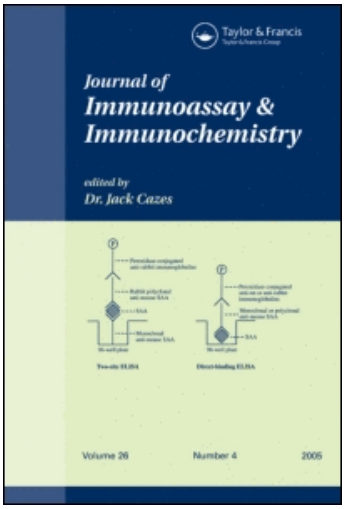

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Evaluation of a Microsphere-Based Flow Cytometric Assay for Diagnosis of Celiac Disease

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ABSTRACT

The multiplexed particle-based flow cytometric technology proposes a new approach for the diagnosis of autoimmune diseases combining the advantages of conventional methods with the ability to quantitatively determine multiple autoantibodies in the same sample, simultaneously and rapidly. Recently, a commercial kit (FIDIS Celiac, Biomedical Diagnostics, Marne la Vallé, France) was introduced for the simultaneous detection of IgA anti-tissue

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transglutaminase (anti-tTG), IgG, and IgA anti-gliadin antibodies (AGA). This study was undertaken to evaluate and compare the FIDIS Celiac kit with standardized commercial ELISAs (QUANTA Lite, INOVA Diagnostics Inc., San Diego, CA). A disease group consisted of 21 samples from untreated patients with biopsy confirmed celiac disease (CD), and two control groups of historical sera (207 from regular blood donors and 181 from chronically infected hepatitis patients) were studied. All control sera were negative for IgA anti-endomysial antibodies (EmA) and had an IgA concentration above the lower normal limit. Concerning the reproducibility, intra- and inter-assay coefficients of variation (CVs) ranging between 2% and 12%, and between 3% and 21%, respectively, were observed. Regarding the diagnostic quality, each assay was compared to the disease diagnosis using the McNemar test and the kappa (*K*) parameter, while ROC analysis was applied. Generally, the performance of FIDIS assay was proved almost equally adequate to that of ELISA in the detection of IgA anti-tTG antibodies, IgA and IgG AGA. However, the performance of FIDIS assay was found surmounting that of ELISA among hepatitis patients, possibly due to the avoidance of debris and unbound cross contaminants and, hence, the "noise" of such materials in samples under analysis. Taking our results together with the simplicity and the high throughput of FIDIS assay, its overall performance in the diagnosis of CD seems better than that of ELISA.

Key Words: Flow cytometry; Celiac disease; ELISA; Microspheres.

INTRODUCTION

The identification of tissue transglutaminase (tTG) as the main antigen of the anti-endomysial antibodies (EmA)[1] led to the development of a series of ELISAs for the detection of IgA anti-tTG antibodies. These tests were currently evaluated and introduced in the screening and the initial diagnostic workup of celiac disease (CD).[2–5] Previous serologic tests for CD with lower specificity (Sp), like the IgA anti-reticulin antibodies, have already been abandoned. As an exception, the IgG anti-gliadin antibodies (AGA) remain useful for the disease screening among individuals with IgA deficiency and children under 2 years of age, as well as for monitoring the efficacy of a gluten-free diet.[6,7]

However, a lot of problems concerning the standardization of the different anti-tTG assays remain as yet unresolved, and conflicting results about their diagnostic performance in special disease groups have appeared in the literature.[6,8,9] For example, a high proportion of false positive anti-tTG results have been reported among patients with liver diseases that have been attributed either to the concurrent hypergammaglobulinemia and hypertransaminasemia or to the presence of autoantibodies, which probably interfere with the tTG antigen.[10,11]

Herein, it must be underlined that, among patients with chronic liver diseases, those with HCV infection are also presented with a high prevalance of CD.[12] Given the moderate to high incidence of HCV infection in the general population, a possibly lower diagnostic accuracy of the anti-tTG ELISAs in this group of patients might compromise their use as a screening test for CD.

