Fluoroenzymeimmunoassay to detect systemic sclerosis-associated antibodies: diagnostic performance and correlation with conventional techniques

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Abstract

Objective
Detection of systemic sclerosis-associated antibodies (SSc-Ab) in routine clinical practice is mostly restricted to anti-centromere and anti-Scl-70 antibodies. However, also other antinuclear antibodies have been shown to be valuable diagnostic and prognostic markers for the disease. The aim of this study was to evaluate the diagnostic performance of measuring the most prevalent SSc-Ab with fluoroenzymeimmunoassay (FEIA) as an alternative for the combined conventional techniques (CCT).

Methods
Sera from 144 consecutive systemic sclerosis (SSc) patients previously tested by CCT (indirect immunofluorescence on HEp-2000, western blotting, protein radio immunoprecipitation and a well-documented line immunoassay) and an additional group of 266 disease controls (80 rheumatoid arthritis, 58 systemic lupus erythematosus, 50 spondyloarthropathy, 48 osteoarthritis, 18 polymyalgia rheumatica and 12 ANCA-associated vasculitis) were retrospectively evaluated. Anti-centromere-B, anti-Scl-70, anti-RNA polymerase III and anti-PM/Scl antibodies were measured using FEIA.

Results
Using cut-off values corresponding with likelihood ratios larger than 10 FEIA obtained the following sensitivities: 45.1% for anti-centromere-B, 15.3% for anti-Scl-70, 5.6% for anti-RNA polymerase III and 2.1% for anti-PM/Scl. The overall agreement between combined conventional techniques and FEIA was good for all individual reactivities (kappa>0.800). The overall diagnostic sensitivity of 68.1% and diagnostic specificity of 98.1% were comparable to those obtained by CCT.

Conclusion
FEIA testing for anti-centromere-B, anti-Scl-70, anti-RNA polymerase III and anti-PM/Scl-100 shows good performance and represents an accurate alternative for the time-consuming combined conventional techniques.

Key words
Antinuclear antibodies, extractable nuclear antigen, systemic sclerosis, fluoroenzymeimmunoassay

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Introduction

Systemic sclerosis (SSc) is a rare connective tissue disease characterised by widespread fibrosis, vasculopathy, which can be retrieved in the skin and several internal organs, and the presence of autoantibodies against various nuclear antigens. Patients with an established disease are classified according to the extent of skin thickening as limited cutaneous SSc or diffuse cutaneous SSc (1). In 2001, Leroy and Medsger proposed the inclusion of an additional third subset (limited SSc) to allow early diagnosis and classification of SSc (2). Besides the presence of Raynaud's phenomenon as a major criterion, SSc-associated positive serology including anti-Scl-70 (otherwise known as anti-topoisomerase I), anti-centromere, anti-PM/Scl, anti-fibrillarin and anti-RNA polymerase III antibodies and/or SSc-type nailfold capillary pattern are requirements for early diagnosis of SSc (2). Apart from their diagnostic value, these SSc associated antibodies (SSc-Ab) carry significant prognostic value as they are associated with particular disease manifestations (3, 4).

It has become common practice to use commercially available enzyme immunoassays to detect the most prevalent SSc-Ab (anti-centromere-B and anti-Scl-70) (5). In contrast, the identification of the less frequently observed SSc-Ab (anti-RNA polymerase III, anti-PM/Scl and anti-fibrillarin) is generally not part of the routine laboratory repertoire. Nowadays, several enzyme immunoassays covering the whole serological SSc-spectrum are becoming available (6-11). However, these assays are not standardised and it is often not known how diagnostic performance of these new technologies correlates with the conventional techniques (12). The purpose of this study was to evaluate the diagnostic performance of measuring the most prevalent SSc-Ab (anti-centromere-B, anti-Scl-70, anti-RNA polymerase III and anti-PM/Scl-100) with fluoroenzymeimmunoassay (FEIA) as an accurate alternative for the combined conventional techniques (CCT).

