Autoantibody profiles in systemic sclerosis; a comparison of diagnostic tests

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ORIGINAL ARTICLE

Autoantibody profiles in systemic sclerosis; a comparison of diagnostic tests

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ABSTRACT

Objectives: Autoimmune antibody profiling plays a prominent role in both classification and prognosis of systemic sclerosis (SSc). In the last years novel autoantibodies have been discovered and have become available in diagnostic assays. However, standardization in autoimmune serology is lacking, which may have a negative impact on the added value of autoantibodies in diagnosis and prognosis of SSc. In this paper we describe the comparison of commercially available diagnostic assays for the detection of SSc-associated autoantibodies and explored the coexistence of multiple SSc-associated autoantibodies within patients.

Methods: Serum samples of 347 patients from the Nijmegen Systemic Sclerosis Cohort were included in this study. All patients fulfilled the ACR/EULAR 2013 classification criteria for SSc and were classified as DcSSc or LcSSc according to the Leroy and Medsger criteria. All samples were evaluated on standard laboratory diagnostic tests for detection of SSc-specific autoantibodies CENPA and CENPB (ACA), Scl-70 (ATA), RNA Polymerase III (rp11/155) (ARA), and SSc-associated autoantibodies Fibrillarin, Th-To, PM-scl75, PM-Scl100, RNP68/A/C, Ku, NOR90, and PDGFR from suppliers EUROIMMUN, D-tek and Thermo Fisher Scientific.

Results: We found that 79% of the patients was positive for one or more of the SSc autoantibodies. Overall, a high agreement was observed between the diagnostic methods for the SSc-specific autoantibodies listed in the ACR/EULAR criteria (ATA, ACA, and ARA) (Cohen's *kappa* 0.53–0.97). However, a lower agreement was found for SSc-associated autoantibodies PM-Scl, and Ku, as well as for the SSc-specific autoantibodies fibrillarin and Th-To. Furthermore, the data revealed that the presence of ATA, ARA and ACA is predominantly mutually exclusive, with only a fraction of the patients testing positive for both ATA and ARA.

Conclusion: Our data showed high concordance of prevalent SSc-specific autoantibodies between different diagnostic assays. Further standardisation for low prevalent SSc-specific and SSc-associated autoantibodies is needed.

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Autoantibody; diagnostic test; systemic sclerosis; clinical study; standardization

Introduction

Systemic sclerosis (SSc) is a chronic autoimmune disease characterized by inflammation, vasculopathy and fibrosis of the skin and internal organs. SSc can be subclassified in two major subtypes: limited cutaneous SSc (LcSSc) and diffuse cutaneous SSc (DcSSc), based on the extent of skin thickening [1]. DcSSc is associated with more severe organ involvement of the lungs and heart although in LcSSc this is not uncommon. Internal organ involvement is associated with a high morbidity and premature death [2]. To date, no cure is available for SSc but treatment with immunosuppressive drugs can increase the quality of life, decrease progression of organ involvement and increase survival [3]. SSc is a

heterogeneous disease and therefore prediction of disease progression and complications is challenging. For both the patient and the clinician the uncertainty of the disease course and possible development of severe complications is a major concern. Although strong prognostic factors are lacking, autoantibodies have shown to be associated with disease course and occurrence of complications. Anti-centromere (ACA), anti-topoisomerase, also called anti-Scl-70 (ATA) and anti-RNA polymerase III (ARA) are the most prevalent autoantibodies and associated with a certain disease course and/or complication [4–6]. ACA is associated with LcSSc and pulmonary arterial hypertension (PAH) late in the disease course, whereas ATA is associated with

DcSSc, interstitial lung disease (ILD) and digital ulcers (DU) [7]. Patients with ARA antibodies more often have renal crisis, diffuse disease and gastric antral vascular ectasia (GAVE). For the autoantibodies with lower prevalence, the associations with disease phenotype are more difficult to demonstrate. However, some associations, were demonstrated in different studies. Anti-PM-Scl, anti-RNP68/A/C and anti-Ku are associated with overlap SSc and myositis [8]. Anti-fibrillarin autoantibodies are clinically associated with overlap SSc, myositis, joint involvement and PAH [9,10]. In the he ACR/EULAR classification criteria for SSc, the presence of ACA, ATA, and ARA contribute to the classification [11]. Since these autoantibodies are used for classification and are associated to prognosis and the occurrence of complications, it is of the utmost importance that available diagnostic assays have comparable results. Though immunoprecipitation is still perceived as the gold standard, this method is not standard of practice in most diagnostic laboratories. Line blot and enzyme linked immunoassays have shown to serve as a reliable alternative for the detection of autoantibodies. These more robust and less laborious diagnostic tests offer fast parallel (semi)quantitative detection of autoantibodies [12]. Therefore, it is important to investigate and compare the performance of different commercially available test kits. Furthermore, knowledge about the test characteristics of the other SSc-specific and -associated antibodies is essential for appropriate interpretation of these test results.

