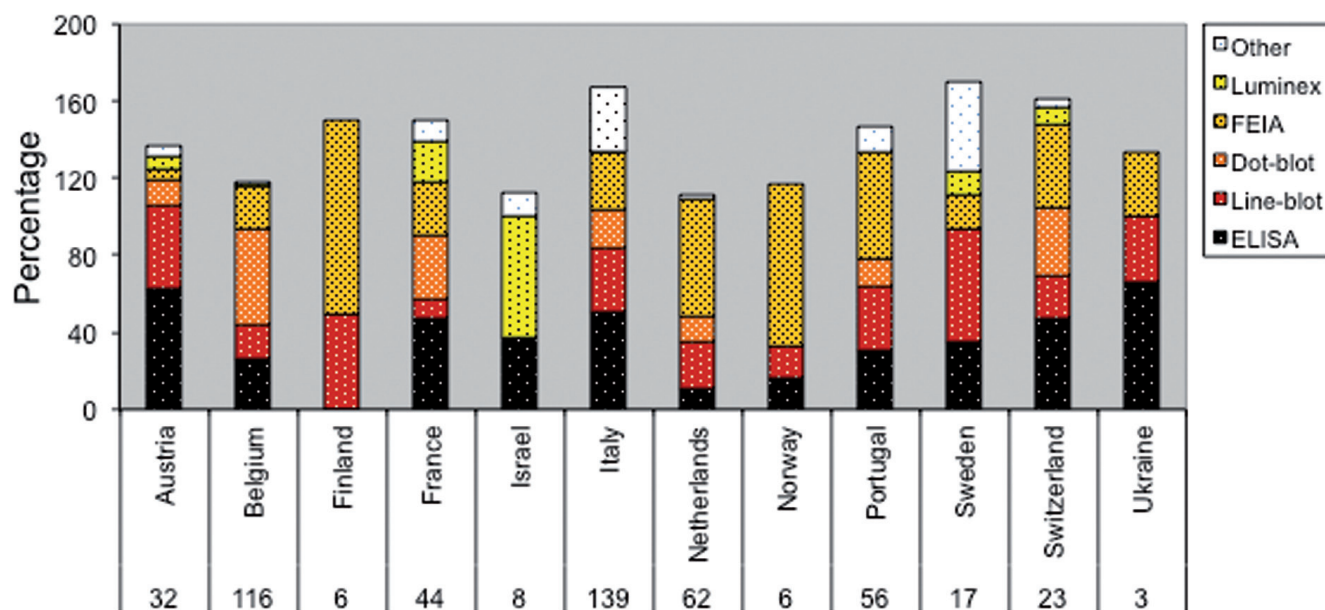
Fig. 1. Methods used for the detection of anti-ENA antibodies as reported by the participating laboratories in different European countries.
Note that about one-third of the laboratories use >1 technique for detection of anti-ENA antibodies (Table IV); therefore total percentages exceed 100%.

Table IV. Anti-ENA antibody testing.

	Austria	Belgium	Finland	France	Israel	Italy	Netherlands	Norway	Portugal	Sweden	Switzerland	Ukraine
Laboratories[1]	36	127	6	45	15	149	66	6	67	18	26	5
Anti-ENA ab[2]	32 (88.9%)	116 (91.3%)	6 (100%)	44 (97.8%)	13 (87.7%)[7]	139 (93.2%)	62 (93.9%)	6 (100%)	56 (83.6%)	17 (94.4%)	23 (88.5%)	3 (60.0%)
>1 techniques[3]	12 (37.5%)	21 (18.1%)	3 (50.0%)	22 (50.0%)	1 (12.5%)[7]	93 (67.3%)	7 (11.3%)	1 (16.7%)	26 (46.4%)	12 (70.6%)	14 (60.8%)	1 (33.3%)
CENP-B[4]	30 (93.8%)	92 (79.3%)	6 (100%)	29 (65.9%)	13 (87.7%)[7]	100 (71.9%)	52 (83.9%)	6 (100%)	47 (83.9%)	8 (47.1%)	18 (78.3%)	2 (67.7%)
Other[5]	8 (25.0%)	48 (41.4%)	3 (50.0%)	13 (29.5%)	0 (0%)[7]	25 (18.0%)	8 (12.9%)	1 (16.7%)	10 (17.9%)	3 (17.6%)	11 (47.8%)	1 (33.3%)
SSA60 vs Ro52												
Detection[6]	12 (37.5%)	54 (46.6%)	3 (50.0%)	21 (47.7%)	4 (50.0%)[7]	55 (39.6%)	17 (27.4%)	1 (16.7%)	18 (32.1%)	14 (82.4%)	9 (39.1%)	0 (0%)
Reporting[6]	6 (18.8%)	31 (26.7%)	3 (50.0%)	10 (22.7%)	NR[7]	42 (30.2%)	8 (12.9%)	1 (16.7%)	9 (16.1%)	5 (29.4%)	4 (17.4%)	0 (0%)

