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Combination testing for antibodies in the diagnosis of coeliac disease: comparison of multiplex immunoassay and ELISA methods

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SUMMARY

Background—Tissue transglutaminase (TTG) antibodies and newly developed deamidated gliadin peptide (DGP) antibodies have better accuracy than native gliadin antibodies. Multiplex immunoassay (MIA) measures multiple antibodies simultaneously providing a complete antibody phenotype with reduced turnaround time and cost.

Aim—To evaluate the agreement between MIA and enzyme-linked immunosorbent assay (ELISA) test results for coeliac antibodies in biopsy-proven coeliac patients and controls and to model the diagnostic utility of combination testing.

Methods—We compared the sensitivity, specificity and accuracy of MIA and ELISA methods for TTG and DGP antibodies in mainly adult untreated coeliac patients ($n = 92$) and controls ($n = 124$).

Results—There was excellent agreement and a significant correlation between the results of MIA and ELISA methods ($\kappa > 0.8$, $r > 0.7$) for all tests, except TTG IgG. Diagnostic indices of individual and combination tests measured by the MIA method did not differ significantly from those measured by ELISA. The combination tests slightly increased sensitivity (if any test was positive) and specificity (if all tests were positive) compared to the individual tests.

Conclusions—Multiplex immunoassay testing for antibodies is as accurate as ELISA for coeliac disease diagnosis and has practical advantages over ELISA method. Rational combination testing can help identify patients who need intestinal biopsy and may reduce unnecessary biopsies.

INTRODUCTION

Coeliac disease (CD) is a gluten sensitive enteropathy that is diagnosed by demonstration of villous atrophy in histopathological examination of a small intestinal biopsy and clinical or histological response to gluten exclusion.¹ Less-invasive tests for detection of CD are desirable because of the high prevalence and diverse clinical manifestations of CD and the expense and inconvenience associated with small intestinal biopsy.^{2–4} Serological tests are often used to screen for CD and to identify those patients who need small intestinal biopsy. There is controversy regarding the ideal serological test(s) for the diagnosis and follow-up of CD. This often tempts clinicians to order multiple tests simultaneously, which can lead to increased costs.

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In addition, the clinician may be faced with uncertainty regarding how to interpret some possible combinations of test results.

Multiplex immunoassay (MIA) is a new technology, which enables measurement of multiple antibodies simultaneously. This technology uses a series of antigen-coated particles with distinct fluorescent signatures to detect simultaneously multiple antibodies in a single sample. MIA technology uses smaller sample volumes and much less technologist time to provide a series of results.⁵ However, there are no reports evaluating the results of MIA technology for antibodies in the diagnosis of CD except for a single small study.⁶

The most common serological tests for initial screening of CD are tissue transglutaminase (TTG) and gliadin antibodies used in various combinations with no clear standardization.^{7, 8} Because of the limited diagnostic accuracy of gliadin antibodies, new guidelines recommend using only TTG immunoglobulin A (IgA) as the initial test for CD screening.⁹ Recent studies have suggested that antibodies reactive with deamidated gliadin peptides (DGPs) are more sensitive and specific than conventional gliadin antibody testing, and are comparable to TTG IgA.¹⁰⁻¹⁵ Nonetheless, the additional diagnostic value of this new test over TTG IgA and the diagnostic value of combination testing have not been fully validated in a large population of CD patients with a wide range of mild and severe histological damage.^{16, 17} The aim of this study was to evaluate the agreement between MIA and enzyme-linked immunosorbent assay (ELISA) test results for TTG and DGP IgA and IgG antibodies in a large series of untreated biopsy-proven CD patients and controls. We also modelled the diagnostic utility of combination testing for TTG and DGP antibodies by both methods.

METHODS AND MATERIALS

Study population

The study population included patients who had undergone small intestinal biopsy at the Mayo Clinic Rochester between January 1999 and December 2006 because of gastrointestinal (GI) symptoms, unexplained anaemia or weight loss, or risk factors for CD. Serum samples were collected from all patients and stored at -70°C . The study was approved by the Institutional Review Board of Mayo Clinic, Rochester, MN.