Patients and methods
Samples
The patient group consisted of a consecutive SSc cohort fulfilling LeRoy and Medsger criteria (1, 2, 13). One hundred and forty four sera were available for further analysis (limited SSc (n=41), limited cutaneous SSc (n=83) and diffuse cutaneous SSc (n=20) (13). The control samples originated from 266 patients with established clinical diagnosis and represented the following connective tissue diseases (classified according to ACR criteria where applicable): 80 rheumatoid arthritis (RA), 58 systemic lupus erythematosus (SLE), 50 spondyloarthropathy (SpA), 48 osteoarthritis (OA), 18 polymyalgia rheumatica (PMR) and 12 ANCA-associated vasculitis (AAV) (14-17). This study was conducted after approval by the Ethics Committee of the Ghent University Hospital and all SSc patients signed informed consent. Control samples used for this study were from the hospital serum bank and were obtained mostly in the context of previously reported studies (18-20).

Detection of SSc-Ab by fluoroenzymeimmunoassay
FEIA (EliA Phadia, Uppsala, Sweden) was used for the detection of the SSc-Ab. The antigens represented on the test wells were human recombinant centromere-B, Scl-70, RNA-polymerase III/RP155 and PM/Scl-100. All assays were performed automatedly according to the instructions of the manufacturer using the Immunocap 250 (EliA Phadia, Uppsala, Sweden). In brief, specific antibodies present in the serum will bind to the antigens coated on the solid phase (EliA caps). In the subsequent reaction step, a β-galactosidase labelled secondary antibody against human IgG antibodies is added to form an antibody-conjugate complex. After incubation and washing, the enzyme-bound complex is incubated with 4-methylumbelliferyl-β-D-galactoside and transforms into a fluorescent product. The fluorescence in the reaction mixture is measured. The higher the response values, the more specific antibody is present in the serum. To evaluate test results, sample responses are converted in U/mL using correction factors obtained by analysis of calibrators. Every sample was analysed in

duplicate; averages of the duplicates were used in the analysis. Imprecision characteristics were evaluated based on the analysis of a positive control material included in each run. For antibodies against centromere-B and Scl-70, control material was provided by the manufacturer (EliA Phadia, Uppsala, Sweden). For anti-RNA polymerase III and anti-PM/Scl antibodies, no commercial control material was available. Alternatively, positive patient samples (as confirmed by combined conventional techniques, see below) were used to calculate assay variability.

Detection of SSc-Ab by combined conventional techniques
All SSc patients (n=144) were analysed with a combination of conventional techniques. These techniques were performed as described in detail previously by Van Praet *et al*. (13).
In brief, indirect immunofluorescence (IIF) for antinuclear antibodies (ANA) on HEp-2000 cells was performed according to the manufacturer's instructions (Immunoconcepts, Sacramento, CA, USA), using a serum dilution of 1:40.
Western blotting (WB) was performed on nuclear extract from K562 cells. The nuclear extract was electrophoresed on a 10% SDS-PAGE and subsequently blotted on nitrocellulose membranes. After overnight incubation with pre-diluted serum (1:100), antibody binding was visualised using HRP-labelled goat anti-human IgG in combination with enhanced electrochemiluminescent detection substrate (Supersignal West Dura Extended Duration Substrate, Pierce, Rockford, IL, USA), using the Versadoc Imaging System (Biorad).
Protein radio-immunoprecipitation (P-IP) was performed with cell extract of 35S-methionine and 35S-cystine labelled K562 cells. A total cell extract of these labelled cells was used for protein-A assisted immunoprecipitation (protein A-Sepharose beads, Sigma). After washing, precipitated proteins were fractionated by 8% SDS–PAGE and visualised by autoradiography.
The INNO-LIA ANA Update (LIA) (Innogenetics NV, Zwijnaarde, Bel-

Table I. Technical performance characteristics of SSc-Ab detection by FEIA.

