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Assessment of Deoxyribonuclease Activity in Serum Samples of Patients With Systemic Lupus Erythematosus: Fluorescence-Based Method Versus ELISA

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Background: We report the improvement of previously described method for determining deoxyribonuclease (DNase) activity in serum samples that uses a fluorescently labeled DNA fragment as a substrate *Methods:* Activity of serum DNase was analyzed in 31 patients with systemic lupus erythematosus (SLE) and 13 healthy individuals by fluoresence-based method and ELISA test *Results:* We found a mean decrease in DNase activity between cases and controls of 12.46% measured by the fluoresence-based method and of 12.21% measured by ELISA method. High level of positive correlation between two methods for DNase activity was observed: $P < 0.001$ and Pearson correlation coefficient 0.740. Decreased DNase activity was found in 25 of 31 SLE patients (81%) by fluoresence-based method and in 24 of 31 SLE patients (77%) by ELISA test. We also observed the significant positive correlation between titer of anti-dsDNA antibodies and DNase activity measured by both methods ($P < 0.05$). *Conclusions:* The key improvement is the use of internal control in the fluorescence-based method, which diminishes the influence of technical errors on the obtained results and increases reliability of the assay. This improved fluorescence-based method, with additional validation, may provide an alternative to more expensive and time-consuming conventional methods, such as ELISA. J. Clin. Lab. Anal. **30**:797–803, 2016. © 2016 Wiley Periodicals, Inc.

Key words: deoxyribonuclease activity; fluorescence; ELISA; systemic lupus erythematosus

INTRODUCTION

Overall deoxyribonuclease (DNase) activity in blood serum is dependent on the concentration and composition of DNA-degrading enzymes and factors influencing their activity. The most prominent serum DNase is DNase I, which is responsible for 90% of the total hydrolytic activity. The remaining 10% is contributed mainly to the acid DNase II, phosphodiesterase I, DNA hydrolyzing antibodies, and lactoferrin (1). DNases are involved in a wide range of cellular functions, including but not limited to apoptosis, DNA repair, replication recombination, and natural transformation (2, 3). In addition, blood DNase activity maintains the physiological level of circulating DNA via clearance of DNA that is released from dying or microbial cells, preventing immune stimulation. Impaired DNase function has been implicated in the pathogenesis of several autoimmune disorders: systemic lupus erythematosus (SLE), Sjogren's disease, thyroid autoimmunity and inflammatory bowel diseases (4–7). On the other hand, elevation of serum DNase activity has been

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observed in the early stage of acute myocardial infarction and may serve as a marker for diagnosis of cell death due to ischemic heart disease (8). Altered serum DNase activity has also been associated with several malignant diseases, such as lymphomas and breast, oral, stomach, colon, and pancreatic cancers (9–11). Apart from its use as a disease-related biomarker, measurement of DNase activity in human serum is helpful for adjusting the therapeutic dose of recombinant human DNase I used in the treatment of patients with SLE and cystic fibrosis (12).

SLE is a complex autoimmune disease characterized by a broad spectrum of autoantibodies directed to ubiquitous intracellular antigens (13). As many complex human diseases, SLE involves multiple, interacting genetic and environmental determinants. The role of DNase I in SLE has been investigated for decades, with growing interest since the description that apoptotic cells can be the source of self-antigens in SLE and the fact that DNase1 plays a key role in the clearance of apoptotic debris (13, 14). Decreased DNase I activity was reported in SLE patients in comparison to healthy controls (4, 5). In some SLE cases, the low DNase I activity can be explained by the presence of mutations in the gene encoding this enzyme, but they are very rare and do not explain the low DNase I activity found in most patients with SLE (15).

Given its diverse diagnostic and therapeutic applications, there is a clear necessity for a reproducible and nonlaborious method for DNase activity measurement. Previously described assays used for assessing the DNase activity in serum samples are mostly discontinuous and based on an enzyme-linked immunosorbent assay (ELISA), colorimetric assay, radial immunodifusion (RID) assay, or radial enzyme diffusion (RED) assay (16–19). All these assays are relatively expensive and time-consuming, and thus not suitable for processing a large number of samples. Continuous DNase activity assays have also been described, but these are not suited for use with complex biological samples (20).

The fluorescence-based method we have previously described was characterized by high reproducibility, high sensitivity, and relatively low cost, which could provide a supplement to currently available methods (21). The aim of this study was to improve previously described fluorescence-based method for assessment of DNase activity in serum samples and to compare it with ELISA test as validated method in routine clinical use.