Patients who had a saved serum sample within 6 months before and 3 months after intestinal biopsy and had histopathological evidence of CD with some degree of villous atrophy (enteropathy type IIIa or greater based on currently accepted Marsh criteria)^{18, 19} were categorized as biopsy-proven coeliac group. Of these, patients who had started a gluten-free diet for more than 2 weeks prior to serum sample collection were excluded (all patients were totally untreated except one who was treated for only 2 weeks).

Controls were selected randomly from patients who did not have any degree of enteropathy based on histopathological examination of small intestinal biopsy (Marsh 0) using a frequency matching for age and gender. Patients with high clinical suspicion for CD despite a normal biopsy ($n = 1$) and those who did not authorize research use of their information ($n = 2$) were excluded from the control group. As isolated intraepithelial lymphocytosis (Marsh I) is neither specific for CD nor a normal condition, patients with Marsh I enteropathy ($n = 8$) were excluded from both the coeliac group and control group.²⁰⁻²²

Based on these inclusion and exclusion criteria, 92 biopsy-proven untreated coeliac patients and 124 biopsy-proven controls were identified. The following characteristics of all subjects were extracted from the medical records: age, gender, presence of GI symptoms (diarrhoea, abdominal pain, bloating, dyspepsia or constipation), malnutritional disorders (anaemia, osteoporosis, osteopenia or osteomalacia), diabetes (both type-1 and type-2 diabetes), thyroid

disorders (hypothyroidism, hyperthyroidism, Hashimoto thyroiditis, Grave's disease, thyroid nodules, goiter or thyroid cancer), dermatitis herpetiformis and family history of CD.

Serology

All samples were stored at -70°C until testing. All samples were vortexed upon thawing, and maintained at 4°C when not actively being analysed. Samples were tested in accordance with the specifications of the kit's manufacturers and each run was checked against stated quality control requirements. All patients had previously been tested for antibodies against DGP (QUANTA Lite Gliadin-IgA II and Gliadin-IgG II; INOVA Diagnostics Inc., San Diego, CA, USA; cut-off value, 20 units) and TTG (BINDAZYME human IgA and IgG anti-tissue transglutaminase EIA kit; The Binding Site, Ltd, Birmingham, UK; cut-off value, 4 and 6 unit/mL for IgA and IgG respectively) by ELISA methods.¹²

Multiplex immunoassay testing was performed using two prototype kits, specific for either IgA or IgG antibodies, which were provided by the manufacturer (INOVA Diagnostics Inc.) for research use only.^{23, 24} The IgA kit contained four polystyrene microsphere populations (bead sets), conjugated to DGP, recombinant human TTG, anti-human-IgA antibody (control), or human IgA (control) (see Figure S1, published online). The IgG kit contained three bead sets, conjugated to DGP, TTG or anti-human-IgG antibody (control). Each bead set was uniquely labelled with a combination of fluorescent dyes, allowing it to be distinguished from the other bead sets used in the same kit.

Assays were run in batch format, using 96-well plates. All pipetting steps were performed by a Tecan 75 Miniprep instrument (Tecan Trading AG, Zurich, Switzerland), using settings that ensured identical incubation timings in each well. Calibrator, prediluted controls or diluted patient sera were added to bead mixes in each well and incubated for 30 min. Fluorescently labelled conjugate (goat anti-human-IgA, or anti-human-IgG) was then added to each well and incubated additionally for 30 min. After completion of the conjugate incubation, plates were read using a Luminex 100 instrument (Luminex Corporation, Austin, TX, USA). Median fluorescence intensity (MFI) values of bead sets in each well were compared to MFI of corresponding bead sets in control wells, allowing for a semi-quantitative determination of serum antibody concentration. Values ≥ 20 adjusted fluorescence units were considered positive for DGP and TTG IgA and IgG isotypes.

Control bead sets in each kit (anti-human-IgA or anti-human-IgG) assisted in the detection of operational error by generating abnormally low fluorescence responses in wells where there was a failure to add serum. Low response from the anti-IgA bead set can also be an indicator of selective IgA deficiency. An additional control bead set in the IgA kit (human-IgA) served as a control for addition of conjugate.

