Coeliac Disease Screening in Children: Assessment of a Novel Anti-Gliadin Antibody Assay

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Coeliac disease (CD) screening has progressed rapidly with tissue transglutaminase (TTG), the screening tool of choice. However, TTG may be unreliable in young children and advances in CD etiology understanding have seen improvements in anti-gliadin (AGA) assay technology. The aim of this study was to investigate the utility of an updated and refined AGA (Neogliadin) assay for CD screening in children with gastrointestinal symptoms. Children attending the Sydney Children's Hospital, Randwick, with gastrointestinal symptoms had sera collected and assayed by Neogliadin and commercial TTG assays in addition to the usual clinical work-up. One hundred and fifteen children were

recruited in which 32 were diagnosed with CD. AGA-IgA screening by Neogliadin showed improved sensitivity (83%) and specificity (91%) but did not eclipse the sensitivity (93%) and specificity (90%) of TTG-IgA screening. In the children diagnosed with CD, 7 were identified as younger than 5 years of age with 4/7 AGA-IgA positive, 5/7 AGA-IgG positive, and 6/7 TTG-IgA positive. The updated Neogliadin IgA assay does not improve on the accuracy achieved by TTG screening. TTG appears to be a suitable screening tool for children younger than 5 years of age although this preliminary finding requires confirmation. *J. Clin. Lab. Anal.* 22:327–333, 2008. © 2008 Wiley-Liss, Inc.

Key words: AGA; gliadin; pediatric; coeliac screening; TTG

INTRODUCTION

Coeliac disease (CD) is a common lifelong disorder with significant morbidity caused by an immune-mediated enteropathy to gluten (a protein component of common grains including wheat, rye, barley) in genetically susceptible individuals (1). CD can be effectively managed with lifelong adherence to a gluten-free diet, thereby obviating symptoms and morbidity. Consequently, prompt and accurate detection and diagnosis of individuals with CD are essential to their well-being.

The prevalence of CD varies although it is now estimated that it may affect 0.3–1% of populations derived from European ancestry (2). CD meets the Wilson and Junger (3) principles for early disease detection and hence the concept of population screening for CD has been suggested (2). However, to date mass screening programs have not been implemented and remain controversial. In the absence of mass screening, a diagnosis of CD generally originates from a clinical suspicion followed by serological screening. The

presence of antibodies against gliadin (anti-gliadin, AGA) (a glycoprotein that associates with glutenin to form gluten), endomysium (EMA), or most recently tissue transglutaminase (TTG) are used as the primary tools of CD serological screening (4). These tests, however, are not diagnostic. Following a positive serology result, a definitive diagnosis of CD is arrived at by observing characteristic small bowel biopsy histological changes while the patient is on a diet that includes gluten (5).

Serological screening provides the clinician with a strong indication of CD warranting further more invasive investigation; therefore, a highly accurate screening test is desirable. AGA screening, although

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initially widely used, is considered to have limited utility owing to low sensitivity (6,7). EMA screening provides high specificity but only moderate sensitivity (7) with TTG reportedly providing superior sensitivity combined with high specificity in detecting CD (7). Further, with the widespread use of TTG there is the suggestion that AGA testing should be considered redundant and superseded by TTG (6–8). However, teleological thinking suggests that AGA antibodies may be present in the large majority of CD patients as an immune response to gluten (or more specifically deaminated gliadin) is central to the etiology of CD (9). Once ingested by a susceptible individual, gliadin is deaminated by TTG and it is this deaminated gliadin–transglutaminase complex that activates T cells to initiate an immune response (9). Therefore, previous limitation of AGA screening may not be because of the absence of AGA antibodies in coeliac patients, but perhaps owing to suboptimal assays used for AGA screening. One limitation may be the use of native or unprocessed gliadin in these assays, which limits the detection of antibodies specific for deaminated gliadin. A recent AGA assay (Neogliadin) has attempted to address this issue by using replicated deaminated gliadin that is subject to acid/enzymatic digestion replicating human gastric digestion.

Therefore, the aim of this study was to investigate the utility of a novel AGA ELISA (Neogliadin) that utilizes processed gliadin to detect AGA, in comparison with a commercially available TTG ELISA, for the screening of CD in a pediatric population with gastrointestinal symptoms.

METHODS

Patient Recruitment and Sample Collection

Consecutive enrollment of children aged between 6 months and 18 years of age, presenting at Sydney Children's Hospital, Randwick, with gastrointestinal complaints requiring endoscopy for investigation of symptoms, occurred prospectively. Venous blood was drawn at endoscopy and centrifuged with serum collected and stored at -80°C until assay.