	n.	Mean	SD	Coefficient of variation (%)
Anti-centromere-B	38	236.3	31.7	13.4
Anti-Scl-70	36	25.4	2.1	8.4
Anti-RNA polymerase III	35	67.1	7.5	11.2
Anti-PM/Scl-100	35	84.0	12.1	14.4

gium) was performed according to the manufacturer's instructions. This assay contains the following recombinant or natural antigens: SmB, SmD, RNP-A, RNP-C, RNP-70k, Ro52/SSA, Ro60/SSA, SSB, centromere-B, Scl-70, Jo-1, ribosomal P, and histones. Each line of the test strip was compared with the respective line on a reference strip, obtained by testing a cut-off control sample in each run.
All patient serum samples were categorised as 'CCT positive' or 'CCT negative'. Global CCT results were considered positive if at least one of the applicable conventional techniques was positive for the specific autoantibody: anti-centromere (IIF or LIA), anti-Scl-70 (P-IP, WB or LIA), anti-RNA polymerase III (P-IP) and anti-PM/Scl (WB or P-IP).

Statistical analysis
Diagnostic sensitivity, diagnostic specificity and positive likelihood ratios (LRs) were evaluated for the individual reactivities using the cut-off values proposed by the manufacturer (21). The positive likelihood ratio of a specific test result for a disease is the likelihood of the test result in diseased individuals divided by the likelihood of the test result in diseased control individuals. This parameter provides an estimation of whether there will be a significant change in pre-test to post-test probability of a disease given the test results (reviewed in (21)). LRs >10 indicate large, often clinically significant differences. When application of the manufacturers' cut-off values did not allow to obtain a LR >10, cut-off values consistent with a LR of at least 10 were selected. These in-house cut-off values were also used to calculate sensitivities and specificities and were further used for dichotomisation of the original continuous data. Overall concordance be-

tween FEIA and CCT was evaluated in 2x2 frequency tables and then quantified using Cohen's kappa (κ) statistics for pair-wise comparison: κ is 1 when there is a perfect agreement; κ is 0 when there is no agreement better than chance (22). Venn diagrams were constructed to visually demonstrate positive concordance between FEIA and CCT for each individual reactivity. For comparison of proportions, Chi-square testing with Yates' correction for continuity was applied. Two-sided p-values <0.05 were considered significant. Statistical analysis was performed with PasW 18.0 statistical package (SPSS Inc., Chicago, IL, USA).

Results
Technical performance characteristics
Inter-assay imprecision characteristics were based on the results of the commercial control material for anti-Scl-70 and anti-centromere. For anti-RNA polymerase III and anti-PM/Scl antibodies, a 'CCT positive' patient sample was used. Co-efficients of variation (CV) ranged from 8.4% to 14.4% (see Table I).

