Diagnostic accuracy of currently available anti-double-stranded DNA antibody assays. An Italian multicentre study

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Abstract

Objective

An Italian multicentre study was promoted in order to assess the accuracy of four anti-double-stranded DNA (dsDNA) antibody assays for SLE diagnosis and monitoring.

Methods

Two hundred and twenty-three patients with established SLE according to ACR classification criteria were enrolled from 9 centres. They included 59 patients at first evaluation (disease duration <12 months) and 164 with longer disease duration (median disease duration 120 months). The sera from 55 healthy subjects and 161 patients with rheumatic, infectious or neoplastic diseases were tested as controls. SLE activity was measured by ECLAM score. Anti-dsDNA antibodies were detected in serum by means of Farrzyme™ assay, fluoroenzymeimmunoassay (EliA™), Crithidia luciliae indirect immunofluorescence (CLIFT) or Farr radioimmunoassay (Farr). Cut-off values of quantitative assays were chosen by ROC curves analysis. Statistics were conducted by SPSS software package.

Results

Sensitivity for SLE diagnosis ranged between 66% with Farrzyme to 95% with Farr, with about 90% specificity for all the methods tested. Farrzyme assay was more specific than the others towards patients with non-SLE connective tissue disease. The four methods were moderately concordant and correlated among them, all showing a positive association with active disease, renal or haematologic involvement, and a negative association with central nervous system disease. Whatever the assay used, anti-dsDNA antibody levels correlated with disease activity with r correlation coefficients ranging from 0.336 to 0.425 ($p<0.0001$).

Conclusion

The diagnostic accuracy for SLE of evaluated anti-dsDNA antibody assays is comparable and potentially improvable especially in terms of specificity. The clinical adherence of the assays confirms the value of anti-dsDNA antibody for SLE monitoring.

Key words

anti-double stranded DNA antibody, systemic lupus erythematosus, laboratory methods, diagnostic accuracy

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Introduction

The detection of autoantibodies to nuclear self antigens in patient's serum is an important tool in the diagnostic algorithm of systemic autoimmune diseases. Antibodies targeting double stranded DNA (dsDNA) are so important for the diagnosis of systemic lupus erythematosus (SLE) that they are included in the established ACR classification criteria (1, 2). They are considered specific markers for SLE (3), being generally related to disease activity and tissue/organ damage, especially nephritis (4). Their potential in predicting the disease, disease relapses and consequently efficacy of traditional or new therapeutic approaches, has been assessed (5-10). However, the clinical value of anti-dsDNA antibodies largely depends on the assay principle and analytical variables of the methods used to quantitate and immunologically characterise them (11-13). In fact, the diversity and polyclonality of the autoimmune response to native DNA in individual patients may contribute to divergent results among assays and makes it difficult to establish a unique laboratory strategy for the detection of these antibodies.

Nowadays, currently used techniques in clinical laboratories to determine anti-dsDNA antibody vary from the in house performed *Crithidia luciliae* indirect immunofluorescence (CLIFT) to radioimmunologic or easily automatised immunoenzymatic assays.

FIRMA (Forum Interdisciplinare per la Ricerca nelle Malattie Autoimmuni) is an Italian interdisciplinary study group promoting research on standardisation of laboratory diagnostics in autoimmune diseases. Multicentre collaborative studies may contribute to the measure of variability in results obtained by different test procedures applied in a clinical setting.

On behalf of FIRMA group, the results of a multicentre study on the clinical performance of four currently available anti-dsDNA antibody assays for SLE diagnosis and monitoring is presented.