Subsequent to endoscopy, children diagnosed with CD (coeliac group), inflammatory bowel disease (IBD group), or no organic intestinal disease (control group) were included in this study. Diagnosis of IBD was based upon standard endoscopic, histologic, and radiologic investigations (10). The diagnosis of CD was based upon standard consistent histological features and a context of appropriate symptoms and abnormal serology (5). Children with previously diagnosed CD or with current treatment with gluten-free diet were excluded. The control group was composed of children who had no

endoscopic or histologic evidence of inflammation or of CD and had normal systemic inflammatory markers.

Informed consent was obtained from patients/parents. The study was reviewed and approved by the Research Ethics Committee, South-East Sydney Illawarra Area Health Service—Eastern Section.

Neogliadin IgA and IgG ELISA

Neogliadin ELISA kits (Vital Diagnostics, Sydney, Australia) were used to measure AGA–IgA and –IgG serum levels according to the manufacturer's instructions. Briefly, a standard, a control, a blank, and stored serum samples were thawed and diluted to 1 in 100 in sample diluent with 100 μl added, in duplicate, to wells coated with acid/enzyme digested gliadin in a 96-well plate. The plate was covered and incubated at room temperature for 30 min. The plate was washed 4 times with wash buffer and blotted dry. One hundred microliters of alkaline phosphatase-conjugated anti-human IgA or anti-human IgG was added to each well of the plate and incubated for a further 30 min at room temperature. The plate was washed 4 times, blotted dry, and 100 μl of substrate was added to the plates and incubated for approximately 30 min. Fifty microliters of 1 M NaOH was added to each well and the plate absorbance was read at 415 nm on an ELISA plate reader (BioRad, Hercules, CA). AGA–IgA and –IgG levels were calculated using the following equation: test sample AGA = $(A/X) \times S$, where A is the standard AGA level in units, X the absorbance of standard, and S the absorbance of the test sample.

TTG–IgA ELISA

Anti-TTG–IgA levels were assessed using a commercial TTG–IgA ELISA kit (AESKU Diagnostics, Wendelsheim, Germany) according to the manufacturer's instructions. This assay employs human recombinant transglutaminase crosslinked with gliadin-specific peptides to increase sensitivity. Briefly, stored serum samples were thawed and diluted 1 in 101 in sample buffer. One hundred microliters of each calibrator, positive control, negative control, and samples were added in duplicate to the plate and the plate was incubated at room temperature for 30 min. The plate was washed 3 times with wash buffer with 100 μl of conjugate added to each well and the plate was incubated for a further 15 min at room temperature. The plate was washed 3 times, 100 μl of substrate was added to each well with the plate incubated for 15 min in the dark. One hundred microliters of stop solution was added to each well and the plate absorbance was read at 450 nm on an ELISA plate reader (BioRad). TTG–IgA levels were estimated by extrapolating from a standard

curve constructed using the logistic four-parameter line of best fit.

Statistics

Chi-square with Fisher Exact test was used to assess gender distributions. *T*-test was used to assess age. The Mann–Whitney test was used to assess differences between groups. Correlations were tested by Spearman's correlation. Receiver operator characteristic (ROC) curves, sensitivities [TP/(TP+FN)], specificities [TN/(TN+FP)], positive predictive value (PPV) [TP/(TP+FP)], and negative predictive value (NPV) [TN/(TN+FN)] (with TP being true positive, FP being false positive, TN being true negative, and FN being false negative) were calculated for each of the screening assays. All statistics were generated with GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). Significance was accepted if $P < 0.05$.

RESULTS

Patient Groups

A total of 115 children were enrolled in the study in which 32 were diagnosed with CD (of which 2 were identified as IgA deficient), 39 with IBD, and 44 with no indication of organic disease and designated as controls. There was no difference in age or gender between the groups (Table 1).

Results of Neogliadin AGA (neoAGA) Assays

NeoAGA–IgA (neoA), neoAGA–IgG (neoG), and TTG–IgA (TTG) levels were measured for all individuals included in the study. NeoA was elevated in CD (median 25.9, range 0–128 units) compared with controls (median 2.1, range 0–50.9 units, $P < 0.0001$) (Fig. 1). NeoA levels in patients with IBD (median 2.3, range 0–17 units) were not elevated compared with control patients ($P > 0.05$).

NeoG was also elevated in CD (median 43.6, range 1.1–61.3 units) compared with controls (median 8.7, range 0.9–31.2 units, $P < 0.0001$) (Fig. 2). Furthermore, neoG was elevated in IBD (median 14, range 0.7–32, $P = 0.0043$) compared with controls. TTG was elevated in CD (median 300, range 0–300 units)

compared with controls (median 3, range 0–144 units, $P < 0.0001$) (Fig. 3) but not in IBD (median 3.5, range 0–31 units).

Fig. 1. Serum neoA levels. NeoA was elevated in sera from children with CD ($P < 0.0001$) compared with controls. There was no significant difference in neoA levels between children with IBD and controls. CD, coeliac disease; IBD, inflammatory bowel disease.