Diagnostic performance of fluoroenzymeimmunoassay in the detection of SSc associated antibodies
With the cut-off values proposed by the manufacturer (11 U/mL for anti-centromere-B, 10 U/mL for anti-Scl-70, 15 U/mL for anti-PM/Scl and 16 U/mL for anti-RNA polymerase III) FEIA identified 65 anti-centromere-B, 22 anti-Scl-70, 8 anti-RNA polymerase III and 3 anti-PM/Scl positive patient sera. This resulted in a diagnostic sensitivity of 45.1%, 15.3%, 5.6% and 2.1%, respectively. For anti-centromere-B and anti-Scl-70, the diagnostic specificity reached more than 99%. For anti-RNA polymerase III and anti-PM/Scl antibodies, 6/266 (diagnostic specificity=
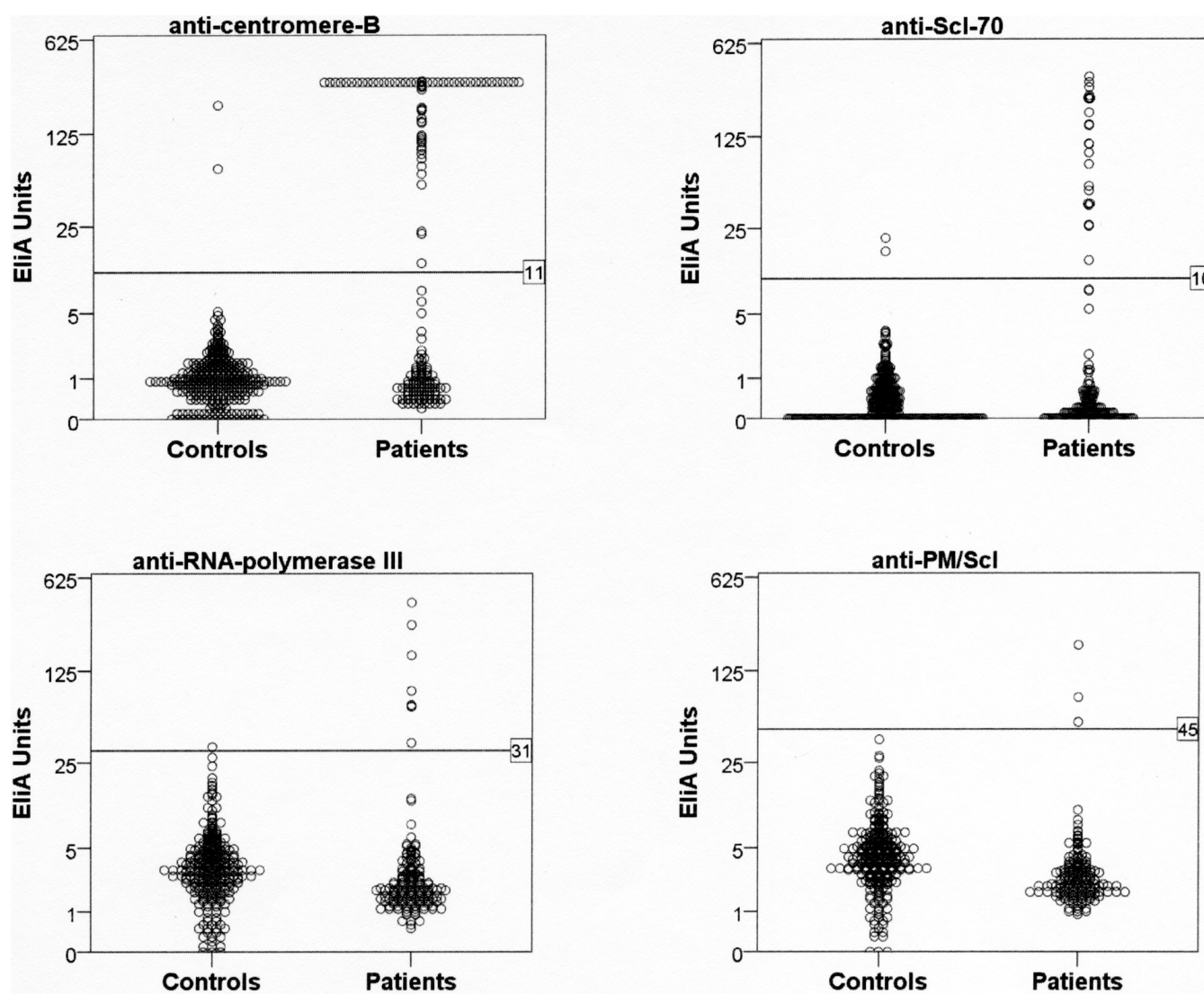
Fig. 1. Distribution plots on log scale of the signals (EliA Units) for the different SSc-Ab on FEIA in SSc patients and controls. Cut-off values matching LR>10 are marked with a horizontal reference line on each plot.

97.7%) and 12/266 (diagnostic specificity = 95.5%) control sera were found positive, respectively. Figure 1 shows distribution plots on signal responses (in EliA Units) for the individual reactivities. Our strategy was to select cut-off values compatible with the major criterion of a minimal LR of 10. For anti-Scl-70 and anti-centromere-B, at manufacturers' cut-off values LRs >10 were found (20 and 60 respectively). For anti-RNA polymerase-III and anti-PM/Scl, lower LRs were found when manufacturers' cut-off values were applied (2.4 and 0.5 respectively). Selected cut-off values were found to be higher than those proposed by the manufacturer. The >10 LR criterion could be obtained at a cut-off value >31U for

RNA-polymerase-III antibodies and at a cut-off value >45 U for anti-PM/Scl antibodies. When these cut-off values were applied, we identified 1 positive control and 8 positive patients for anti-RNA polymerase III (LR=15). For anti-PM/Scl, we found 3 positive patient samples and no positive controls (LR= ∞). Comparing with the results we obtained with the manufacturers' cut-off values, sensitivity did not change for both reactivities, but specificity increased (anti-RNA polymerase-III specificity=99.6% and anti-PM/Scl specificity=100%). An overview of the results is shown in Table II.