[1] Number of laboratories that responded.
[2] Number of laboratories (percentage of responding laboratories) that perform anti-ENA antibody testing.
[3] Number of laboratories that perform >1 technique for the detection of anti-ENA antibodies (percentage of laboratories that detect anti-ENA antibodies)[7].
[4] Number of laboratories that specifically type for anti-CENP-B antibodies (percentage of laboratories that detect anti-ENA antibodies)[7].
[5] Number of laboratories that specifically type for anti-ENA specificities other than SS-A/Ro, SS-B/La, Sm, RNP, CENP-B, Scl-70, and Jo-1 (percentage of laboratories that detect anti-ENA antibodies)[7].
[6] Number of laboratories that distinguish between anti-SSA60 and anti-Ro52 antibodies in their detection and reporting, respectively (percentage of laboratories that detect anti-ENA antibodies)[7].
[7] Only 8 out of 13 Israeli laboratories responded on follow-up questions about anti-ENA antibodies; therefore percentages of follow-up question refer to 8 instead of 13 laboratories
(NR: not reported).

tories that detect anti-ENA antibodies, 213 participating laboratories (41.2%) use more than one method. This varies from 11.3% (the Netherlands) to 70.6% (Sweden). It was not questioned if multiple methods were routinely used for all samples.

When it comes to the ENA available in the typing-assays it is apparent that basically all the participating laboratories test for antibodies to SS-A/Ro, SS-B/La, Sm, and RNP. Overall, a minority of laboratories (n=22; 4.3%) do not test for antibodies to Scl-70 and Jo-1. This is most apparent in the Netherlands (n=5; 8.1%) and Portugal (n=5; 8.9%). There

is more variation with respect to typing for anti-CENP-B antibodies. In total 403 laboratories (77.9%) test for antibodies to CENP-B. This is in particular less common in participating laboratories of France (n=29; 65.9%), Sweden (n=8; 47.1%), and Ukraine (n=2; 67.7%). Beside these antigen-specificities, some laboratories (n=131; 25.3%) test for additional ENA-specificities. Basically, this is a minority of the participating laboratories within each country (range: 0–50%). With respect to the distinction between SSA60 and Ro52 antibodies it appears that 208 of the participating laboratories (40.2%) discriminate in their

test-systems between both specificities, while only 119 laboratories (23.0%) also report both entities separately to the clinician. Typically, in almost all countries about half of the participating laboratories that make this distinction also report as such.

The algorithm for ANA, anti-dsDNA, and anti-ENA antibody testing
The questions on the algorithm for autoantibody testing addressed the issue if ANA are considered as an essential screening test, leading automatically to refusing (in case of a negative ANA) or adding (in case of a positive ANA)

Table V. Algorithm for ANA, anti-dsDNA, and anti-ENA antibody testing.

	Austria	Belgium	Finland	France	Israel	Italy	Netherlands	Norway	Portugal	Sweden	Switzerland	Ukraine
Laboratories[1]	36	127	6	45	15	149	66	6	67	18	26	5
if ANA neg[2]	7 (22.6%)	112 (99.1%)	0 (0%)	13 (30.2%)	3 (23.1%)	44 (32.1%)	11 (17.5%)	3 (50.0%)	14 (22.6%)	0 (0%)	2 (8.7%)	4 (80.0%)
if ANA neg[3]	10 (31.3%)	114 (98.3%)	0 (0%)	8 (18.2%)	1 (12.5%)[8]	23 (16.7%)	21 (33.9%)	1 (16.7%)	23 (41.1%)	0 (0%)	0 (0%)	2 (66.7%)
dsDNA if ANA pos[4]	19 (61.3%)	89 (78.8%)	1 (20.0%)	43 (100%)	NR	56 (40.6%)	36 (57.1%)	4 (66.7%)	26 (41.9%)	14 (77.8%)	19 (82.6%)	0 (0%)
ENA if ANA pos[5]	27 (84.4%)	100 (86.2%)	1 (16.7%)	40 (90.1%)	NR	34 (24.6%)	41 (66.1%)	3 (50.0%)	33 (58.9%)	15 (88.2%)	16 (69.6%)	0 (0%)
ANA if dsDNA req[6]	13 (41.9%)	74 (65.5%)	0 (0%)	18 (41.9%)	NR	4 (2.9%)	10 (16.1%)	2 (33.3%)	26 (41.9%)	0 (0%)	5 (21.7%)	0 (0%)
ANA if ENA req[7]	15 (46.9%)	79 (68.1%)	0 (0%)	17 (38.6%)	NR	19 (13.7%)	17 (27.4%)	3 (50.0%)	28 (50.0%)	2 (11.8%)	4 (17.4%)	0 (0%)