Histopathology

Upper GI endoscopy and small intestinal biopsy was performed as part of each patient's clinical evaluation. Patients with an increased number of intraepithelial lymphocytes, crypt hyperplasia, inflammation and partial, subtotal or total villous atrophy (Marsh IIIa, IIIb or IIIc respectively) comprised the biopsy-proven coeliac group. Patients who did not have any evidence of enteropathy (Marsh 0) comprised the biopsy-proven noncoeliac control group. Patients who had only intraepithelial lymphocytosis without crypt hyperplasia or shortening of the villi (Marsh I) were excluded from both groups and were analysed separately.

Statistics

Statistical analysis was conducted using JMP version 6.0.0 software (SAS Institute Inc., Cary, NC, USA). Student's *t*-test assuming equal variances was used to compare age between the

two groups. To compare binary variables, chi-squared test or Fisher's exact test was employed as appropriate. Correlations between MIA and ELISA methods for each antibody titre (DGP and TTG IgA and IgG) were assessed by Spearman's correlation coefficients. The sensitivity, specificity and accuracy were calculated for each test using conventional formulas. The agreement between antibodies for positive and negative results was measured using Kappa statistics. Good agreement and excellent agreement were defined as a κ -coefficient ≥ 0.6 and ≥ 0.8 respectively. The significance of difference in sensitivity, specificity and accuracy between MIA and ELISA methods for each antibody was tested using McNemar or McNemar exact test (as appropriate). The chi-squared test was used to compare the proportion of positive test results between coeliac patients with partial villous atrophy vs. those with total villous atrophy. Statistical significance was inferred at P -values < 0.05 for all comparisons.

RESULTS

Subjects' characteristics

Table 1 shows the demographic characteristics of the study population. The study population consisted mainly of adult patients (90% older than 18 years of age). Coeliac patients and controls were similar in age and gender. The median (range) of age was 47 (4–80) years for coeliac group and 45 (1–85) years for controls. There was no significant difference between the two groups for the frequency of GI symptoms, weight loss, anaemia, thyroid disorders, diabetes and positive family history of CD indicating that our coeliac and control patients had similar risks of CD before endoscopic evaluation. Dermatitis herpetiformis was exclusively seen in coeliac patients ($P = 0.002$). Bone disorders were also significantly more frequent in the coeliac group than in the controls ($P = 0.002$). To calculate the diagnostic indices of serological tests, the eight Marsh I patients [mean (range) of age 37 (22–52) years, 75% female] were excluded from both coeliac group and control group. Four of these patients were completely seronegative with both ELISA and MIA methods. The other four patients had TTG and/or DGP antibodies (see Table S1, published online).

Agreement between MIA and ELISA methods

The MIA method was used to measure antibodies against TTG and DGP in the sera of patients that were previously tested with ELISA method.¹² There was a statistically significant correlation between the MIA and ELISA methods for measurement of all antibody titres ($P < 0.0001$). The strongest correlation between the two methods was for TTG IgA ($r = 0.81$) followed by DGP IgG ($r = 0.76$), DGP IgA ($r = 0.71$) and TTG IgG ($r = 0.41$) (Figure 1). Four coeliac patients were detected by MIA as possibly IgA deficient and the test results for TTG IgA and DGP IgA in these patients were removed from the analysis. The sensitivity, specificity and accuracy of each antibody measured by MIA are shown in Table 2. There was no significant difference between the MIA and ELISA methods regarding the sensitivity, specificity and accuracy of TTG IgA, DGP IgA and DGP IgG. The sensitivity and accuracy of TTG IgG was greater when measured by ELISA compared with MIA ($P = 0.0005$ and $P = 0.025$ respectively). Nonetheless, both methods showed a very low sensitivity and accuracy for TTG IgG. There was excellent agreement between the two methods for all antibodies ($\kappa > 0.8$) except for TTG IgG ($\kappa = 0.48$).