Materials and methods

Cohort enrolment

This study was performed at the Radboud University Medical Centre, Nijmegen, the Netherlands, which is a tertiary referral centre for SSc in the Netherlands. Data of 347 consecutive patients from the Nijmegen Systemic Sclerosis Cohort were included in this study [13]. All patients fulfilled the ACR/EULAR 2013 classification criteria for SSc [11].

Patients were subclassified as having DcSSc or LcSSc according to the Leroy and Medsger criteria [1]. Informed consent was obtained from all patients and study protocol was reviewed and approved by the local ethical committee. Blood samples of patients were collected at scheduled outpatient clinic visits. Serum aliquots were stored at -80°C . Disease duration was defined as time from the first non-Raynaud phenomenon. We collected the main disease complications in our cohort namely: ILD, PAH, and DU. ILD was defined as more than 20% lung involvement (fibrosis/ground glass) on High Resolution CT scan according to the Goh et al. algorithm [14]. PAH was diagnosed by right heart catheterization. DU is ulcers present distal from the PIP joint due to vasculopathy. Data from patients with current or past DU were collected. Because of the low prevalence of renal crisis, this data is not shown. Cardiac involvement is not displayed, as the diagnostics for this are currently under debate. Table 1 displays the baseline characteristics of the cohort.

Table 1. Baseline characteristics of the cohort.

	DcSSc (n = 111)	LcSSc (n = 236)
Age, mean (S.D.)	57 (11.6)	60 (12.3)
Female, n (%)	65 (59%)	161 (69%)
Disease duration in years (mean (SD))	8.17 (6.1)	8.35 (6.8)
Maximal mRSS, mean (S.D.)	18.16 (8.8)	5.27 (4.4)
ILD, n (%)	60 (54%)	85 (36%)
PAH, n (%)	8 (7%)	35 (15%)
DU, n (%)	36 (32%)	82 (35%)

DcSSc: diffuse cutaneous systemic sclerosis; LcSSc: limited cutaneous systemic sclerosis; mRSS: modified Rodnan Skin Score; PAH: pulmonary arterial hypertension; DU: digital ulcers; S.D.: standard deviation; IQR: interquartile range.

Analysis for prevalent autoantibodies

Sera were analyzed for prevalence of circulating autoantibodies using tests from three commercial suppliers, EUROIMMUN (Lübeck, Germany), D-tek (Mons, Belgium) and Thermo Fisher Scientific (Uppsala, Sweden). The choice of these suppliers was guided by the availability of these assays in the current hospital routine and availability of resources to acquire additional reagents, and not based on a-priori assumption of higher quality of these assays over other commercially available assays.

From EUROIMMUN, the Systemic Sclerosis [Nucleoli] Profile EUROLINE [IgG]. Antigens tested were: RNA polymerase III (subunits RP11 and RP155), Centromere protein (CenpA and CenpB), Topoisomerase 1 (Scl70), fibrillarin, NOR-90, Th-To, PM-Scl100, PM-Scl75, Ku, tripartite motif-containing protein (TRIM)-21 (Ro 52) and PDGFR. Sera were analyzed at a dilution of 1:100 and autoantibodies were detected using alkaline phosphatase-labeled anti-human IgG. Blots were quantified using the EUROLINE flatbed scanner. Signal intensities of <11 intensity units were regarded as negative.

From D-tek, the nitrocellulose blots containing scleroderma associated antigens (SCL10DIV-24) from D-tek (Mons, Belgium) were used. The dot blot contains the antigens Topoisomerase 1 (Scl-70), Centromere protein CenpA and CenpB, PM-Scl75, PM-Scl100, Ku, RNA Polymerase III, Th-To, RNP68/A/C and Fibrillarin. Ten μL of patient serum was used for performing the assay according to manufacturers' protocol. Signal intensities of <11 intensity units were regarded as negative.

From Thermo Fischer, the following available (EliA) tests, for Topoisomerase 1 (Scl-70), Ro52, RNA Polymerase III, PM-Scl100, Fibrillarin, Centromere protein (CenpB) were analyzed on a Phadia 250 analyzer according to the manufacturers' protocol. Signal intensities of <10 U/ml intensity units were regarded as negative.

