

Multiplex Assays to Diagnose Celiac Disease

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ABSTRACT: Patients with celiac disease are sensitive to the gluten fractions of wheat. Symptoms include gastrointestinal problems and a failure to thrive in children, but may range from headaches to general malaise in adults. Thus, it is difficult to diagnose celiac disease by symptoms alone. The standard diagnostic criteria include the presence of the characteristic anti-gliadin or anti-tissue transglutaminase antibodies (anti-tTG) in serum, flattened mucosa on intestinal biopsy, and improved symptoms on a gluten-free diet. Because of the ease of use of the tTG enzyme-linked immunosorbent assay (ELISA) compared to endomysial by indirect immunofluorescence assay, there has been much more screening for celiac disease in recent years. This increased screening showed that celiac disease was more prevalent than previously believed. We compared a new multiplex assay that includes a novel form of deamidated gliadin and recombinant human tTG as the antigens to other assays using standard antigens. In addition, the new assay detects the presence of selective IgA deficiency, which shows a 10-fold increase in prevalence in patients with celiac disease compared to the general population. The combination of sensitivity and specificity of the new multiplex assay was equal or better than those for standard assays. Thus the performance, ease of use, and ability to measure three clinically important parameters in a single test make the new multiplex assay a viable alternative to standard assays in a clinical lab.

KEYWORDS: celiac disease; immunopathology; deamidated gliadin; multiplex assay; ALBIA

Celiac disease (CD) is an immune-mediated enteropathy caused by sensitivity to wheat gluten in genetically susceptible individuals.¹⁻³ Clinical manifestations range from typical gastrointestinal problems and failure to thrive in children, to a diverse range of symptoms in adults. The most prominent pathological change is flattened villi (FIG. 1) observed in biopsy of the small intestine.

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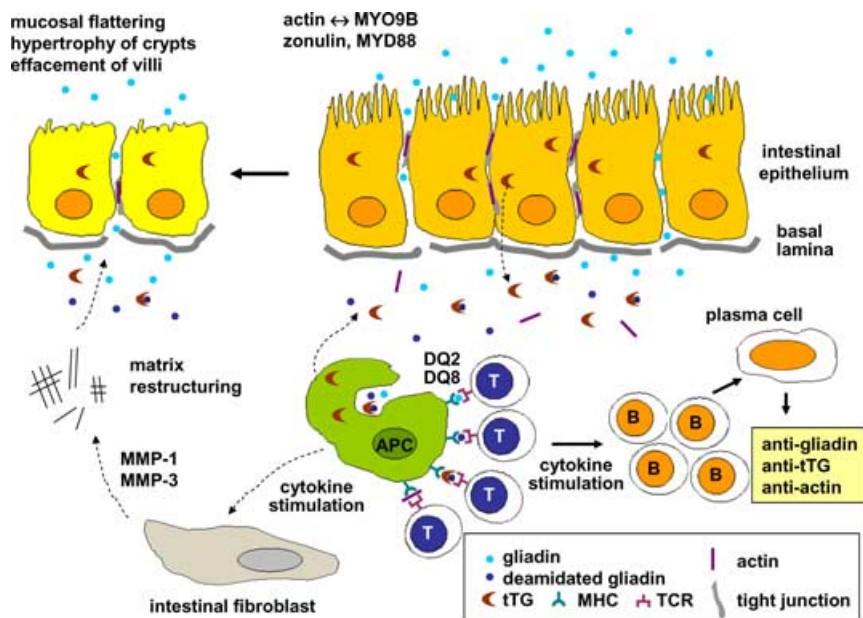


FIGURE 1. Pathophysiology of celiac disease.

The cause of this change is immune mediated since all symptoms and pathology disappear with a gluten-free diet. Besides biopsy, the laboratory diagnosis and monitoring of CD is based on the determination of IgA anti-gliadin and anti-tissue transglutaminase (anti-tTG) antibodies by enzyme-linked immunosorbent assay (ELISA), and detection of anti-endomysial antibodies (EMA) by indirect immunofluorescence (IIF). EMA has the same diagnostic value as the determination of anti-tTG, because tTG is the antigen in the endomysium.⁴ Selective IgA deficiency has to be excluded when negative results of IgA anti-gliadin and anti-tTG or EMA are found. If there is IgA deficiency, then IgG antibodies to those antigens are determined. Antibodies against common gliadin are not as sensitive or specific as anti-tTG in adult patients with CD. However, young children with CD are often positive for antigliadin and negative for anti-tTG. Assays measuring T cell responses⁵ as well as antibody responses⁶ to deamidated gliadin peptides (DGP) are more sensitive and specific than assays using unmodified gliadin.