Diagnostic values of tests in combination

Table 3 shows the diagnostic indices of antibody test combinations by MIA and ELISA methods. The test results for 'OR' combinations were considered positive if any test in the combination was positive and were considered negative if all tests in that combination were negative. Based on this definition, the sensitivity for 'OR' combinations increased over any single test, although not dramatically. There was excellent agreement between the MIA and ELISA results for all of the 'OR' combinations ($\kappa > 0.8$). The combination of 'TTG IgA or

DGP IgG' increased the sensitivity without significantly decreasing the specificity; and this combination had the best accuracy amongst other combinations when measured by ELISA method. With both MIA and ELISA methods, 'TTG IgA or DGP IgG' was able to identify more coeliac patients compared with the combination of 'TTG IgA or IgG'.

Table 4 shows the test results for 'AND' combinations measured by MIA and ELISA methods. The test results were considered positive if all of the tests in the combination were positive and were considered negative if any test in the combination was negative. The combination of DGP IgG 'AND' TTG IgA and/or DGP IgA maximized specificity. There was only one control patient with normal intestinal biopsy that was positive for all three antibodies. This patient had fibromyalgia and an undifferentiated collagen vascular disorder. Except for the combination that incorporated TTG IgG, there was excellent agreement between MIA and ELISA results for all 'AND' combinations. Using TTG IgG in combination with other tests significantly decreased the sensitivity and accuracy compared to other combinations ($P < 0.0001$).

Difference in diagnostic sensitivity by degree of mucosal damage

Figure 2 shows the percentage of positive test results in coeliac patients (sensitivity) for single and combination tests measured by the MIA method. Patients were stratified by degree of villous atrophy. For simplicity of analysis, patients with Marsh IIIb or IIIc were categorized as 'total villous atrophy' group ($n = 42$) and those with Marsh IIIa were categorized as 'partial villous atrophy group' ($n = 50$). There was a significant difference in the sensitivity of TTG IgA and DGP IgA between the two groups ($P < 0.01$ and $P < 0.05$ respectively). The sensitivity was also significantly greater in total villous atrophy patients in all of the 'OR' combinations that incorporated either IgA isotype, as well as in the combination of DGP IgA and TTG IgA ($P < 0.05$).

DISCUSSION

In this study, we have assessed the diagnostic values of DGP and TTG antibody results measured by MIA in a large group of biopsy selected CD patients with both mild and severe degrees of mucosal damage. We demonstrated that the MIA performs as well as conventional ELISA method in CD diagnosis for either individual DGP or TTG antibodies or various combinations of these tests.

The MIA method has the advantage of measuring several antibodies simultaneously with reduced costs and turnaround time. MIA also incorporates reagents, which allow the operator to identify specimens that have selective IgA deficiency and can identify errors in pipetting that can lead to false negative results.⁶ In our study group, four CD patients were presumptively identified as having IgA deficiency. Two of these patients were truly IgA deficient with total IgA levels < 1 mg/dL (normal range: 50–400 mg/dL). These patients had negative DGP IgA and TTG IgA test results and high titres of DGP IgG and TTG IgG with both MIA and ELISA methods. However, the other two patients were IgA sufficient and had positive DGP IgA and TTG IgA with the ELISA method. The MIA gave negative results for all IgA and IgG antibodies in these two cases, suggesting that a technologist error occurred in the handling of these specimens.

The specificity of serological markers in our study was similar to that in previous reports for both individual and combination testing; however, the sensitivity of the tests was not as high as previously reported.^{15, 16, 25} All of our seronegative coeliac patients had either a positive clinical or histopathology response to gluten exclusion or carried a coeliac predisposing HLA (DQ2 or DQ8) that supported their CD diagnosis. Nonetheless, our study population included a large number of CD patients with partial degrees of mucosal damage. When we evaluated the MIA results in CD patients with total and subtotal villous atrophy, the sensitivity increased

to the same level as other reports in which a majority of patients had severe histological damage.^{14, 17} Several studies have demonstrated that the degree of intestinal damage is a major indicator of seropositivity.^{12, 19, 26-29} The inclusion of a high number of seronegative CD patients with mild histological damage in our sample reflects the fact that the selection of subjects in this study was based on pathology and not a previous positive serology. Typically, serological tests and endoscopy are ordered simultaneously at our centre when there is a suspicion for CD; therefore, we were able to avoid a selection bias, which would occur if the CD patients are biopsied on the basis of their positive serology. Such a bias would result in an overestimation of the performance of serological tests.