Fig. 2. Serum neoG levels. NeoG was elevated in sera from children with CD ($P < 0.0001$) and IBD ($P = 0.0034$) compared with controls. CD, coeliac disease; IBD, inflammatory bowel disease.

TABLE 1. Patient Demographics

Group	N	Age: mean (SD)	P statistic (compared with control)	Gender: male:female (%)	P statistic (compared with control)
Control	44	8.94 (4.03)		27:17 (61:39)	
Coeliac	32	8.46 (3.79)	$P = \text{NS}$	16:16 (50:50)	$P = \text{NS}$
IBD	39	10.62 (4.33)	$P = \text{NS}$	22:17 (56:44)	$P = \text{NS}$

NS, not significant; IBD, inflammatory bowel disease.

Correlations Between neoA, neoG, and TTG

Significant correlations were made between titers of neoA, neoG, and TTG ($P < 0.0001$). The weakest correlation was observed between neoA and neoG ($R^2 = 0.4358$) followed by the correlation between neoA and TTG ($R^2 = 0.4563$). The strongest correlation observed was between neoA and TTG ($R^2 = 0.6353$).

ROC Curve Analysis

NeoA, neoG, and TTG results were further analyzed by ROC, with sensitivities, specificities, PPV, NPV, and

Fig. 3. Serum TTG levels. TTG was elevated in sera from children with CD ($P < 0.0001$) compared with controls. There was no significant difference in TTG levels between children with IBD and controls. TTG, tissue transglutaminase; CD, coeliac disease; IBD, inflammatory bowel disease.

area under the curve calculated for a range of cut-off values (Table 2). For neoA and neoG an equivocal cut-off of 25 units with a true positive cut-off of 30 units was suggested. The respective sensitivities and specificities for neoA were 53.3 and 97.6% for a cut-off of 25 and 46.7 and 97.6% for a cut-off of 30. In comparison, TTG gave a sensitivity of 93.3% and a specificity of 90.9% using the recommended cut-off of 15 units. NeoG gave sensitivities and specificities of 84.4 and 90.9% for the equivocal cut-off of 25 units and 71.9 and 97.7% for a cut-off of 30 units.

To further analyze these results, the cut-off for neoA was adjusted and summarized in Table 2. NeoA with a cut-off of 18 units increased the sensitivity to 56.7% without any decrease in specificity; however, with a cut-off of 10 units the sensitivity increased to 83.3% with specificity of 91.5%. In addition, the neoA result was added to neoG result to provide a combined AGA level. This combined score also improved sensitivity and specificity. With a cut-off of 35, the combined score gave a sensitivity of 90.6% and a specificity of 89.2% (Table 2).

AGA and TTG Levels in Patients Younger Than 5 Years of Age

Seven children with CD, six children with IBD, and seven control children, younger than 5 years of age, were identified in our patient cohort. From these young children with CD, only four were neoA positive, five were neoG positive, and six were TTG positive (Table 3). None of these children were identified as IgA deficient.

Sensitivities, specificities, PPV, and NPV for each screening test, for the age group of 0–5 years, have been summarized in Table 4.

TABLE 2. Summary of Results of CD Screening Assays

	Cut-off	Sensitivity (%)	Specificity (%)	PPV	NPV	Area under ROC (%)	95% CI
NeoA	10	83.3	91.5	78.1	93.8	90.3	82.7–97.9
	18	56.7	97.6	89.5	86.2		
	22	53.3	97.6	88.9	85.3		
	25	53.3	97.6	88.9	85.3		
	30	46.7	97.6	87.5	83.5		
NeoG	25	84.4	90.9	69.2	93.4	94.1	87.9–100.2
	30	71.9	97.7	85.2	89.8		
TTG	15	93.3	90.9	71.8	97.3	95.9	89.6–102.2
Combined AGA	23	96.9	80.7	66.0	98.5	94.2	87.9–100.4
	25	90.6	81.9	65.9	95.8		
	35	90.6	89.2	76.3	96.1		
	45	75.0	95.2	85.7	90.8		

CD, coeliac disease; TTG, tissue transglutaminase; AGA, anti-gliadin; PPV, positive predictive value; NPV, negative predictive value; ROC, receiver operator characteristic curve; CI, confidence interval.

TABLE 3. Coeliac Patients Under 5 Years of Age

Age	Gender	NeoA	Interpretation	NeoG	Interpretation	TTG	Interpretation
3.0	F	128	Positive	56	Positive	> 300	Positive
4.2	M	27	Positive	29	Positive	> 300	Positive
4.2	M	4	Negative	21	Negative	85	Positive
1.7	M	13	Positive	46	Positive	> 300	Positive
4.3	M	11	Positive	27	Positive	223	Positive
4.2	F	3	Negative	33	Positive	> 300	Positive
2.4	M	0	Negative	1	Negative	0	Negative

TTG, tissue transglutaminase.