PATIENTS AND METHODS

Study Samples

Our study included 31 consecutive SLE patients attending Departments of Dermatovenereology, Allergy and Clinical Immunology, and Nephrology at the Clinical Center of Serbia in Belgrade, Serbia, from December 2011 to April 2012. All patients fulfilled at least four revised criteria for SLE defined by the American College of Rheumatology (ACR) (22). The control group consisted of 13 healthy blood donors. The Ethical Committee of the Clinical Center of Serbia approved the study protocol and the informed consent was obtained from all subjects. Blood samples were centrifuged after collection and the sera were stored at –80°C until analysis.

Fluorescence-Based DNase Assay

Two fluorescently labeled fragments were prepared by polymerase chain reaction (PCR). The VIC dye labeled fragment was prepared by amplifying 214-bp-long fragment of ANKRD2 promoter cloned into pGL4.10 vector using the following primers: 5′-VIC-GGGTTTCCAGG CATCCAGCAGGTGGCACT-3′ and 5′-AGCAGAGC CAGTTGCCCCCCCAACTCCTG-3′. The 6-FAM dye labeled fragment was prepared by amplifying 200-bp-long fragment of SMAD4 promoter cloned into pGL4.10 vector using the following primers: 5′-ATCTTTTCC CAAGTAGTCAG-3′ and 5′-6-FAM-TGTTCAAGTTT TTCCTTTTA-3′. The VIC dye labeled fragment (green) was used as a substrate for serum DNase, while the 6-FAM dye labeled fragment (blue) was used as an internal size standard.

Amplification of both fragments was performed in a reaction mixture containing in a total volume of 100 μl: 50 ng of plasmid DNA, 2 U of FIREPol DNA Polymerase (Solis BioDyne, Tartu, Estonia), 1× Buffer B (Solis BioDyne), 2.5 mM $MgCl_2$, 0.2 mM of each of deoxynucleoside triphosphates (dNTPs), and 20 pmol of each primer. The amplification was performed under the following conditions: initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with the final elongation step at 72°C for 10 min. The obtained PCR product was purified using the GeneJET PCR Purification Kit (ThermoFisher Scientific, Waltham, MA).

Activity of serum DNase was determined by incubation of the sera samples with VIC dye labeled PCR fragment (green), followed by detection of digestion products by capillary electrophoresis on 3130 Genetic Analyzer (Applied Biosystems Corporation, Carlsbad, CA). The assay was performed in a reaction mixture containing in a total volume of 10 μl: 2.5 ng of VIC dye labeled fragment and 0.3 μL of serum sample. The negative control contained only the fragment, with no source of DNase activity. The reaction mixture was incubated for 75 sec at room temperature, after which the reaction was stopped by incubation at 75°C for 10 min. The obtained products were purified using the GeneJET PCR Purification Kit (ThermoFisher Scientific). Each sample subjected to fragment analysis contained 5 μl of the purified reaction

mixture, 0.3 μl of GeneScan-500 LIZ Size Standard (Applied Biosystems Corporation), 0.25 μl of the 6-FAM dye labeled fragment (blue), and 15 μl of HiDi Formamide (Applied Biosystems Corporation). Capillary electrophoresis was performed with POP-7 Polymer (Applied Biosystems Corporation), using the default genotyping module for the G5 dye set. The results were analyzed using the GeneMapper Software, version 4.0 (Applied Biosystems Corporation). DNase activity of serum samples measured as the fluorescence intensity of the undigested VIC dye labeled fragment (green) was normalized with the intensity of the 6-FAM dye labeled fragment (blue) as an internal control.

TABLE 1. Demographic and Serological Data of SLE Patients

	$n = 31$
Demographic data	
Age (years, mean ± SD)	34.7 ± 12.4
Age range (years)	13–56
Male gender (%)	6 (19.4)
New-onset SLE (%)	4 (12.9)
Duration of disease (years, mean)	3.2
Duration of disease (years, range)	0–14
Serological data	
Presence of antibodies against dsDNA-CLIFT (%)	29 (93.5)
Median 1/titer of antibodies against dsDNA (range)	80 (0–1,280)

ELISA

Activity of DNase I in all study samples was measured by a commercial ELISA kit (Orgentec Diagnostika, Mainz, Germany). In brief, the samples were added to a DNA-coated microplate, incubated for 1 hr at 37°C, and then washed. The horseradish peroxidase conjugated anti-DNase I substrate was added and incubated for 15 min at room temperature to react with the remaining DNA. After adding the substrate (TMB), the intensity of color developed during the enzymatic reaction was measured spectrophotometrically at 450 nm. Standard sera, as well as positive and negative controls, were included in the analysis. The amount of color was inversely proportional to the DNase I activity. According to the manufacturer's instructions, study with serum samples from healthy blood donors defines the cut-off value for the significant enzyme activity reduction. Therefore, the DNase I activity below 61.82 ± 12.51%/mL was considered as decreased.