Patients and methods

Two hundred and twenty-three unselected patients with established SLE according to revised ACR classification criteria (1, 2), followed in 9 specialised units in Italy, were studied, including 59 at first diagnosis, therapy-free or under treatment with low-doses steroids (52 F, 7 M, mean age±SD 29±9 years, disease duration <12 months) and 164 with longer follow-up (151 F, 13 M, mean age±SD 38±12 years, median disease duration 120 months, range 18-360). The sera from sex-age matched 55 healthy subjects and 161 patients with rheumatic, infectious or neoplastic diseases were tested as controls. Disease controls comprised: 64 patients with viral infections (hepatitis C virus, hepatitis B virus, cytomegalovirus, Epstein Barr virus), 64 non-SLE connective tissue disease (CTD) classified according to established criteria (27 primary Sjögren's syndrome, 21 systemic sclerosis, 4 poly/dermatomyositis, 8 overlap syndrome, 2 vasculitis, 1 rheumatoid arthritis, 1 primary antiphospholipid syndrome), 27 cryoglobulinemia and 6 malignancies. In addition, 45 rheumatoid arthritis patients under treatment with anti-TNF-α agents were tested.

At the time of serum sampling, overall SLE activity was evaluated by the European Consensus Lupus Activity Measurement (ECLAM) score (14). An ECLAM score of >2 was arbitrary and considered indicative of active disease. Organ-specific involvement was defined according to revised ACR classification criteria (1). Patient and control clinical and laboratory data pertinent to the study were retrospectively collected and recorded in a computer-assisted database.

The study was approved by the local Ethics Committee and all patients gave written consent.

Serum anti-dsDNA antibodies were measured by means of the following assays: Farrzyme™ (The Binding Site, Birmingham, UK, distributed by Radim, Pomezia, Italy), fluoroenzymeimmunoassay (EliA™, Phadia, Freiburg, Germany), *Crithidia luciliae* indirect immunofluorescence (CLIFT, INOVA, S. Diego, USA) and in-house Farr radioimmunoassay (Farr). The anti-dsDNA antibody assays largely differ in their analytical characteristics

(Table I), in terms of characteristics of methods, antigenic source, or specificity for some antibody characteristics such as avidity, isotype, or quantitation modalities.

The anti-dsDNA antibody assays were each performed independently in different laboratories, blinded to the clinical status of serum aliquotes to be tested.

Table I. Analytical variables of anti-double stranded (ds)DNA antibody assays.

	Farrzyme	Farr	EliA	CLIFT
Principle	ELISA	RIA	Fluoro-immunoassay	IIF
dsDNA source	Calf thymus	Plasmidic	Plasmidic	Crithidia luciliae kinetoplast
Ab. avidity	High	High	Low-high	Medium-high
Ab. isotype	IgG	IgG, IgM, IgA	IgG	IgG
Ab. quantitation	IU/ml	AU	IU/ml	Titer

ELISA: enzyme-linked immunosorbent assay; RIA: radioimmunoassay; IIF: indirect immunofluorescence; Ab: antibody.

Farrzyme

It was performed according to the manufacturer's instructions (The Binding Site, Birmingham, UK, distributed by Radim, Pomezia, Italy). Briefly, microwells are precoated with calf thymus dsDNA antigen. The calibrators, controls and diluted patient sera (1:100) are added to the wells and autoantibodies recognising the dsDNA antigen bind during the first incubation. After washing out the unbound proteins, purified peroxidase labelled rabbit anti-human IgG (γ chain specific) conjugate is added and bound to the captured human autoantibody. After a further washing step, the bound conjugate is visualised with 3,3´,5,5´-tetramethylbenzidine (TMB) substrate and the colour intensity is photometrically measured at 450nm.

Fluoroenzymeimmunoassay (EliA™)

The assay principle is based on a modular reagent system (EliA™, Phadia, Freiburg, Germany). EliA dsDNA is to be used together with the EliA IgG method on the instrument ImmunoCAP 100 Software version 2.0. A dilution 1:10 of the samples is required and it is a default setting in ImmunoCAP 100 Instrument Software. Briefly, enter lot-specific code of the EliA dsDNA Well, EliA IgG Calibrator Well and IgG Conjugate. The calibration curve is obtained with EliA IgG Calibrators which are run in duplicate. The curve is stored and subsequent tests are evaluated against the stored curve using only the EliA IgG curve control. The IgG Calibrators are traceable via an unbroken chain of calibrations to the International Reference Preparation 67/86 of Human Serum Immunoglobulins from World Health Organisation. Antibodies to dsDNA are detected fluourometrically using a mouse monoclonal β-galactosidase conjugated anti-IgG antibody, and 4-methylumbelliferyl-β-D-galactoside as substrate. ImmunoCAP 100 measures specific IgG concentrations in μg/ml, by using a conversion factor given by the lot-specific code of the EliA dsDNA Well, the results are automatically converted to IU/ml.