Data analysis

All data were processed and analyzed in the statistical package R (<https://www.r-project.org>), version 3.6.1. Packages for visualization and modelling were tidyverse (1.0.0), dplyr (0.8.3) and ggplot2 (3.2.1). Data were used as measured values (autoantibody levels). Autoantibody data were converted to "positive" (present) and "negative" (absent) values using the cut-off values recommended by the manufacturers, i.e. lower limit of normal, recommended by the supplier. The

concordance between the various assays were calculated and expressed as Cohen's kappa coefficient.

Results

Prevalence of SSc-specific and -associated autoantibodies

In total 347 serum samples of individual patients with SSc were evaluated for the presence of SSc autoantibodies (Figure 1(A)). It appeared that there was a difference in prevalence between the autoantibodies in LcSSc and DcSSc patients. The autoantibodies ATA, ARA, fibrillarin and Th-To antibodies revealed a higher prevalence in the diffuse cutaneous form, whereas ACA, Ro52, PM-Scl75, and PM-Scl100 and Ku were more prevalent in the limited cutaneous form patients, although for the latter the difference between LcSSc and DcSSc was small. The overall prevalence in the three assays of the antibodies included in the ACR/EULAR classification criteria, namely ACA, ATA, and ARA, ranged between 29.2–30.3%, 21.4–24.0%, and 3.8–6.9%, respectively. The prevalence of PM-Scl autoantibodies in the cohort was between 3.2 and 6.4%. Fibrillarin and Ku autoantibodies could be detected in, respectively, 2.3–3.8% and 1.4–2.6% of

the patients. Autoantibodies directed against Th-To, NOR90 had a low prevalence (<2%) and for PDGFR no positive results were observed. The exact prevalence of autoantibodies per antigen per provider is shown in Table 2.

Subsequently we investigated the prevalence of specific autoantibodies per disease complication group (Figure 1(B)), where we only focussed on the ACA, ATA and ARA autoantibodies. These data showed that ACA autoantibodies associate mainly with PAH and DU complications whereas ATA associates with ILD. The prevalence of ARA is largely comparable in all three complication groups. The data obtained with the assays from all three suppliers showed high agreement.

Concordance between tests

To assess the agreement between the three assays, we calculated the Cohen's kappa coefficient for the comparisons (Table 3). The concordance for the autoantibodies with the highest prevalence, ATA and ACA, was very high with a Cohen's kappa coefficient of 0.95, dependent on the specific antigen used, whereas for ARA, a lower concordance of around 0.70 was observed. Using the EUROLINE and EliA assays, respectively 8 and 13 sera were tested positive for the presence of fibrillarin autoantibodies ($k = 0.67$). In

Figure 1. (A) Overview of prevalence of autoantibodies per tested antigen in LcSSc and DcSSc patients. (B) Prevalence of ACA, ARA, and ATA per tested antigen in patient sub-populations with different disease complications.

Table 2. Overview of prevalence of autoantibodies (per tested antigen) in SSc patients.

Autoantibody	Antigen	Supplier		
		EUROLINE (EUROIMMUN) (%)	Dot-blot (D-tek) (%)	EliA (Thermo Fisher Scientific) (%)
ACA (centromere)	CENPA	29.2	29.5	
	CENPB	29.8	30.3	29.8
ATA (Scl70)	Scl70	22.3	21.4	24.0
ARA (RNA polymerase III)	Rp11	6.6		
	Rp155	6.9		
	RNAP3		3.8	3.8
PM-Scl	PM75	6.4	3.2	
	PM100	6.4	4.0	4.0
	Fibrillarin	2.3	ND	3.8
Ku	Ku	2.6	1.4	
Th/To	Th/To	1.4	1.7	
NOR90	NOR90	0.9		
RNP68/A/C	RNP/68/A/C		7.5	
PDGFR	PDGFR	0		
Ro52	Ro52	21.7		12.4

Prevalence of autoantibodies per provider/test were evaluated using 347 serum samples of individual SSc patients. ND indicates that result was not evaluated due to technical issues with the antigen present in the assay.

Table 3. Concordance between tests.