Detection of Anti-dsDNA Antibodies

The presence and concentration of IgG anti-dsDNA antibodies were determined by an indirect immunofluorescence (IIF) assay on *Crithidia luciliae* substrate (CLIFT, Viro-Immun Labor-Diagnostika, Oberursel, Germany) where the pure, circular dsDNA of the kinetoplast was the antigen. Anti-dsDNA titers higher than 1:10 were considered positive. The specimens were analyzed using fluorescence microscope with incident light illumination (Euroimmune, Germany) at magnification 200× and 400×.

Statistical Analysis

The data were analyzed using the statistical program SPSS 17.0 for Windows (SPSS, Chicago, IL). The data were first analyzed by descriptive statistics and values were expressed as mean ± SD. Distribution of values was checked using the Kolmogorov–Smirnov test. Since the parameter DNase I activity was found not to have a normal distribution in the investigated groups, Mann–Whitney *U* test was used for comparison of DNase I activity between patients and healthy controls. For the characterization of the correlations between two DNase I activity methods and between DNase I activity and anti-dsDNA IgG concentration, the Pearson correlation coefficient was determined. Statistical associations were considered significant if *P*-values were less than 0.05.

RESULTS

The activity of serum DNase was determined in 44 samples (31 from SLE patients and 13 from healthy healthy blood donors) by two different methods, fluorescence-based method and ELISA. The main characteristics of all study subjects are shown in Table 1.

The fluorescence assay was based on use of VIC dye labeled fragment as a substrate for serum DNase and capillary electrophoresis of the obtained products. Signal intensities of 214-bp-long VIC dye labeled fragment (green) were registered and the values were normalized with the signal intensity of the 196-bp-long 6-FAM dye labeled fragment (blue) used as internal standard (Fig. 1). DNase activity was assessed based on reduction in normalized signal intensity. The mean normalized signal intensity was 40.39 ± 13.83 for SLE patients and 52.85 ± 19.26 for the controls (Fig. 2A). The decrease of 12.46% was observed in DNase activity in patients with SLE compared to healthy individuals and the observed difference between the two groups was statistically significant ($P < 0.05$). Decreased DNase I activity was found in 25 of 31 SLE patients (81%). The average DNase I activity in SLE patients' sera obtained by ELISA test was also significantly lower than in healthy controls: 49.61 ± 16.90%/mL versus 61.82 ± 12.51%/mL, $P < 0.05$ (Fig. 2B). The decrease of 12.21% was observed in DNase activity measured by ELISA in patients with SLE compared to healthy individuals. Decreased DNase I activity was found in 24 of 31 SLE patients (77%).