Recently, a microsphere-based flow cytometric assay was introduced for the simultaneous measurement of the IgA anti-tTG antibodies, IgA AGA, and IgG AGA (FIDIS Celiac kit, Biomedical Diagnostics, Marne la Vallée, France). Beyond its technical simplicity and rapidity, the principle of this assay has raised hopes that many of ELISAs' problems and limitations can be overcome.[13,14] However, the diagnostic performance of this assay has not been evaluated in the context of the clinical diagnosis.

The aim of this study was to evaluate the performance of the above assay in CD diagnosis, in parallel with three commercial ELISAs for the quantification of the same antibodies (QUANTA Lite, INOVA Diagnostics Inc., San Diego, CA).

EXPERIMENTAL

Patients

Three groups of historical sera conserved in $-80^{\circ}C$ were studied. The disease group consisted of 21 samples collected from patients with biopsy confirmed CD before gluten withdrawal. Three of these patients were children with a medium age of 6 years (range 2–10) and 18 were adults (12 women and 6 men) with a medium age of 35 years (range 18–53). All these sera were positive for IgA anti-EmA antibodies.

Two sera groups served as controls. The first one included 207 regular blood donors (119 men and 88 women with a medium age of 38 years, range 18–55) and the second consisted of 181 chronically infected hepatitis patients (125 with HBV and 56 with HCV infection; 78 men and 103 women with a medium age of 42 years, range 16–58). In both control groups, CD was excluded on the basis of IgA EmA negativity and individuals' clinical history, which was available as their sera were collected in the context of a relevant epidemiological study. IgA deficiency was also excluded after the nephelometric determination of IgA (BN II, DADE-Behring, Marburg, Germany).

Detection of Anti-EmA

IgA EmA were detected by indirect immunofluoresence, using commercial slides of primate distal esophagus (INOVA Diagnostics, Inc.). All sera were

tested according to the manufacturer instructions, at a 1 : 5 and 1 : 10 dilution, with the induction of positive and negative controls in every batch of tests.

FIDIS Microsphere-Based Flow Cytometric Assay

The principle and the procedure of the FIDIS microsphere-based flow cytometric assays have been recently described in details by Rouquette et al.[15] In brief, the FIDIS technology employs uniform polystyrene microspheres as a solid phase for the capture of the antigens. Microspheres are internally dyed with a distinct mixture of two fluorophores, so that different microsphere sets can be discriminated on the basis of their emission signal intensities. In FIDIS Celiac kit, the three target antigens are coupled on different microsphere sets forming, in this way, the basis of the individual assays. Product formation of the antibodies with their respective capture antigens on the different microsphere sets can be detected with a fluorescence-based reporter system. Anti-tTG antibodies are measured against human recombinant tTG. The detection of IgA anti-tTG antibodies and IgA AGA is performed using two combined bead sets, while IgG AGA is detected in another well, adding a different conjugate.

Sera samples, positive and negative controls are prediluted (1 : 201) in PBS–Tween buffer. Microsphere reagent is added into each one of the 96 wells of a plate designed to work as a strainer for holding within the microspheres. Then, prediluted sera and controls, as well as the calibrator, are added in each well and left, covered, to interact with the microspheres for 30 min at room temperature. Two washing steps follow into a reaction tray. Thereafter, 1 : 51 diluted conjugate is added in each well, the plate is covered, incubated for 30 min at room temperature, and finally, counted by a Luminex 100 system (Luminex, Austin, TX). A XYP plate handler is programmed to analyze each 96 well plates.

ELISAs

Three commercial ELISAs for the quantification of the IgA anti-tTG, IgA, and IgG AGA ELISA antibodies were used (QUANTA Lite, INOVA Diagnostics Inc.). The IgA anti-tTG ELISA by INOVA uses human tTG isolated from red blood cells and has been proven as an acceptable assay.[16–18]

The IgA anti-tTG, IgA, and IgG AGA ELISA tests for each serum sample were performed in different plates but at the same time, using a fully automated system (ELISA analyzer DSX, Labtechnics, Thermolab System, Virginia, USA) according to the manufacturer instructions.