Crithidia luciliae immunofluorescence test (CLIFT) assay

The CLIFT assay was performed according to the manufacturer's instructions (CLIFT, INOVA, San Diego, USA). All sera were diluted 1:10 with diluted PBS buffer (1:40). Antibodies to dsDNA were detected under a fluorescence microscope (40x and 100x magnification) using a ready to use fluorescein isothiocyanate-labelled anti-human IgG conjugate (INOVA). CLIFT positivity was four-fold scored from 1 to 4 as suggested by the manufacturer, and score 1 or above was defined as abnormal.

Farr radioimmunoassay (Farr)

It was a home-made radioassay based on ^{14}C labelled plasmidic dsDNA (^{14}C-DNA). ^{14}C-DNA (Amersham, GE Healthcare, UK) is diluted in borate buffer (final conc. 1ng/μl). Scomplemented sera together with a positive and negative control serum (1:10 diluted in borate buffer, 100 μl) are added in duplicate to 100 μl ^{14}C-DNA (100ng = 6000 cpm) and after overnight incubation at 4°C, ammonium sulphate 55.55% is added for separating the antibody bound to ^{14}C-DNA from free ^{14}C-DNA. After centrifugation (2500 rpm, 30', at 4°C), precipitates are washed two times, diluted in distilled water, and warmed at 70°C for 10'. Then, the radioactivity of the dsDNA antibody complex in the precipitate is counted for 5' in a β-counter. Farr anti-dsDNA antibody level in the sample is expressed as Arbitrary Units (AU) calculated as the ratio between average sample counts per minute (cpm) and total ^{14}C-DNA tracer cpm. Farr anti-dsDNA antibody cut-off level was calculated as the mean value + 3 standard deviations obtained in the sera from 50 healthy subjects, and assessed to 8 AU. Results were arbitrarily expressed in a dichotomatous mode (pos/neg) because calibrators were not stable over time, thus a reliable calibration curve could not be created.

Specific antinuclear antibodies or antiphospholipid antibodies were determined locally according to established laboratory protocols.

Statistical analysis was performed by means of SPSS software package for Windows (version 15.0) using Receiver Operating Characteristics (ROC) curves analysis, chi-square test and parametric or non-parametric statistics for continuous variables.

Concordance in the methods results was evaluated by contingency tables with kappa measure of agreement (15, 16).

Sensitivity for SLE = (number of anti-dsDNA positive patients with SLE at first visit/total patients with SLE at first diagnosis) x 100. Specificity for SLE (with respect to healthy subjects) = (number of anti-dsDNA negative healthy subjects/total healthy subjects) x 100. Specificity for SLE (with respect to disease controls) = (number of anti-dsDNA negative disease controls/ total disease controls) x 100.

Table II. Correlation among the anti-dsDNA antibody assays in SLE sera (Pearson or Spearman tests).

Assays	r	*p*
Elia *vs*. CLIFT	0.706	<0.0001
Elia *vs*. Farrzyme	0.676	<0.0001
Farrzyme *vs*. CLIFT	0.597	<0.0001

EliA: fluoroenzymeimmunoassay; CLIFT: indirect immunofluorescence on Crithidia luciliae; Farrzyme: enzyme linked immunosorbent assay. Farr results were not included in the analysis because dichotomically expressed.

Pearson's or Sperman's rank correlation tests were used to evaluate the correlation between the anti-dsDNA serum levels detected by each quantitative method and ECLAM disease activity score.