[1] Number of laboratories that responded.
[2] Number of laboratories (percentage of laboratories that detect anti-dsDNA antibodies) that refuse an anti-dsDNA antibody test if ANA is negative.
[3] Number of laboratories (percentage of laboratories that detect anti-ENA antibodies[7]) that refuse an anti-ENA antibody test if ANA is negative.
[4] Number of laboratories (percentage of laboratories that detect anti-dsDNA antibodies) that add an anti-dsDNA antibody test if ANA is positive.
[5] Number of laboratories (percentage of laboratories that detect anti-ENA antibodies[7]) that add an anti-ENA antibody test if ANA is positive.
[6] Number of laboratories (percentage of laboratories that detect anti-dsDNA antibodies) that add an ANA test if anti-dsDNA antibodies are requested.
[7] Number of laboratories (percentage of laboratories that detect anti-ENA antibodies) that add an ANA test if anti-ENA antibodies are requested.
[8] Only 8 out of 13 Israeli laboratories responded on follow-up questions about anti-ENA antibodies; therefore percentages of follow-up question refer to 8 instead of 13 laboratories (NR: not reported/reported as not relevant).

follow-up tests for anti-dsDNA and anti-ENA antibodies (Table V). Of note, most questions were reported as not relevant by the Israeli laboratories. It appears that if ANA IIF is negative 213 of the participating laboratories performing ANA IIF (43.1%) refuse further testing for anti-dsDNA antibodies. This is strongly dominated by Belgium (n=112; 99.1%). Similarly, 180 of the participating laboratories performing ANA IIF (41.1%) do not allow testing for anti-ENA antibodies in case of a negative ANA result. Again, this approach is by far most apparent in Belgium (n=114; 98.3%). In particular in the participating laboratories of Finland, Sweden, and Switzerland requests for anti-dsDNA and anti-ENA antibodies are (hardly) not refused if ANA IIF is negative. On the other hand, if ANA IIF is positive, reflex testing for anti-dsDNA (n=307; 64.0%) and anti-ENA (n=310; 64.6%) antibodies is quite common in the participating laboratories performing ANA IIF of most countries, except Finland and Ukraine. Finally, the responses revealed that, in case of a request for anti-dsDNA or anti-ENA antibodies, ANA IIF was added in 152 (31.7%) and 184 (38.3%) of the participating laboratories performing ANA IIF, respectively. Both approaches were most common in Belgium (n=74; 65.5% and n=79; 68.1%, respectively), while not apparent in Finland and Ukraine.

Discussion

In the current study we have presented the results of a questionnaire on testing for ANA, anti-dsDNA, and anti-ENA antibodies in 12 European countries. This survey was initiated by EASI in order to ultimately harmonise autoimmune diagnostics for SARD (5). Although some countries, like Finland, Norway, and Ukraine, participated with only few laboratories, the results reveal not only apparent differences between countries, but also within countries.

Although a matter of discussion (8-10), it is still advocated that IIF remains the gold-standard for ANA-testing (4). The choice for IIF is primarily based on the inclusion of ANA in the classification criteria of systemic lupus erythematosus (SLE) (1, 2). Since ANA are considered to be present in almost 100% of the SLE patients, a negative ANA result has a very high negative predictive value. However, this is at the cost of low specificity especially when low titers are being used for screening (11). Our survey revealed that the majority of the participating laboratories screens for ANA in a 1/80 dilution, or one two-fold dilution step below or above 1/80. Although it should be realised that a positive test result not only depends on the serum dilution, but also on the substrate, the conjugate, as well as the microscope, a higher dilution may negatively impact on the suspected high negative predictive value for SLE. On the other hand, while ANA by IIF may be the optimal choice for SLE, this may not be the case for other SARD. For example, antibodies to SSA/Ro and Jo-1, associated with Sjögren's syndrome and myositis, respectively, might reveal negative ANA results. Antigen-specific assays might have better test charac-

teristics for these SARD (12, 13). If a laboratory uses an ENA screening system that employs a limited number of autoantigens, instead of ANA by IIF, the limitations of the test should be known by the ordering clinicians. Indeed, false negative results may be obtained with this kind of tests due to the fact that relevant antigens are lacking or are present in too low concentration to be detected. These differences in assays further emphasise the necessity to report the method of choice to the ordering clinician.