Based on our results, TTG IgG does not have any additional diagnostic value over DGP IgG in routine CD diagnosis. DGP IgG had a significantly higher sensitivity and accuracy than and a similar specificity to TTG IgG. The two IgA-deficient patients in our sample were identified with both DGP IgG and TTG IgG. Although the usefulness of both IgG antibodies in detecting IgA-deficient CD patients needs to be assessed in a large sample, our results suggest that DGP IgG performs at least as well as TTG IgG for detecting IgA-deficient CD patients.

We assessed the diagnostic values of various combinations of DGP and TTG antibodies in CD diagnosis. As expected, the sensitivity increased with 'OR' combinations at the expense of a decrease in the specificity. Based on our results, the best accuracy for a combination of tests was achieved if we considered the final result to be positive if either TTG IgA or DGP IgG was positive. The combination of DGP IgG or TTG IgA is particularly useful because in addition to detection of IgA-deficient CD patients, DGP IgG was able to detect a few more IgA-sufficient patients who were missed by TTG IgA alone. Nonetheless, panels of tests often leave clinicians with ambiguous possibilities. Therefore, it is important that physicians not only assess the performance of each test, but also be able to interpret the results in different clinical settings with different pretest probabilities of disease. For example, in a referral population with very high pretest prevalence of CD,¹⁷ the positive predictive value of a combination test is very high when two or more coeliac-related antibodies are positive. In this setting, the likelihood of CD is very high on the basis of serology. Nevertheless, biopsy affords both confirmation and baseline information about the degree of intestinal damage, which is useful in the follow-up of patients and assessment of response to a gluten-free diet.³⁰ However, in such a high risk population, a substantial number of seronegative patients may still have CD. Hence, a small intestinal biopsy should be performed when there is a high clinical suspicion for CD even in the face of negative test results. Conversely, in a situation with a lower prevalence of CD, for example, in general population or in patients being screened only because of history of another autoimmune disease,³¹⁻³⁵ the combination testing has a very high negative predictive value when all the tests in that combination are negative. However, the lower specificity of combination testing may lead to a substantial number of false positives in a low risk population. Therefore, in the setting where the pretest probability of CD is low, a single TTG IgA test may be sufficient to rule out the disease.

Despite several strengths of our study, such as biopsy-based selection of subjects and inclusion of a large number of patients with a wide range of histological damage, there are also a few limitations. To have a similar case and control group, we used frequency matching to select potential controls. We did not include Marsh I patients in the analysis because of diagnostic uncertainty.²⁰⁻²² Therefore, we were not able to calculate the positive and negative predictive values of the diagnostic tests in this study. Further large prospective cohort studies are essential to validate the diagnostic and predictive values of these serological tests for CD diagnosis and screening.

In conclusion, the MIA method we evaluated is practical and useful for the measurement of multiple CD-related antibodies. Considering the pretest probability of disease and performance

of each test, combination testing helps physicians to decide whom to refer for biopsy and may reduce the number of unnecessary intestinal biopsies.

Supplemental Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

This figure shows a statistically significant correlation between multiplex immunoassay (MIA) and enzyme-linked immunosorbent assay (ELISA) for all antibodies ($P < 0.0001$). Correlations were assessed by Spearman's correlation coefficients.

Figure 2.

This figure shows the difference in the percentage of positive serologic test results measured by multiplex immunoassay method among coeliac patients with total villous atrophy (TVA, black bars, $N = 42$) vs. partial villous atrophy (PVA, white bars, $n = 50$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; P -values were obtained by chi-squared test.