Results

Global concordance rates between anti-dsDNA antibody test results were low to moderate, the lowest being 71% (kappa 0.4) between Farrzyme and Farr, the highest 87% (kappa 0.7) between Farr and CLIFT. Correlations in antibody levels between different assays were good (r≥0.6, p<0.0001) (Table II).

Optimal cut-off values of quantitative tests were chosen by ROC curves analysis (Fig. 1) and compared with those suggested by the manufacturer. Farrzyme anti-dsDNA antibody cut-off value was assessed to 24 IU/ml, obtaining 66% sensitivity for SLE diagnosis at first evaluation and 91% overall specificity. The chosen cut-off value was lower than that suggested by the manufacturer (30 IU/ml), by which specificity increased to 95% but sensitivity lowered to 52%. EliA anti-dsDNA antibody cut-off corresponded to the value proposed by the manufacturer (15 IU/ml), obtaining a sensitivity and specificity for SLE of 81% and 92%, respectively. Farr cut-off value, assessed to 8 AU, gave 95% sensitivity with 89% specificity. Overall sensitivity and specificity obtained by CLIFT were 83% and 91%, respectively.

Diagnostic accuracy of the evaluated assays is comparatively visualised in Figures 2 and 3, expressed as sensitivity and specificity for the diagnosis of SLE. As you can see, Farr and CLIFT

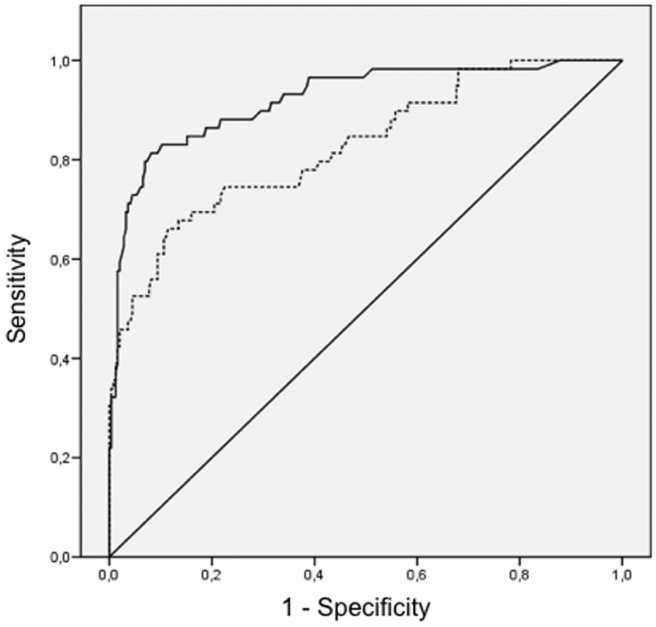

Fig. 1. ROC curves of EliA (continuous line) and Farrzyme (hatched line) test results in SLE patients at first diagnosis (n=59) and healthy/disease controls (n=161). Sensitivity/100 and (1- Specificity)/100 are plotted.

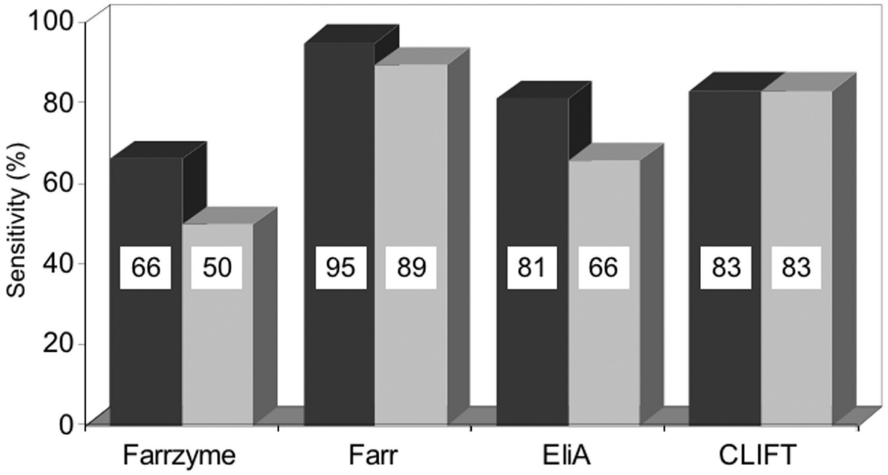

Fig. 2. Sensitivity (percent values) for SLE diagnosis of the anti-dsDNA antibody assays in 59 SLE patients at first visit (black bars) and 164 SLE patients during follow-up (grey bars).