Statistical Analysis

The reproducibility of all assays was determined by measuring a low and a high concentration sample 20 times within the same run and in 20 consecutive runs.

The diagnostic quality of each method was separately evaluated in both a continuous scale and in binary form. In the binary form, cutoff points were defined independently in the group of blood donors, as well as in the group of hepatitis patients. A cutoff point was determined as the concentration value that maximizes the sum of the Sp and sensitivity (Sn), weighting equally their significance.[19] Based on cutoff points, each assay was compared to the disease diagnosis using the McNemar test and the kappa (K) parameter.[20] In the continuous scale, ROC analysis was applied to the individual concentrations.[21] All these procedures were performed using SPSS (release 11, SPSS Inc., Chicago, IL).

RESULTS

The intra- and inter-assay coefficients of variation (CVs) were ranged between 2% and 12%, and between 3% and 21%, respectively. In general, the reproducibility of ELISAs was found somehow better than that of FIDIS and CVs; this is evidenced by the fact that the values for high concentration sample were lower than those of the low one.

The results of the evaluation are presented in Tables 1, 2, 3. Only ROCs referring to the determination of the IgA anti-tTG antibodies by both assays are presented in Fig. 1. It must be underscored that the cutoff values estimated in the setting of this study were substantially different from those proposed by both manufacturers, giving as cutoff value for all tests the concentration of 20 AU/mL. Generally, the performance of FIDIS assay was proved almost equally adequate to that of ELISA in the detection of IgA anti-tTG antibodies, IgA, and IgG AGA. Titers of antibodies lower than their cutoff values were not found in any of the celiac patients. Finally, the performance of FIDIS assay was found surmounting that of ELISA among hepatitis patients.

DISCUSSION

In this study, the evaluation of a newly introduced microsphere-based flow cytometric assay (FIDIS Celiac kit) was performed for the diagnosis of CD in parallel with ELISA. Taking our results, together with the simplicity (fewer washing steps, etc.) and the high throughput of FIDIS technology, its

Table 1. The characteristics of diagnostic quality of the FIDIS assay and of INOVA ELISAs for the detection of IgA anti-tTG antibodies in blood donors and hepatitis patients groups.

	Blood donors		Hepatitis patients	
	ELISA	FIDIS	ELISA	FIDIS
AUC	0.998 (0.994–1.00)	0.992 (0.983–1.00)	0.99 (0.98–1.00)	0.995 (0.988-1.00)
Cut off (AU/mL)	25.43	69.5	26.2	74
Sn	1	1	1	1
Sp	0.99 (0.976–1.00)	0.95 (0.92–0.98)	0.95 (0.927–0.974)	0.96 (0.931–0.989)
PPV	0.91 (0.793–1.00)	0.68 (0.516–0.844)	0.68 (0.516–0.844)	0.75 (0.59–0.91)
NPV	1	1	1	1
LR(+)	100	20	20	25
LR(−)	0	0	0	0
Efficiency	0.99 (0.977–1.00)	0.96 (0.935–0.986)	0.95 (0.92–0.98)	0.97 (0.974–0.994)
K value	0.95 (0.88–1.02)	0.79 (0.65–0.92)	0.78 (0.65–0.91)	0.84 (0.72–0.95)
Agreement[a]	Excellent	Excellent	Excellent	Excellent

Note: Sn, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; LR, likelihood ratio. Numbers in parentheses signify 95% confidence intervals (CI).

[a]Excellent agreement stands for *K* values $\geq$0.75, good for *K* values between 0.40 and 0.75, and poor for *K* values $\leq$0.40.