With the cut-off values corresponding to minimal LRs of 10 for each individual antibody, global diagnostic

performance of FEIA in the serological work-up of SSc was evaluated. Diagnostic sensitivity and specificity of the combination of the four SSc-Ab were 68.1% (95% CI 63.2-71.4%) and 98.1% (95% CI 95.8-99.3%), respectively.

Five control sera tested positive on FEIA for the following antigens: 2 for centromere-B, 2 for Scl-70 and 1 for RNA polymerase III (Fig. 1). Most of the positive control sera (n=3/5, 60%) were derived from SLE patients. The remaining two positive control sera were from patients with RA (1 anti-RNA polymerase III and 1 anti-Centromere-B). None of the sera showed multiple SSc-Ab reactivity on FEIA. Also the two samples that were identified with double reactivity on CCT

Table II. Comparison of the diagnostic performance characteristics of FEIA using cut-off values proposed by the manufacturer *versus* calculated cut-off values corresponding with a minimal likelihood ratio (LR) of 10.

	Manufacturers' cut-off values				Optimised cut-off values (>10 LR)			
	Cut-off (U)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	LR (95% CI)	Cut-off (U)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	LR (95% CI)
Anti-centromere-B	>11	45.1 41.5-46.3	99.3 97.3-99.9	60.0 15.2-353.0	–	–	–	–
Anti-Scl-70	>10	15.3 12.0-16.4	99.3 97.5-99.9	20.3 4.8-124.6	–	–	–	--
Anti-RNA polymerase III	>16	5.6 2.9-7.9	97.7 96.3-99.0	2.5 0.8-7.9	>31	5.6 2.9-7.9	99.6 98.3-100	14.8 1.9-315.0
Anti-PM/Scl-100	>15	2.1 0.6-4.9	95.5 94.7-97.1	0.5 0.1-0.7	>45	2.1 0.6-4.9	100 99.2-100	∞ 0.835-∞

Table III. Analytical performance characteristics of FEIA and correlation with CCT.

	Anti-centromere	Anti-Scl-70	Anti-RNA polymerase III	Anti-PM/Scl
Overall concordance (%) (95% CI)	96.5 (91.4-97.8)	95.1 (90.1-96.5)	97.9 (94.0-97.9)	99.3 (96.6-99.3)
Analytical sensitivity (%) (95% CI)	94.1 (88.7-95.5)	77.8 (64.2-81.3)	72.7 (47.3-72.7)	75.0 (26.1-75.0)
Analytical specificity (%) (95% CI)	98.7 (93.8-99.9)	99.1 (96.1-100)	100 (97.9-100)	100 (98.6-100)
Kappa agreement (κ)	0.930	0.828	0.831	0.854

(1 sample with anti-centromere-B and anti-Scl-70, 1 sample with anti-centromere-B and anti-RNA polymerase III) showed monoreactivity on FEIA (both anti-centromere positive).

Relationship between SSc antibody reactivity by fluoroenzymeimmunoassay and conventional techniques

To validate analytical accuracy of the results obtained by FEIA, a combination of conventional techniques was used as a 'golden standard'. All patient serum samples (n=144) were categorised as 'CCT positive' or 'CCT negative'. Global CCT results were defined positive if at least one of the applicable conventional techniques was positive for the specific autoantibodies. When this CCT positive/CCT negative definition was applied, 68 sera were found positive for anti-centromere, 26 for anti-Scl-70, 11 for anti-RNA polymerase III and 4 for anti-PM/Scl antibodies.