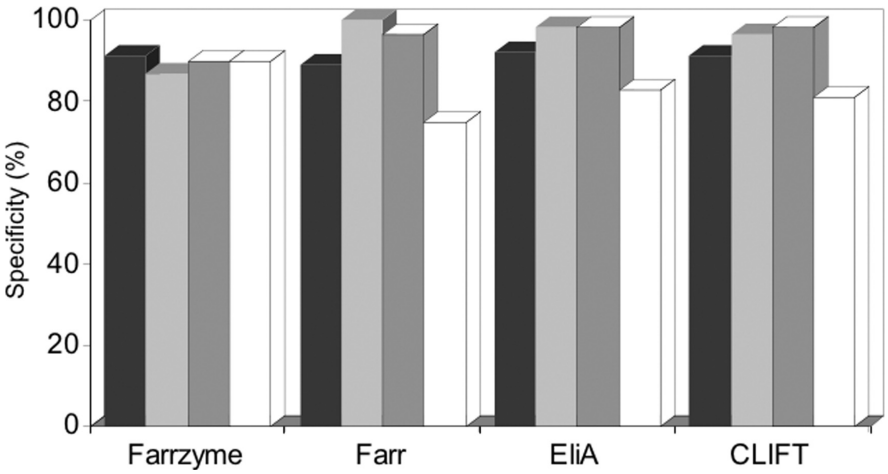

Fig. 3. Specificity (percent values) for SLE diagnosis of the anti-dsDNA antibody assays in overall controls (black bars), healthy subjects (grey bars), other diseases (dark grey bars) or other CTDs (white bars).

Table III. Farrzyme, EliA and CLIFT anti-dsDNA antibody levels in SLE patients with and without active disease (ECLAM > 2) or organ-specific involvement.

	Active disease		Renal involvement		Haematologic involvement		CNS involvement	
	Yes, n=146 mean±SD	No, n=74 mean±SD	Yes, n=82 mean±SD	No, n=129 mean±SD	Yes, n=39 mean±SD	No, n=154 mean±SD	Yes, n=24 mean±SD	No, n=188 mean±SD
Farrzyme (IU/ml)	300.7 ± 611.2*	32.5 ± 44.2	341.7 ± 729.5§§	131.2 ± 302.7	447.7 ± 825.2§§	169.9 ± 424.0	55.0 ± 171.4§	232.1 ± 545.9
EliA (IU/ml)	136.5 ± 151.7*	42.9 ± 68.3	148.7 ± 157.8§	79.6 ± 114.7	186.9 ± 160.6§	93.3 ± 126.7	32.8 ± 64.8*	115.4 ± 140.8
CLIFT (semiqu.)	2.7 ± 1.4*	1.8 ± 1.3	2.9 ± 1.3*	2.1 ± 1.5	3.1 ± 1.2§	2.4 ± 1.4	1.6 ± 1.5§	2.6 ± 1.4

EliA: fluoroenzyme immunoassay; CLIFT: indirect immunofluorescence on *Crithidia luciliae*; n.s.: not significant. Statistical significance: *$p<0.0001$; §$p<0.002$; §§$p<0.05$. Farr results were not included in the analysis because dichotomically expressed.