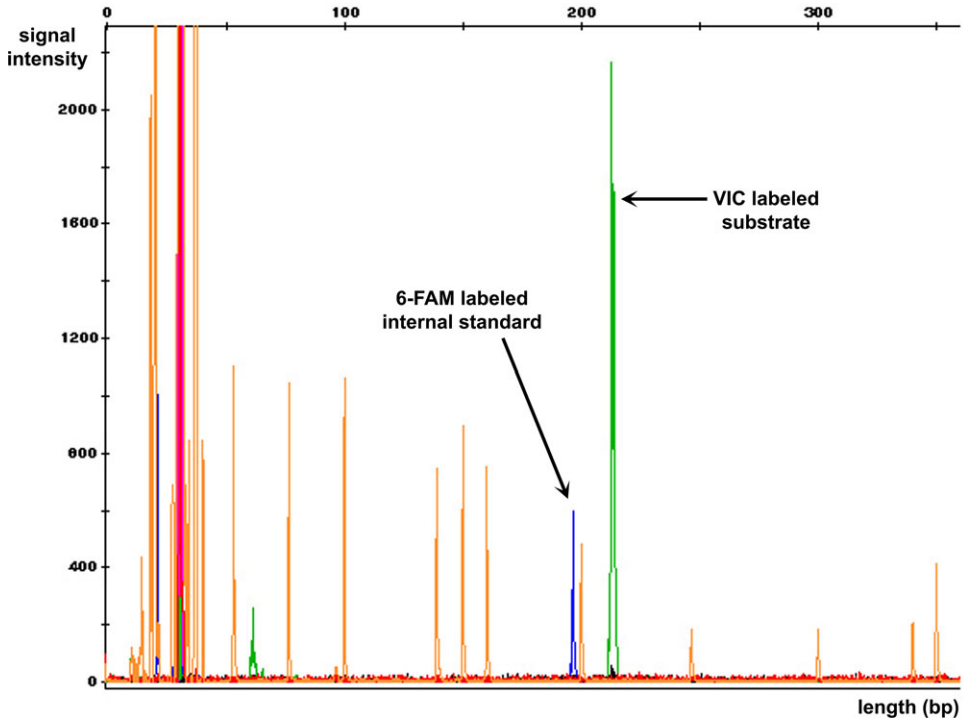

Fig. 1. Output result of the fluorescence-based assay for one representative sample.

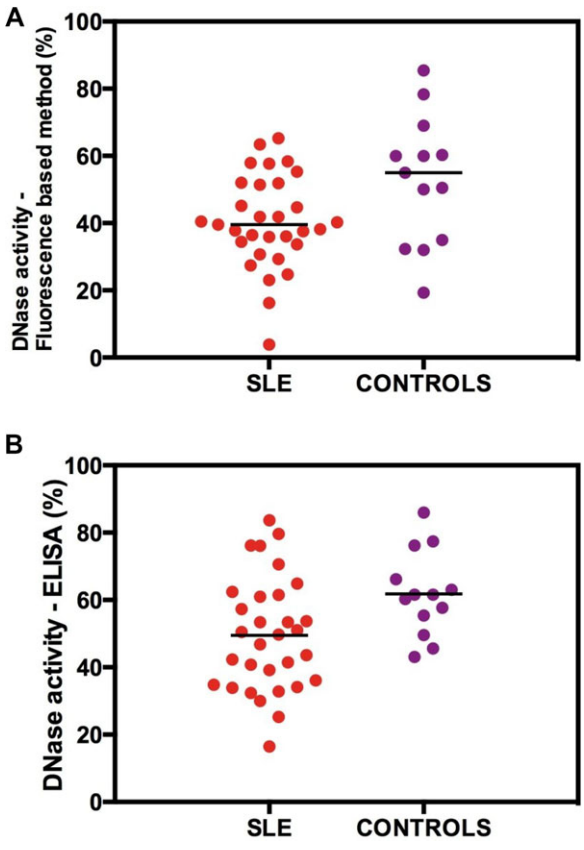

Fig. 2. The average DNase activity in serum of SLE patients and controls obtained by fluorescence-based method (A) and ELISA (B).

We found high level of positive correlation between the two methods for measurement of DNase activity: $P < 0.001$ and Pearson correlation coefficient 0.740 (Fig. 3). We also observed the significant positive correlation between titer of anti-dsDNA antibodies and DNase I activity measured by both methods, fluorescent assay ($P < 0.05$, Pearson correlation coefficient 0.364) and ELISA ($P < 0.05$, Pearson correlation coefficient 0.434; Fig. 4).

DISCUSSION

We have previously described the method for determining DNase activity in serum samples that uses a fluorescently labeled DNA fragment as a substrate for DNase (21). Here we report the improvement of this method and its validation by comparison with commonly used ELISA method.

The key feature of the developed assay is the use of fluorescently labeled fragment as a substrate for DNase, which enables highly sensitive measurements (10^{-3} U/mL), as reported previously (20). Substrates used for other assays, namely DNA-methyl green for the colorimetric assay, ethidium bromide or SYBR green for RED assay, and biotinylated DNA bound to avidin-coated wells for ELISA, provide significantly lower sensitivity (16–19). The RED assay the highest sensitivity of 10^{-4} U/mL; however, this assay is susceptible to interference by DNase I inhibitor, which can affect the accuracy of the measurement and it