By evaluating the 2x2 tables for each individual reactivity on the total cohort of SSc patients samples, the overall concordance and kappa-agreement between CCT and FEIA was calculated, as well as the analytical sensitivity and specificity. Overall concordance between CCT and FEIA ranged from 95.1– 99.3%. There was a good agreement (κ-values ranging 0.828-0.930) between CCT and FEIA for all four reactivities. Analytical sensitivities and specificities of FEIA for the identification of SSc-Ab are given in Table III.

Then we evaluated which method showed a better association with the different disease subsets (lSSc, LcSSc and DcSSc) for the different reactivities. No significant differences between frequencies obtained by CCT and FEIA were found as shown in Table IV.

Of the 144 sera tested by CCT and FEIA, 96 (66.7%) tested positive for both techniques. By means of CCT, SSc-Ab (anti-Scl-70, anti-centromere-B, anti-RNA polymerase III and anti-PM/Scl) were detected in 110 (76.4%) of the 144 SSc patients previously described (13). In contrast, FEIA identified SSc-Ab in 98 (68.1%, 95% CI 63.2–71.4%) of the 144 SSc patients. The relationship between CCT and FEIA for the individual antibodies is shown visually in Figure 2. Fourteen CCT positive sera were not picked up by FEIA. For all anti-Scl-70 and anti-PM/Scl CCT positive sera identification was confirmed with 2 out of 3 conventional techniques. For the anti-centromere CCT positive samples (n=4), three were positive on IIF only. Inversely, only two FEIA positive samples could not be confirmed on CCT. These sample expressed high intensity value for anti-Scl-70 (242.5 U/mL) and anti-centromere-B (22.0 U/mL),

Table IV. Frequencies within disease subsets of the SSc-Ab detected by FEIA and CCT.

	Anti-centromere-B*			Anti-Scl-70*			Anti-RNA polymerase III*			Anti-PM/Scl*		
	lSSc	DcSSc	LcSSc	lSSc	DcSSc	LcSSc	lSSc	DcSSc	LcSSc	lSSc	DcSSc	LcSSc
FEIA	66 (27)	15 (3)	42 (35)	0 (0)	30 (6)	19 (16)	0 (0)	25 (5)	4 (3)	0 (0)	0 (0)	4 (3)
CCT	71 (29)	15 (3)	43 (36)	2 (1)	40 (8)	22 (18)	0 (0)	30 (6)	6 (5)	0 (0)	0 (0)	5 (4)
p-value	0.8123	0.6579	0.9787	0.8417	0.7403	0.7745	–	1.000	0.8142	–	–	0.9493

*Fiindings are reported as % of positivity within disease subsets: lSSc (n=41), DcSSc (n=20) and LcSSc (n=83) (number of positive patients)
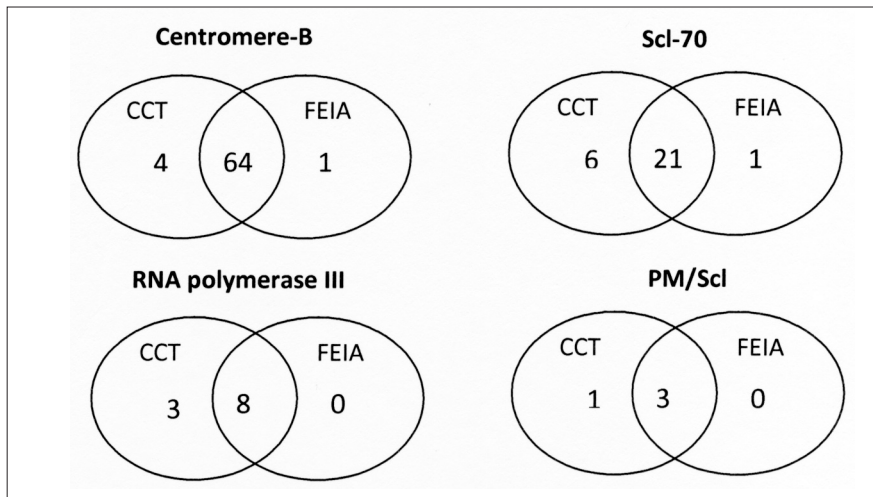
Fig. 2. Relation between CCT and FEIA in patient samples with positive results in one of both techniques.