gave the highest sensitivity compared to Farrzyme and EliA; moreover, Farr and CLIFT sensitivity remained almost constant in SLE patients at first diagnosis and in patients with longer disease duration. Farrzyme and EliA sensitivity were lower compared with those of Farr or CLIFT and even reduced in SLE patients at follow-up compared with those at first evaluation.
As regards diagnostic specificity, approximately 10% false positives within controls was found with any one test, generally at levels approaching respective cut-off values; however, Farrzyme resulted more specific than the other assays towards patients with CTD other than SLE (92% by Farrzyme *vs*. 75% by Farr, 83% by EliA, 81% by CLIFT). Otherwise the other assays were more specific than Farrzyme with respect to healthy subjects or patients with other diseases (Fig. 3). Noteworthy, a proportion of positive anti-dsDNA antibody was found in the group of patients with rheumatoid arthritis under treatment with anti-TNFα agents, ranging from 7% by EliA, 13% by Farrzyme or Farr to 20% by CLIFT.
The clinical significance of serum anti-dsDNA antibody levels in SLE patients is reported in Table III. Antibody levels detected by Farrzyme, EliA or CLIFT were significantly higher in patients with active disease (ECLAM >2), renal or haematologic involvement during serum sampling, and significantly lower in patients with central nervous system involvement despite the small number of patients affected. In addition, anti-dsDNA level quantitation by Farrzyme or EliA, and titration by CLIFT were all significantly correlated with ECLAM score (correlation coefficients between 0.336 and 0.425, $p<0.0001$).
Whatever the method employed, anti-dsDNA antibodies did not demonstrate any relationship neither with other organ-specific involvements including thromboembolic manifestations, nor with specific antinuclear or antiphospholipid antibodies.

Discussion

The present study comparatively evaluated the diagnostic accuracy of two recently developed commercial ELISA assays, and two traditional methods (Farr and CLIFT) for anti-dsDNA antibody detection, differing with regard to their principle and performance design, and independently performed. A multicentre collaborative project was planned in order to update general guidelines for anti-dsDNA antibody detection as a serological criterion for SLE. To our knowledge, only few studies compared more than 2 independent anti-dsDNA assays in their clinical performance on multicentrally enrolled large patient cohorts (12, 17, 18).
Different tests have different specificity/selectivity towards multiple anti-dsDNA antibody types. The choice of DNA source and preparation in anti-DNA assays is crucial: human genomic DNA or pure dsDNA of adequate length (from 40 to hundreds of base pairs) have the best performance (19, 20). Anti-dsDNA antibody pathogenicity is strongly related to affinity maturation, IgG class or IgG/IgM ratio, complement activation, cross reactivity with glomerular basement membrane components (21-23). Anti-dsDNA antibody-mediated trafficking of nucleic acid fragments towards plasma membrane causing activation and secretion of inflammatory cytokines has been also hypothesized (24). The clinical usefulness of anti-DNA assays depends on their ability to determine specifically pathogenic autoantibodies and to measure them by a standardised quantitative approach (25, 26).
Besides the adoption of international guidelines, standard protocols and reference materials, there is no unique established approach to laboratory diagnostics of anti-dsDNA antibodies and new techniques are continuously developed for implementing the serological panel.
Solid-phase compared to liquid-phase immunologic techniques offer both advantages and limitations to autoantibody determination. Antigen-antibody interaction in liquid/solution phase has in general to be preferred because epitope conformational structures are preserved and antibody recognition optimised. In this context, Farr assay is generally considered the reference method for anti-dsDNA antibody: it preferentially, if not exclusively, measures levels of high avidity anti-dsDNA antibodies, and antibody fluctuations strictly correlate with disease relapses, especially nephritis (4, 27). However, the isotype is not defined and histone or nucleosome-containing immune complexes may also be responsible for