Table 1

Subject characteristics

	Coeliac patients (<i>n</i> = 92)	Noncoeliac controls (<i>n</i> = 124)	<i>P</i> -value
Age (years), mean $\pm$ s.d.	46.7 $\pm$ 18.7	43.7 $\pm$ 20.0	0.279 [*]
Female	65 (71)	90 (73)	0.756
Children	8 (9)	13 (10)	0.661
Gastrointestinal symptoms	71 (77)	98 (79)	0.743
Anaemia	24 (26)	29 (23)	0.648
Osteoporosis/osteopenia	32 (35)	20 (16)	0.002
Thyroid disorders	19 (21)	20 (16)	0.393
Diabetes	6 (7)	7 (6)	0.789
Dermatitis herpetiformis	7 (8)	0 (0)	0.002 [†]
Family history of CD	16 (17)	20 (16)	0.806
Partial villous atrophy	50 (54)	—	—
Subtotal villous atrophy	4 (4)	—	—
Total villous atrophy	38 (41)	—	—

Values are expressed as *n* (%). Continuous variable is presented as means $\pm$ s.d. and categorical variables as counts and percentages.

^{*} *P*-values were obtained using chi-squared test, Student's *t*-test

[†] Fisher's exact test as appropriate.

Table 2
Diagnostic indices of single serological tests for coeliac disease by MIA vs. ELISA method

Test	κ -Statistics	Sensitivity (%)		Specificity (%)		Accuracy (%)	
		MIA	ELISA [†]	MIA	ELISA [†]	MIA	ELISA [†]
DGP IgA	0.89	71.6 [*]	75.0 [*]	92.7	95.2	83.9 [*]	86.8 [*]
DGP IgG	0.86	66.3	65.2	96.8	98.4	83.8	84.3
TTG IgA	0.97	81.8 [*]	79.3 [*]	98.4	98.4	91.5 [*]	90.5 [*]
TTG IgG	0.48	13.0	28.3	100.0	96.8	63.0	67.6

^{*}The MIA and ELISA test results for TTG IgA and DGP IgA of four CD patients were excluded from the analysis because of potential IgA deficiency detected by the MIA method.

[†]The results of ELISA test from previously published data are shown for easy comparison.¹²

Table 3
Diagnostic indices of combination of serological tests for coeliac diseases by MIA vs. ELISA method

Test	κ -Statistics	Sensitivity (%)		Specificity (%)		Accuracy (%)	
		MIA	ELISA	MIA	ELISA	MIA	ELISA
DGP IgA or IgG	0.87	77.8	78.3	90.2	94.4	85.0	87.5
TTG IgA or IgG	0.93	82.2	80.4	98.4	96.0	91.5	89.4
DGP IgA or TTG IgA	0.93	83.0	83.0	92.7	95.2	86.6	90.1
DGP IgG or TTG IgA	0.96	84.4	82.6	95.9	97.6	91.1	91.2
DGP IgA or IgG or TTG IgA	0.91	85.6	83.7	90.2	94.4	88.3	89.8
DGP IgA or IgG or TTG IgA or IgG	0.88	85.6	83.7	90.2	91.9	88.3	88.4

MIA, multiplex immunoassay; ELISA, enzyme-linked immunosorbent assay.
The combination test was considered positive if any test in that combination was positive and was considered negative if all of the tests in that combination were negative.

Table 4
Diagnostic indices of combination of serological tests for coeliac disease by MIA vs. ELISA method

Test	κ -Statistics	Sensitivity (%)		Specificity (%)		Accuracy (%)	
		MIA	ELISA	MIA	ELISA	MIA	ELISA
DGP IgA and IgG	0.90	60.0	61.4	99.2	99.2	82.7	83.5
TTG IgA and IgG	0.50	11.1	25.3	100.0	99.2	62.6	68.7
DGP IgA and TTG IgA	0.96	70.5	71.3	98.4	98.4	86.7	87.2
DGP IgG and TTG IgA	0.88	63.3	60.9	99.2	99.2	84.1	83.4
DGP IgA and IgG and TTG IgA	0.91	60.0	58.6	99.2	99.2	82.7	82.5
DGP IgA and IgG and TTG IgA and IgG	0.57	11.1	20.7	100.0	99.2	62.6	66.8

MIA, multiplex immunoassay; ELISA, enzyme-linked immunosorbent assay.
The combination test was considered positive if all of the tests in that combination were positive and was considered negative if any test in that combination was negative.