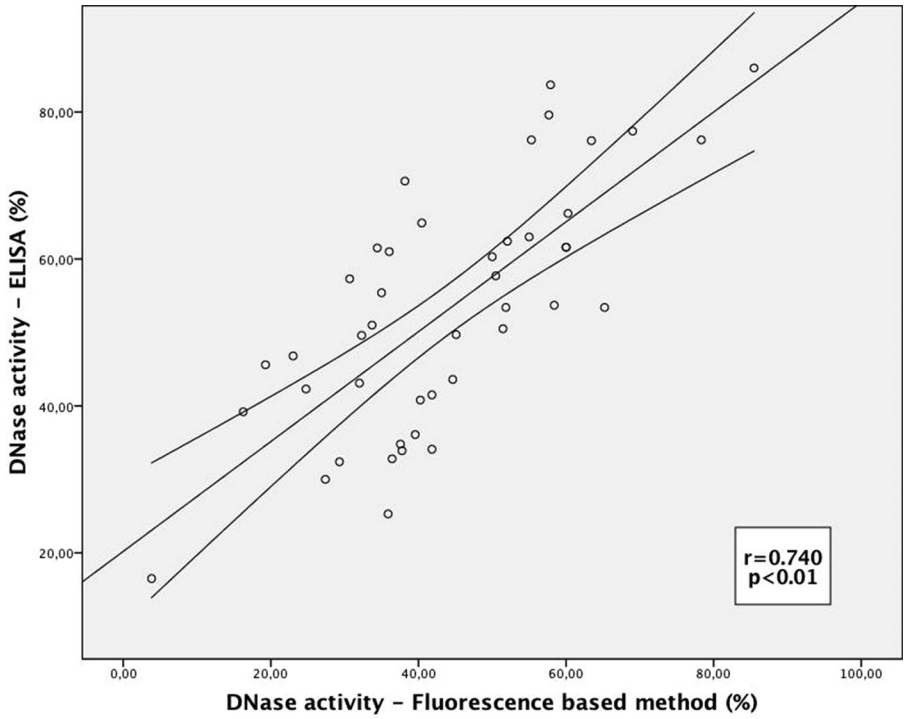

Fig. 3. Correlation between DNase activity measured by fluorescence-based method and ELISA.

takes 16 hr to perform (19). In comparison to the previously reported assay, a significant improvement has been made regarding the fluorescence detection, with internal control fragment used for normalization of the results (21). The use of internal control diminishes the influence of technical errors, such as pipetting, on the obtained results and increases reliability of the assay. This improvement has enabled the use of fluorescence-based method not only for analysis of large sets of samples with the same sensitivity as ELISA test, but also its potential use as a screening tool in clinical practice. The purification of the obtained fragments before analysis by capillary electrophoresis adds time and cost to the method, but is necessary due to high sensitivity of the detection technique applied in order to avoid potential interference of the impurities remained in the mixture. However, further validation of the method on larger sets of samples is required in order to verify if the method is applicable in clinical practice and to estimate its cost benefit.

In this study, DNase activity measured by the two different methods (fluorescence-based method and ELISA) in patients diagnosed with SLE was significantly lower ($P < 0.05$) than in the control group. We have found a mean decrease in Dnase activity of 12.46% measured by the fluoresence-based method and of 12.21% measured by ELISA method. High level of positive correlation between two methods for DNase activity was observed ($P < 0.001$, Pearson correlation coefficient 0.740). This result indicates that fluorescence based method for DNase activity measurement is as sensitive as ELISA to detect differences between the patients with SLE and healthy individuals. The advantage of the fluorescence-based method over the ELISA method is the small volume of sample necessary for the analysis and less time required for processing of the samples. Therefore, this method can be used as an additional tool for assessment of DNase activity in patients with SLE.

A significant positive correlation between DNase activity and the titer of anti-dsDNA antibodies was also obtained. This correlation is important because it was demonstrated that the monitoring of DNase I activity, together with anti-dsDNA antibodies, might be a useful tool in the followup of SLE patients (4). Antibodies with catalytic properties (abzymes) that could hydrolyze DNA have been isolated from the sera of patients with SLE and of other autoimmune diseases (23). Abzymes could belong to the group of anti-dsDNA antibodies. This could explain our finding that there was a statistically significant positive correlation between the serum DNase I activity and the concentration of anti-dsDNA antibodies.