respectively. In addition, we examined whether the sera missed by FEIA (n=14) were derived from a particular disease subset. Eight of the 14 sera missed by FEIA were derived from patients belonging to the LcSSc subset (n=83), 2 to the DcSSc (n=20) subset and 4 to the lSSc subset (n=41). However, we were not able to identify a significant difference in SSc-Ab detection by FEIA and CCT for the different disease subsets: CCT 80 % *versus* FEIA 70% for DcSSc (*p*=0.7150), CCT 76 % *versus* FEIA 66% for lSSc (*p*=0.4506), CCT 24 % *versus* FEIA 14% for DcSSc (*p*=0.1487).

Discussion

Serum antinuclear antibodies (ANA) are detected in most of the SSc patients (4, 13). Apart from their diagnostic value, these ANA contribute in the prognosis due to their association with particular internal organ involvement (3, 4, 23). Despite their importance, routine testing algorithms are often limited to testing for anti-centromere-B and anti-Scl-70 (5, 20, 24, 25). As these two major autoantibodies cover only about 65% of the serological spectrum of SSc, it is suggested that assays targeting anti-RNA polymerase III and anti-PM/SCl should be added to the routine test repertoire (13). Until recently, these autoantibodies could only be detected by the use of a combination of laborious and time-consuming conventional techniques (9, 26). Nowa-

days, several immunoassays targeting these additional SSc-Ab are becoming commercially available. Most of these immunoassays use highly purified recombinant antigens immobilised on a solid phase. In contrast, conventional techniques employ native antigens. Due to this differences in antigen sources, discordant results in comparative evaluations are self-evident and not only an effect of antibody heterogeneity alone (9, 12, 27).

In this study, we identified the most prevalent SSc-Ab in parallel by FEIA and CCT. The imprecision characteristics of FEIA for all four SSc-Ab, expressed as CV, were all less than 20% (Table I).

With optimised cut-off values for anti-RNA polymerase III and anti-PM/Scl, a highly specific diagnostic performance (>99%) was obtained for all parameters. For anti-centromere and anti-Scl-70, the cut-off values proposed by the manufacturer were found to be acceptable. For anti-RNA polymerase III and anti-PM/Scl, cut-off values fulfilling the selected LR>10 criterion were higher than the cut-off values proposed by the manufacturer, but did not influence diagnostic sensitivity (Table II). Individual diagnostic sensitivity of the four SSc-Ab were in agreement with those reported in previous studies (3, 7, 10, 28-30). In addition, we tested SSc serological profile in other connective tissue diseases. Most of the SSc-Ab positive sera were derived from

SLE patients (3/58=5%). All samples showed single reactivity on FEIA, confirming that SSc-Ab are serologically independent and mutually exclusive antibodies (4).

After optimisation of the FEIA cut-off values, results obtained by FEIA were compared with CCT. The agreement between FEIA and CCT results was good (κ>0.800) for the four parameters tested. No significant difference in the SSc-Ab frequencies calculated within the disease subsets (LcSSc, DcSSc and lSSc) as detected by both methods was observed. The combination of conventional techniques (see Table III) was selected as a 'golden standard' to evaluate analytical accuracy, as they were historically used to evaluate their specificity for SSc and to characterise association with clinical manifestations (31-34). However, It should be noticed that nowadays some of these golden standard test are considered less disease specific than the newer technologies using recombinant proteins. For instance, IIF for the detection of centromere antibodies is less SSc-specific than using recombinant centromere-B in immunoassays. This is illustrated by autoantibodies to centromere-F, which are associated with cancer instead of SSc , but show a fluorescence pattern similar to the classical anti-centromere pattern (reviewed in (35)).