Gliadin is not a normal physiologic substrate for tTG.⁷ However, gliadin can be deamidated by tTG, and tTG can be cross-linked to gliadin, under the right conditions *in vitro*.^{8–10} It is hypothesized that these same reactions occur in the intestinal submucosa in patients with CD, and the modified molecules are recognized as foreign by the immune system. Not only is deamidated

gliadin more reactive with antibodies from patients with CD,^{11–14} but DGP are more reactive with their T cells. The deamidated gliadin epitopes have up to 400 times higher affinity for some HLA DQ2 and DQ8 molecules. This leads to more efficient presentation of these epitopes to T cells and makes individuals with these HLA markers susceptible to higher risk of CD.^{1–3,9,15,16} Besides HLA genotype and selective IgA deficiency, another genetic marker for increased risk of CD is a variant of myosin IXB (MYO9B), which has a role in actin remodeling of epithelial enterocytes, and can lead to impairment of the intestinal barrier.¹⁷

It is well accepted that activated T cells are involved in the intestinal pathology in CD patients. However, based on studies with organ and tissue culture, and genetic analysis of MHC class I genes, additional mechanisms have been proposed. Some gliadin peptides, like p31–43, delay inactivation of epidermal growth factor receptor (EGFR) through interference with the endocytic pathway in the intestinal cells and thus amplify the effects of trace amounts of EGF on actin cytoskeleton.¹⁸ They also lead to overexpression of MHC chain-related molecules (MIC) on mucosa, and production and release of IL-15, which induces expression of its ligand NKG2D on intraepithelial lymphocytes (IEL). Interaction of NKG2D and MICA tends to destroy mucosa epithelial cells.¹⁵

Transglutaminases (TGs) are ubiquitous enzymes with many functions. There are several forms of TGs with high homology. Each of them is localized in different compartments of different tissues. The main form of TG in the small intestine is TG2, also called tTG. It has deamidation and transamidation activity as well as GTPase activity.^{7,19,20} Inflammatory processes induced by the presence of gliadin in the submucosa may lead to the release of tTG from participating cells and amplification of destructive mechanisms mediated by cytokines and with metalloproteinases leading to mucosa transformation (Fig. 1). Anti-tTG antibodies produced by B lymphocytes that were presumably activated in the intestine may be involved in pathological processes in other tissues and organs.^{7,21–23}

IgA anti-F-actin antibodies are another serological marker for CD.^{24–26} Raised titers may have prognostic value because they are found in CD patients with severe intestinal mucosa damage.^{24–26} These antibodies do not correlate closely with the levels of EMA or anti-tTG.²⁷ An active area of research is to determine if there is correlation between anti-F-actin antibody levels and the degree of intestinal damage rated according to Marsh criteria.²⁸ It will be interesting to see if there is any correlation between IgA anti-F-actin and the MYO9B mutation of myosin, a protein that interacts with actin, in patients with CD.

Multiplex analysis is a process in which several analytes are measured in one reaction chamber at the same time. Generally, the assays use less time, labor, and primary sample volume, and cost less than the equivalent classical one-analyte assays, yet yield the same information. Addressable laser bead

immunoassays (ALBIA) using the LuminexTM flow cytometer and xMAPTM technology (Luminex, Oosterhout, The Netherlands) and line or dot analysis (LDA) are the most used multiplex techniques in clinical immunology laboratories today.

The determination of IgA anti-tTG or EMA is the most common single screening test for CD. More complete analysis includes IgA anti-gliadin or anti-DGP tests. The lab should test all negative samples for total IgA concentration or for IgG anti-tTG and anti-DGP to rule out a negative result because of selective IgA deficiency. Because no individual test for CD is perfect, the combination of several serological tests is more reliable for diagnosis and monitoring than any one test. Even measuring anti-tTG by ELISA and EMA by IIF is not the best strategy because this is just measuring anti-tTG by two different techniques.

The Luminex flow cytometer, which allows simultaneous measurement of reactions on the surface of up to 100 separate bead sets, is a good platform for multiplex assays.²⁹ Because the determination of several analytes is useful to diagnose and monitor CD, an ALBIA was developed that included a complete set of assays to screen for CD. Four beads are used. One is coated with recombinant human tTG, another with DGP, one with IgA, and one with anti-IgA. The ALBIA is simple to perform. Diluted patient sample is added to the well of a microtiter plate and incubated with the beads. Then goat anti-human IgA conjugated with phycoerythrin is added. Finally, the fluorescence on the beads is read in the Luminex flow cytometer.