Autoantibody	Compared test systems (antigen used in the test)	Concordance (Cohen's κ)
ACA	EUROLINE (CENPA) vs. Dot-blot (CENPA)	0.99
	EUROLINE (CENPB) vs. Dot-blot (CENPB)	0.99
	EUROLINE (CENPB) vs. EliA (CENPB)	0.96
	Dot-blot (CENPB) vs. EliA (CENPB)	0.97
ATA	EUROLINE (Scl70) vs. Dot-blot (Scl70)	0.94
	EUROLINE (Scl70) vs. EliA (Scl70)	0.94
	Dot-blot (Scl70) vs. EliA (Scl70)	0.91
ARA	EUROLINE (Rp11) vs. Dot-blot (RNAP3)	0.53
	EUROLINE (Rp155) vs. Dot-blot (RNAP3)	0.57
	EUROLINE (Rp11) vs. EliA (RNAP3)	0.65
	EUROLINE (Rp155) vs. EliA (RNAP3)	0.69
	Dot-blot (RNAP3) vs. EliA (RNAP3)	0.76
PM-Scl	EUROLINE (PM75) vs. Dot-blot (PM75)	0.59
	EUROLINE (PM100) vs. Dot-blot (PM100)	0.77
	EUROLINE (PM100) vs. EliA (PM-Scl100)	0.77
	Dot-blot (PM100) vs. EliA (PM-Scl100)	0.85
Fibrillarin	EUROLINE (Fibrillarin) vs. EliA (Fibrillarin)	0.67
Ku	EUROLINE (Ku) vs. Dot-blot (Ku)	0.70
Th/To	EUROLINE (Th/To) vs. Dot-blot (Th/To)	0.17
Ro52	EUROLINE (Ro52) vs. EliA (Ro52)	0.68

The values show the Cohen's kappa coefficient for each comparison.

general, the concordance for the low prevalent SSc-associated autoantibodies, Ku, Th-To and PM-Scl was lower and varied between 0.70 and 0.17.

Quantitative correlation of read outs

The analysis described above was based on classification of a sample as either positive or negative, based on the absolute quantitative read out in the antibody assays and the threshold value. We next investigated the correlation between test results based on raw values as an indicator of test characteristics, instead of converting the values to "positive" or "negative" based on the threshold values (Figure 2). The data showed that for the assays for CenpB, which showed a high concordance (Cohen's kappa >0.9), the correlation between the raw values is also high. In contrast, assays that showed low Cohen's kappa coefficient, like Ku and Th-To and to a lesser extend Fibrillarin, the correlation between the raw values is low or even absent which is related to antigen source or epitope used.

Co-occurrence of autoantibodies in systemic sclerosis

To see the co-association pattern between the autoantibodies in individual patients, we identified for all autoantibody combinations the level of co-occurrence within individual patients (Figure 3). Based on the percentage overlap, clear clusters (indicated in the blue colour) can be observed for co-associations between the antigens for ACA, ATA, ARA, Ro52, PM-Scl, fibrillarin, in agreement with the high concordance between tests (Table 3).

Since ATA, ACA and ARA antibodies are used for disease classification and prognosis, we also examined the possibility of co-occurrence of these SSc-specific autoantibodies with these or other autoantibodies within SSc patients. For this we merged the data from the three suppliers and regarded each patient to be positive for an autoantibody when at least one of the tests showed a positive score. We found that most of the patients (85%) were positive for only a single antibody, predominantly for ATA ($n=66$), ACA ($n=63$) and ARA ($n=23$). Most double positives (71 in total) were found with Ro52/ACA ($n=21$), Ro52/RNP-

Figure 2. Quantitative comparisons between the tests with equivalent read outs. Examples for the antigens CenpB, Scl-70, Th-To, Fibrillarin, and Ku are shown. The raw values as obtained from the tests from different suppliers are plotted. Results for each assay are expressed in arbitrary units and therefore are not commutable.

68Kd-A-C ($n=9$) and Ro52/ATA ($n=8$). For the major antibodies we found 6 double positives: ATA/ARA (5) and ACA/ATA (1). The combination of ACA/ARA was not found. Multiple triple positives were found with ACA/RNP-68Kd-A-C/Ro52 (4). Other triple combinations occurred only once.

Discussion

Assessment of autoantibody profiles is an integral part of the disease classification in SSc [15]. To date, three autoantibodies, ATA, ARA and ACA, are listed in the ACR/EULAR classification criteria for SSc. These autoantibodies are

highly specific for SSc with a specificity of >95% for ACA, >99% for ATA and >99% for ARA, respectively [16]. In the present study, we evaluated the comparability of three different commercially available tests for SSc autoantibodies. Despite technological differences we observed high levels of agreement for both ATA and ACA (Cohen's kappa >0.95). Agreement between assays for ARA and low prevalent SSc autoantibodies was low (a Cohen's Kappa between 0.70 and 0.17).