Also the presumed high specificity of Scl-70 antibodies was recently questioned. Indeed, several groups documented that significantly more anti-Scl70 positivity was found in SLE patients compared to healthy controls (reviewed in (36)). Frequencies up to 25% of the SLE patients have been reported (37). Despite various attempts, no other studies have been able to confirm the high prevalence of anti-Scl-70 in SLE patients. Nevertheless, low frequency of these antibodies (<5%) in SLE-patients was recently confirmed by Mahler *et al*. using three different assay platforms (36). Regarding our own data, we found no documentation in literature of high frequencies of anti-Scl-70 (frequencies reported were all below 3%) in SLE patients using the reference tests we selected in our study (protein immunoprecipitation, western

blotting and line immune assay INNO-LIA™ ANA Update) (36).

For RNA polymerase III antibodies, we selected protein-immunoprecipitation on 35S methionine labeled Hela cell extracts as the reference test. In 1993, this conventional technique was applied by Kuwana at al. for the detection of an autoantibody reactive with all three classes of RNA polymerases (anti-RNA polymerase I, II and III) in 275 SSc patients and 286 control patients (38). In this study, autoantibody response against RNA polymerases was found in 5% of the SSc sera; but in none of the control sera. These results were in contrast with the results previously published by Stetler and co-workers describing RNA polymerase I antibodies in the majority of sera from SLE, mixed connective tissue disease, rheumatoid arthritis and Sjögren syndrome patients (39, 40). Both groups used immunoprecipitation, with the most notable difference the use of the antigen sources (living culture cells *versus* biochemically purified RNA polymerase I). Regarding our own study, we used the reference technique based on living culture cells as proposed by Kuwana *et al.* (38). In a more recent study, the high analytical specificity of this assay for the detection of RNA polymerase III antibodies was confirmed (specificity ≥99%) (41).

Regarding anti-PM/Scl, controversy exists about both their predictive value and the best method for their detection (42-44). Despite their presence in the classification criteria of Leroy and Medsger, it is generally accepted that antibodies are not specific for SSc (2). Apart from their relation with SSc, their association with polymyositis, and the polymyositis/scleroderma overlap syndrome is well documented (reviewed in (10)). In sporadic cases, anti-PM/Scl antibodies have been reported in patients with inclusion body myositis, Sjögren syndrome, SLE and even in a case with acquired haemophilia (reviewed in (10)). Although no international standard method has been defined for their detection, most authors consider P-IP the golden standard test for detecting PM/Scl antibodies (44, 45). Applying this method,

Ghirardello and coworkers identified no PM/Scl antibodies in a total of 230 disease controls (45). In contrast Schnitz *et al.*, observed low frequency of PM/Scl antibodies as determined by P-IP in individuals with no clinical evidence of myositis (46). Our FEIA data using manufacturers' cut-off values confirm the presence of anti-PM/Scl antibodies in disease controls, albeit at low frequency and with low titers. In contrast, anti-PM/Scl positive SSc patients were found to have higher titers. These data were compatible with the observation that low titer reactivity to PM1-alpha antibodies was seen in disease controls (47). As highly specific detection of PM/Scl antibodies was a priority for clinical application of the Leroy and Medsger, we selected a highly specific cut-off value. Sensitivity and agreement with CCT were not influenced (data on CCT not shown). We retained 2.1% anti-PM/Scl positive SSc patients. This percentage is comparable with the prevalence found in the Pittsburgh Scleroderma database, but is somewhat lower than more recently published results (4, 48).

For classification of the patient samples in view of global serological positivity for SSc-Ab, FEIA reached an overall diagnostic sensitivity of 68.1% which is not significant different from that obtained by CCT (76.4%, *p*=0.1495). Overall diagnostic specificity was acceptable (98.1%). In addition, we were not able to identify a significant difference in overall SSc-Ab detection by FEIA and CCT for the different SSc subpopulations.

In conclusion, the use of FEIA for the detection of the most prevalent SSc-Ab shows appropriate performance characteristics. We found good agreement between FEIA and CCT, illustrating that FEIA testing for anti-centromere-B, anti-Scl-70, anti-RNA polymerase III and anti-PM/Scl-100 is highly accurate and represents a valid alternative for the time-consuming combined conventional techniques.

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