anti-dsDNA reactivity, mostly in sera from active SLE patients, thus causing apparent false positive results (28, 29). Moreover, nowadays, radioisotope manipulation is greatly discouraged (30).
Conversely, antigen immunobilisation on solid-phase matrices offers standardisation and automatisation facilities even though it may alter antigen native structure thus compromising antibody recognition (13).
CLIFT assay is likely to be accurate because nearly resembles the liquid-phase tests performance offering dsDNA in a native antigenic form; however, even though easy to perform, it is greatly operator-dependent and semiquantitative in antibody level measurement. Antibody quantitation by serum titration is considered inadequate in measuring changes in anti-dsDNA levels during disease course (4).
It has been demonstrated that Farr and classic ELISA are not equivalent due to their low concordance and correlation with clinical assessment (18, 27). In addition ELISA results are unsuitable for disease monitoring because they are not related to either global or organ-specific activity (27).
In the present study, solid-phase assays are not merely classic ELISAs which generally suffer from poor accuracy (17, 31), but newly developed implemented immunoenzymatic assays with some peculiarities which can increment their clinical performance. Farrzyme has been proposed as more specific for SLE than other ELISAs because it was developed specifically to detect high avidity antibodies by using a high ionic strength buffer as sample diluent (17); EliA is an automated fluorescent enzyme immunoassay in which antibodies to plasmid dsDNA are detected fluorometrically by a fluorocrome-conjugated secondary antibody. EliA performance as a diagnostic and monitoring tool seems to be better than the other tests according to some authors (18, 32-35), with the advantages of being automated, time saving and quantitative. However, there is not a complete agreement about it (36). The ROC curves with our data (Fig. 2) showed that the EliA assay performs better than the Farrzyme dsDNA test.

Besides the important differences in methodological variables summarised above (Table I), the evaluated assays apparently perform quite well in a clinical setting and in a comparable fashion, but in our opinion they should be improved especially in terms of specificity.
In spite of the efforts of many manufacturers to improve the accuracy of the methods for anti-dsDNA antibody detection, commercial assays still seem to suffer from a poor specificity, due to a false positivity ratio almost within low level positive sera. In this regard, Farrzyme could be promising for a better specificity than the other assays with respect to other CTD patients, and this is particularly important in the differential diagnosis of SLE. These data are in agreement with those reported by Jaekel *et al*. (17), the only one paper comparatively analysing the same assays in a similar fashion. Moreover, all the tests detected anti-dsDNA antibodies in about 10-20% of rheumatoid arthritis patients treated with TNF-α blocking agents. It is well known that anti-TNF-α therapy, especially infliximab, can induce both organ and non organ specific autoantibodies in patients with inflammatory arthropathies, only rarely associated with any specific clinical syndrome (37-41). Although the clinical significance of the induction of autoimmune phenomena is still unclear (41, 42), it has to be pointed out that even newly conceived assays can detect anti-dsDNA antibodies in anti-TNF-α treated patients.
As regards SLE clinical monitoring, the assays gave meaningful results, generally concordant one to the other in relation to global activity and organ specific involvement, mostly renal and haematologic, as expected. In addition, antibody levels correlated with global disease activity score measured by ECLAM score, in an independent manner. The association of the autoimmune response against DNA with CNS involvement during SLE is still controversial (43) and our results seem to exclude such a role or at least to show that circulating levels of anti-DNA antibodies are apparently unrelated to the occurrence/history of neurological manifestations.

It has to be pointed out that our study suffers from a limitation which regards the dichotomatous expression of data obtained by Farr assay. However, we decided to include the Farr results since they could contribute to the comparative evaluation of the assays' accuracy.
The multicentre validation of different currently available anti-dsDNA antibody assays for the laboratory confirmation of SLE diagnosis and monitoring showed that different laboratory strategies give comparable but not optimal results from a clinical point of view, mostly for their diagnostic specificity towards non-SLE systemic rheumatic diseases, which is still far from being clinically acceptable, eventhogh we aware that patients with other rheumatic diseases may show up with some borderline anti-dsDNA antibodies. Farrzyme better specificity towards other CTD could be promising in this regard, but its lower sensitivity in comparison to other assays compromises its diagnostic accuracy.
In conclusion, since a "gold standard" method does not exist at present, in interpreting anti-dsDNA antibody test results clinicians should be aware of suboptimal accuracy of currently available assays.

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