The developed and improved DNase activity assay based on fluorescence can be used for research purposes, but is also potentially applicable in clinical practice. The method we describe requires a small volume of sample, is

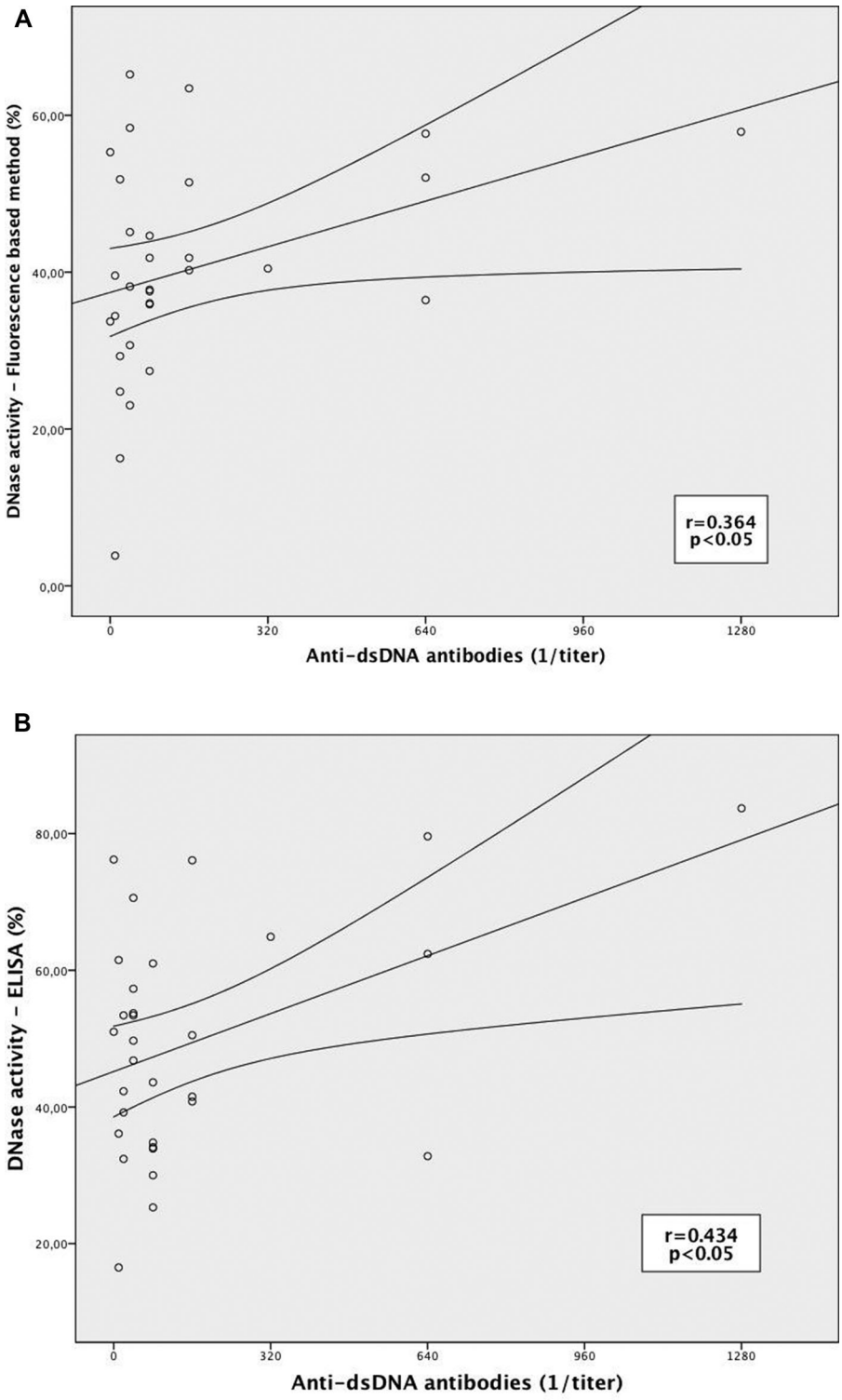

Fig. 4. Correlation between the concentration of anti-dsDNA antibodies and DNase activity in serum of SLE patients obtained by fluorescence-based method (A) and ELISA (B).

simple to perform, and the results are reproducible. This improved fluorescence-based method, with additional validation, may provide an alternative to the conventional methods that are more expensive and time-consuming, such as ELISA. The improvement achieved with the use of internal control for the normalization of results has enabled potential use of this method as a screening tool in clinical practice, especially for followup of DNase activity as a serum biomarker. Although the assay described here was designed for the measurement of DNase activity in serum samples, it may be adaptable to measurement of DNase activity in a variety of biological specimens.

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