Table 2. The characteristics of diagnostic quality of the FIDIS assay and of INOVA ELISAs for the detection of IgA AGA in blood donors and hepatitis patients groups.[a]

	Blood donors		Hepatitis patients	
	ELISA	FIDIS	ELISA	FIDIS
AUC	0.809 (0.703 − 0.915)	0.855 (0.804 − 0.905)	0.837 (0.755 − 0.919)	0.899 (0.857 − 0.942)
Cut off (AU/mL)	4.15	50	4.12	50
Sn	1	1	1	1
Sp	0.33 (0.266 − 0.394)	0.77 (0.712 − 0.828)	0.51 (0.437 − 0.583)	0.85 (0.798 − 0.902)
PPV	0.13 (0.078 − 0.182)	0.75 (0.59 − 0.91)	0.19 (0.116 − 0.264)	0.43 (0.291 − 0.569)
NPV	1	1	1	1
LR(+)	1.49	4.35	2.04	6.67
LR(−)	0	0	0	0
Efficiency	0.39 (0.326 − 0.454)	0.79 (0.737 − 0.843)	0.56 (0.492 − 0.628)	0.86 (0.812 − 0.908)
K value	0.14 (0.05 − 0.23)	0.41 (0.26 − 0.56)	0.18 (0.05 − 0.31)	0.53 (0.37 − 0.69)
Agreement	Poor	Good	Poor	Good

[a]See notes of Table 1.

Table 3. The characteristics of diagnostic quality of the FIDIS assay and of INOVA ELISAs for the detection of IgG AGA in blood donors and hepatitis patients groups.[a]

	Blood donors		Hepatitis patients	
	ELISA	FIDIS	ELISA	FIDIS
AUC	0.886 (0.822 − 0.95)	0.813 (0.715 − 0.911)	0.881 (0.807 − 0.955)	0.874 (0.791 − 0.957)
Cut off (AU/mL)	5.79	27.5	5.79	8.5
Sn	1	0.71 (0.516 − 0.904)	1	0.86 (0.712 − 1.0)
Sp	0.58 (0.512 − 0.648)	0.84 (0.79 − 0.89)	0.46 (0.387 − 0.533)	0.79 (0.731 − 0.849)
PPV	0.2 (0.124 − 0.276)	0.32 (0.187 − 0.453)	0.18 (0.111 − 0.249)	0.32 (0.199 − 0.441)
NPV	1	0.97 (0.945 − 0.995)	1	0.99 (0.974 − 1.0)
LR(+)	2.38	4.44	1.85	4.10
LR(−)	0	0.35	0	1.18
Efficiency	0.62 (0.557 − 0.683)	0.83 (0.781 − 0.879)	0.52 (0.451 − 0.589)	0.79 (0.914 − 1.00)
K value	0.2 (0.07 − 0.34)	0.36 (0.17 − 0.54)	0.15 (0.03 − 0.27)	0.36 (0.19 − 0.54)
Agreement	Poor	Poor	Poor	Poor

[a]See notes of Table 1.