The quality of the diagnostic tests is in this case only inferred from a comparison with each other. It would be interesting to compare these results with an additional non-related quantitative measure, such as e.g. ANA immune fluorescence pattern which has been shown to be related to

SSc antibody associations [17]. However, in our cohort we could not make this comparison, since only the qualitative score for ANA-positivity was available.

cohort is a tertiary referral cohort, including patients in whom the diagnosis of SSc is difficult to make, although they do fulfil the classification criteria.

Our data reveal that there is a high level of agreement between tests from three individual suppliers for ATA and ACA which is reflected in the observed Cohens kappa. The prevalence of ACA in our cohort was approximately 30%, and comparable between the three assays used in our study cohorts and reported elsewhere [18,19]. Of interest, the prevalence of ATA differs between cohorts. We observed a prevalence of 21–24% depending on the assay used. Other cohort studies report prevalence for ATA of 20–40% [18,19]. This higher prevalence reflects the higher number of more severe cases in our tertiary referral cohort. In our cohort ARA positivity was found in 4–7% of SSc patients, however, level of agreement was also lower (Cohens Kappa between 0.53 and 0.76) which could also contribute to the

differences in reported prevalence of ARA in cohorts in different countries from 1.5 to 25% [20]. Also differences of the prevalence dependent on ethnicity have been reported [21,22]. Since our cohort is a predominantly Caucasian cohort, a detailed investigation of the relations between prevalence patterns and demographic or ethnic factors was not undertaken.

We found an association of ACA autoantibodies with PAH (45%, [Figure 1\(B\)](#)) and DU (32%, [Figure 1\(B\)](#)) and of ATA with ILD (33% and DU (32%, [Figure 1\(B\)](#)), which is comparable to published literature [5,7,23]. Since our primary aim was the quantitative comparison of three commercial assays, a further analysis of the association patterns with other complications such as GI, joint and muscular involvement was not done.

Though immunoprecipitation is still perceived as the gold standard, more robust and less laborious diagnostic tests have been developed for fast parallel (semi)quantitative detection of autoantibodies. However, antigens used for the detection of autoantibodies differ between the suppliers, preventing a direct quantitative comparison of the results. An additional challenge for harmonization is the absence of antigen specific controls or calibrators. Availability of proficiency testing initiatives, including less prevalent autoantibodies, and sufficient control material together with close collaboration with manufactures could provide a first step in harmonization of these assays [24].

The presence of ARA is associated with rapid diffuse cutaneous disease with poor prognosis and renal crisis and has therefore important clinical consequences [25–28]. Therefore, we can debate whether a concordance between diagnostic ARA assays of 0.76–0.53 is acceptable. For ACA and ATA the agreement between the tests is sufficient for clinical practice. Results reported on other SSc-associated autoantibodies could be biased, based on the type of assay. Therefore, all types of diagnostic assays should be subject to terms of harmonization and standardization [24]. For autoimmune diagnostics the first step would be to agree on antigen, epitope and alignment of results between different assays. Our results show that this goal is achieved for ACA and ARA autoantibody testing, but for the other SSc-specific and -associated autoantibodies there is room for improvement. There is a clinical unmet need and both a diagnostic and commercial drive to report autoantibodies for diagnostic and prognostic purposes. Furthermore, addressing the issue of the sero-negative gap is warranted. If this harmonization is not achievable, we should rethink how to perform low prevalent autoantibody tests for SSc or how to report the result to our clinicians.

Several studies have examined the co-occurrence of autoantibodies in patients. Combinations of ATA with ACA or ARA have been reported but are rare [16,18]. Here we show that the combination of these autoantibodies is dependent on the assay used and is a result of the assay characteristics and antigens used. Because there is no clinical relevance for this co-association, this finding should be interpreted with care as a laboratory finding. Therefore, we should look at

the dominant autoantibody in relation to the clinical phenotype.

We conclude that the diagnostic performance of commercially available assays described in this paper for detection of ATA and ACA is good. We found a high concordance between tests. However, concordance of PM-Scl, RNA Polymerase III and low prevalent autoantibodies like fibrillarin, Th-To and Ku should be improved. The journey to achieve this important consensus necessitates for laboratory specialists, clinicians and suppliers to join their forces.

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Ethical approval

The study protocol was reviewed and approved by the local ethics committee. All patients gave written informed consent prior to blood collection.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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