Interpretation of the results is straightforward. Any IgA antibodies that bind to either the tTG bead or the DGP bead are detected by the anti-IgA conjugate and yield high fluorescence on that bead. The IgA bead and anti-IgA beads are used together to detect IgA deficiency. If a person has a sufficient amount of total IgA, it will be captured by the anti-IgA bead. In this case the anti-IgA bead and the IgA bead will have similar fluorescence. If the patient has selective IgA deficiency, there will not be human IgA on the anti-IgA bead, and the fluorescence of the IgA bead will be much higher than that for the anti-IgA bead. Note that an error where the patient's sample is omitted from the well will yield the same result as if the patient has IgA deficiency.

Measuring IgA antibodies against DGP is preferred over regular antigliadin because the former is both more sensitive and specific.^{6,11-14} Gliadin is the agent that incites the clinical symptoms of the disease. There have been reports at meetings that IgA titers to the DGP decrease more rapidly than anti-tTG when a patient goes on a gluten-free diet, and increase more rapidly when the diet is broken. It is tempting to speculate that gliadin that has been deamidated *in vivo* is the main antigen that starts the immune response in CD, and tTG cross-linked to deamidated gliadin becomes immunogenic at the site of inflammation.

Human tTG is a preferred antigen compared to the guinea pig tTG that was used in the original assays.^{4,30} In typical CD when intestinal enteropathy and gastrointestinal discomfort are the main symptoms, antibodies in IgA class

TABLE 1. Laboratory methods applied in the study

Manufacturer	Diagnostic kit	Description	Abbreviation
INOVA	Quanta Plex Celiac IgA Profile	ALBIA for simultaneous IgA anti-gliadin and anti-tTG determination with monitoring of IgA deficiency	INOVA P
BMD	FIDIS celiac	ALBIA for simultaneous determination of anti-gliadin and anti-tTG determination in IgA class and anti-gliadin in IgG	FIDIS
DTek	Blue dot Celiac IgA	LDA for simultaneous determination IgA of anti-gliadin and anti-tTG in IgA with simultaneous monitoring of IgA deficiency	DTek IgA
DTek	Blue dot Celiac IgG	LDA for simultaneous determination of IgG anti-gliadin and anti-tTG	DTek IgG
INOVA	Quanta Lite Gliadin IgA II ELISA	ELISA for the determination of IgA antibodies against DGP	INOVA L IgA
INOVA	Quanta Lite Gliadin IgG II ELISA	ELISA for the determination of IgG antibodies against DGP	INOVA L IgG
Euroimmun	Tissue transglutaminase IgA ELISA	ELISA for determination of IgA anti-tTG	Euroimm
Genesis	Tissue transglutaminase IgA ELISA	ELISA for determination of IgA anti-tTG	Genesis
Test line	EIA Gliadin IgA/IgG	ELISA for determination of IgA or IgG anti-gliadin antibodies	Test-Line
Binding site	Antiendomysial antibody IgA	IIF detection of EMA on primate esophagus	EMA

are of higher diagnostic value than the antibodies in the IgG class for the individuals without IgA deficiency.

In the future there may be more serological tests to help diagnose and monitor patients with CD. Determination of zonulin levels may be useful because it has been reported that increased zonulin levels lead to increased intestinal permeability to macromolecules including gliadin.^{31–33} Testing for zonulin is not widely available outside of research laboratories today.

We performed a study for serological diagnosis of CD to compare kits from several manufacturers to verify the statements mentioned above. Sera from 67

TABLE 2. Serological diagnosis of CD

Test	IgA antibodies (Abs)%				IgG Abs%		Multiplex IgA positive%			
	anti-tTG		antigliadin		antigliadin		both tests		single test only	
	sens	spec	sens	spec	sens	spec	sens	spec	sens	spec
Test-Line			60	86	73	32				
INOVA L			79	93	87	93				
INOVA P	100	95	43	93			50	96	100	89
FIDIS	100	93	56	80	88	23	56	96	100	76
DTek	69	98	25	96	67	14	25	100	69	93
Genesis	100	77								
Euroimmun	100	84								
EMA	94	96								

Description of tests is given in TABLE 1; sens = sensitivity; spec = specificity.

patients were examined in our study using the methods and kits specified in TABLE 1. The diagnosis of CD was confirmed in 22 patients by biopsy and improvement in symptoms when adhering to a gluten-free diet. Results of this study are summarized in TABLE 2 and confirm the statements mentioned above.

Based on the study of these patients on 3 multiplex assays and 5 single analyte assays (TABLE 1), we conclude that anti-tTG and anti-DGP can be successfully measured in multiplex assays, and they work as well as standard ELISAs. There are savings of time and space using a single multiplex test compared to several ELISAs. The anti-DGP ELISA is significantly more sensitive and specific for CD diagnosis than standard anti-gliadin ELISAs. Antibodies to the new DGP give improved sensitivity and specificity for diagnosis, and IgA anti-F-actin may be useful for measuring disease activity. Thus, once these new assays become more readily available, the need for biopsy will likely be decreased.

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