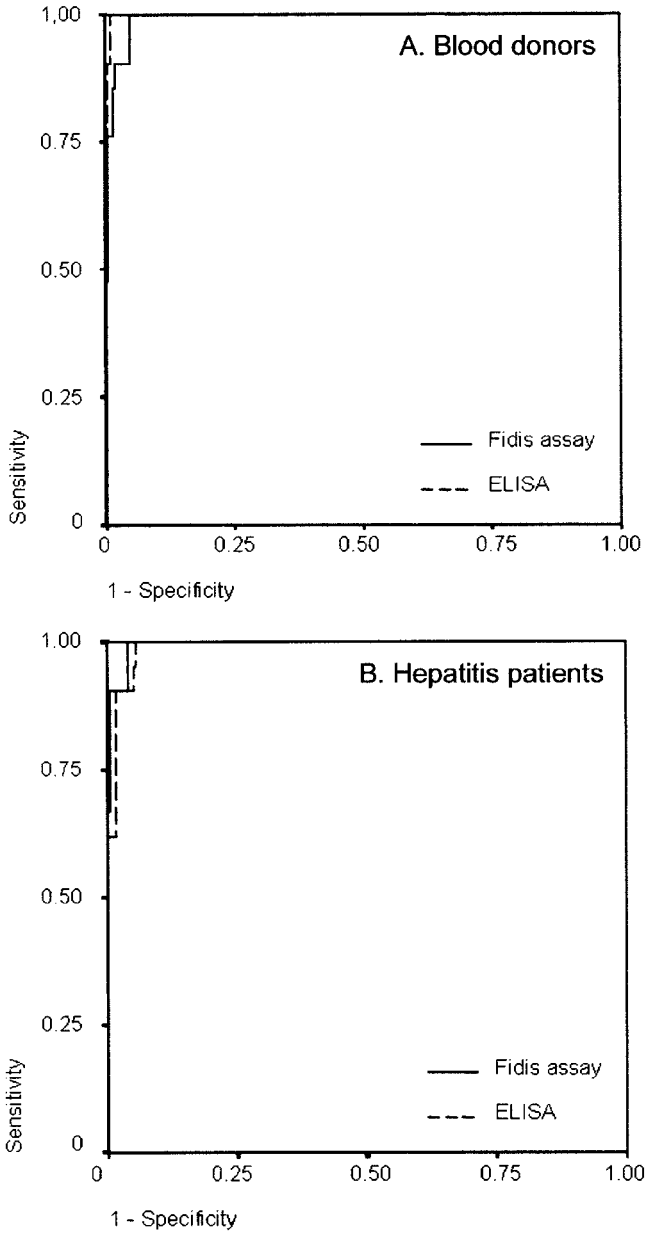

Figure 1. The ROCs of the FIDIS assay and the ELISA for the detection of IgA anti-tTG antibodies.

overall performance in the diagnosis of CD seems better than that of ELISA. Its advantage was revealed while assaying sera from hepatitis patients. In these sera, it is usually difficult to perform immunological assays due to matrix effect, which causes many false positive results.[22] The FIDIS technology avoids debris and unbound cross contaminants and, hence, the "noise" of such materials in samples under analysis. Furthermore, the FIDIS system provides a high signal-to-noise ratio for the detection of low level phycoerythrin fluorescence, an advantage that ELISA cannot attain. In ELISAs, the whole process of reaction, as well as the measurements, take place in the same well, where cross-contamination is rather unavoidable. In an attempt to reduce this defect, the INOVA ELISA for IgA anti-tTG antibodies is using human purified tTG and buffers containing calcium ions. These modifications help the tTG to attain the necessary conformation so as to be recognized by the highest amount of anti-tTG antibodies.

The fact that estimated cutoff values were substantially different than those proposed by manufacturers, underlines the utmost significance of the cutoff determination according to the clinical setting that has to be served by these assays. Obviously, mass screening for CD necessitates lower cutoff values in order to achieve higher Sn. The problem, however, presents quite differently when these assays are used for the purposes of diagnosis. As it is shown from our study, interferences like those produced by the presence of chronic hepatitis, result in antibody titers higher than those observed in normal samples. Despite the fact that FIDIS technology seems released from such interferences, this might also be the reason in the many cases of CD co-morbidity with other common conditions, like rheumatic diseases, thyroid autoimmunity, etc., where similar interferences could be produced by the presence of irrelevant autoantibodies or other substances.

Unfortunately, EmA negative celiac patients were not available in the archive of our Department, neither was it ethical to perform biopsies in the blood donors and in the EmA negative hepatic patients without any other clinical or laboratory indication beyond anti-tTG antibodies. Therefore, the performance of the FIDIS assay in this increasingly recognized group of CD patients[23] remains to be elucidated.

As far as the determination of the IgA and IgG AGA is concerned, our results confirmed their limited usefulness in CD diagnosis, as is already known.[24] So, the multiplexing approach of the FIDIS Celiac assay seems, at this point, inappropriate. A more appropriate design of a FIDIS assay against CD diagnosis could be the coupling of tTG antigen of different sources (species, purified human or recombinant), or parts of this antigen (peptides, neoepitopes, chemically manipulated proteins), to the microspheres. In this way, it might be possible that the whole antibody spectrum against the autoantigen will be detected. Furthermore, bearing in mind the co-morbidity

of organ specific autoimmune diseases, a FIDIS assay designed to concomitantly determine a panel of relevant autoantibodies, including those of CD,[7,25] could also be of diagnostic interest.

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