In case of anti-dsDNA antibodies, the Farr-assay is considered to be the gold-standard (14). This technology appears to be only minimally available in the diagnostic laboratories of the participating countries. While the CLIFT might be second choice because of the high specificity, this test is available in about one-third of the laboratories only. The test characteristics of the Farr-assay and CLIFT, however, do not per definition outperform those of other technologies (15-17). Importantly, since anti-dsDNA antibodies might be relevant for the follow-up of SLE patients (14, 15), this is only worthwhile if results are presented in a quantitative way. This happens not to be the case in a substantial number of laboratories in Belgium, the Netherlands, and Portugal. In Belgium this seems to be associated with the relatively high number of laboratories that utilise a dot-blot for the detection of anti-dsDNA antibodies. Differences between countries with respect to the use of distinct techniques for the detection of anti-dsDNA and

anti-ENA antibodies may primarily be the result of the market position in the respective countries and/or the origin of diagnostic companies.

SSA/Ro is, together with SSB/La, part of a ribonucleoprotein. At first SSA/Ro was considered to consist out of a 60 kDa unit (SSA60) and a 52 kDa unit (Ro52), but the latter component, also known as TRIM21, recently appeared not to be part of the complex (18). In SARD autoantibodies to both SSA60 as well as Ro52 are prevalent, but the clinical significance is clearly different (18). Both these antibodies, but in particular anti-Ro52 antibodies, may cause neonatal lupus and/or congenital heart block (19). Furthermore, they are associated with Sjögren's syndrome and the respective (preliminary) classification criteria do not discriminate between both entities (20). It has been reported that distinction of anti-Ro52 antibodies does influence the classification and clinical characterisation of Sjögren's syndrome (21). The main difference is observed in patients with myositis and systemic sclerosis that have a high prevalence of anti-Ro52 antibodies, but not anti-SSA60 antibodies (18). In subacute cutaneous lupus erythematosus, however, the situation is inversed (22). In this respect it is noticeable that only about 20–25% of the laboratories report these two antibodies as separate entities.

While it is obvious that there is a clear relation between ANA, anti-dsDNA, and anti-ENA antibodies (3), this is not as evident from the test-algorithms used in the laboratories. Anti-dsDNA antibody screening is recommended by the ACR, based on an extensive literature search, to be reserved for patients who have a positive ANA (15). This typically holds for situations where ANA-testing is performed in a setting that reveals high sensitivity, *i.e.* high negative predictive value. On the other hand, it becomes more and more clear that ANA-testing does not cover the full spectrum of anti-ENA antibodies (12, 13). Our results reveal that more than half of the laboratories test for anti-dsDNA antibodies even if ANA are negative (as not recommended by the ACR) (15), and also about half of the laboratories

refuse testing for anti-ENA antibodies if ANA are negative (despite detection limits). The other way around, *i.e.* reflex testing if ANA are positive, is more common (70–80%), but quite heterogeneous between and within countries. In some countries, like Belgium or Finland, there seems to be substantial harmonisation between test-algorithms, but this might primarily be the result of reimbursement policies instead of consisting evidence.

Altogether, the results presented in the current study reveal a snapshot of how ANA, anti-dsDNA, and anti-ENA antibodies are being tested and reported in 12 European countries. In the Netherlands the Dutch results have been published in combination with 15 recommendations formulated by the national EASI-team (7). As a follow-up, the international EASI group has recently formulated 25 recommendations on ANA, anti-dsDNA, and anti-ENA antibodies (23). Although many experts in the field support these recommendations, they do not have the status of criteria. Nevertheless they may help to harmonise test-algorithms for the use of these autoantibodies in the diagnosis of SARD. They even may be of help to change reimbursement policies within countries. Obviously, the recommendations are based on the methodologies currently in use in the diagnostic laboratories. Novel technologies, and in particular additional antigen specificities related to SARD (24-26), may emerge and become integrated in the fast changing world of laboratory diagnostics. Future questionnaires will be needed to evaluate the implementation of the current recommendations (23), and the need for additional recommendations.

Acknowledgement

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