TABLE 4. Summary of Results of CD Screening Assays for 0–5 Years of Age

	Cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
NeoA	10	57.1	100	100	81.3
NeoG	25	71.4	84.6	71.4	84.6
TTG	15	85.7	92.3	85.7	92.3

CD, coeliac disease; TTG, tissue transglutaminase; PPV, positive predictive value; NPV, negative predictive value.

DISCUSSION

The introduction of AGA screening was an important milestone in the history of CD. It changed the initial perception that CD was a rare malabsorption syndrome in children to an understanding that CD is a common disease that may be diagnosed at any age and affects many organs (11). However, the more recent introduction of TTG screening has generated criticism in the literature that AGA screening, owing to its limited sensitivity, is redundant and can be superseded by the newer CD screening tools available (6,7). The aim of this study was to investigate whether an updated and refined neoAGA assay has the utility for CD screening in children with gastrointestinal symptoms.

Utilizing AGA, measured by the Neogliadin assay, for the screening of CD in children with gastrointestinal symptoms provided promising results. Using the IgA assay according to the manufacturer's instructions (equivocal cut-off of 25 units), a poor sensitivity (53%) but excellent specificity (97%) were achieved. By adjusting the cut-off to 10 units, the sensitivity dramatically improved (83%) with only a relatively small reduction to specificity (91%). This discrepancy may be owing to the developmental nature of the Neogliadin assay, indicating that further refinement of suggested cut-off may be required. The neoG achieved a similar sensitivity (84%) and specificity (90%) to neoA when the manufacturer's instructions were adhered to (using the equivocal cut-off of 25); therefore, further refinement of the cut-off was not pursued. Interestingly,

neoG levels were elevated in some IBD patients suggesting that some children with IBD generate AGA-IgG antibodies. However, this did not appear to affect the specificity of neoG in comparison with neoA. Further, by combining the results of the neoA and neoG, there was improvement in sensitivity (90%), whereas comparable specificity was retained. These results indicate there may be an advantage to combining assays. Although comparable sensitivities and specificities were achieved, neither of the individual or the combination of AGA assays eclipsed the sensitivity (93%) and specificity (90%) of the TTG assay.

A strength of this study was the control population. Controls included children with IBD and children with no organic disease, all of whom had gastrointestinal symptoms. This is precisely the patient population that is likely to undergo CD screening; therefore, the calculated sensitivities and specificities are representative of the clinical situation. One drawback, however, was that the study population was limited to children and therefore a similar study in adults is required to determine if the same is true for an older population.

Another important issue in CD screening is the screening of young children. CD has been reported to be underdiagnosed in children at 7 years of age in the United Kingdom population (12). Further, there has been limited evidence to suggest that TTG may be generally unreliable in children less than 5 years of age (13), with more specific evidence indicating that it should not be used for children younger than 18 months of age (8,14). This may be because of an immature immune system, especially in the very young, or owing to a delay in the appearance of TTG antibodies (8,14) compounded with the acute nature of the disease in these young children. Therefore, there is the suggestion that AGA may be a better screen owing to the more immediate production of AGA following gluten challenge (15). The results of this study, however, indicate that a TTG humoral response is present in the majority (6/7) of children less than 5 years of age with CD. Further, the youngest child with CD at 1.7 years of age had a very high TTG titer of >300 units. In addition,

TTG provided a good sensitivity (85%) and specificity (92%) for the detection of CD within this age range. One limitation of these findings is the limited group size. Of the 20 children under 5 years of age screened for CD, only seven had biopsy confirmed disease and no children younger than 18 months of age were identified with CD. To confirm these preliminary findings, further studies that specifically investigate levels in children less than 5 years of age are required. Nevertheless, the results of this study indicate that a single TTG assay may be suitable for screening children, including children less than 5 years of age but older than 18 months, for CD.

Correlating antibody titers provided some interesting results. As may be expected, all three antibody titers were significantly correlated, indicating that their expression is related. Interestingly neoG, and not neoA, was most closely correlated to TTG. However, these results indicate that a common factor (i.e., likely the gliadin–transglutaminase antigen complex) has the major influence in driving antibody production; however, the production of each antibody also appears to be significantly influenced by individual specific factors. Therefore, the results of one assay may not accurately reflect the results of another assay; indicating that one antibody may be superior for CD screening.

Despite the limitations of the current neoAGA assay, further improvements in AGA screening can still be achieved. Improved results with AGA screening are seen with an assay that uses synthetic peptides that replicate deaminated gliadin (16–18). Deaminated gliadin peptide (DGP) assays differ from the neoAGA assay in that the DGPs are synthetically produced, whereas the neoAGA assay utilizes gluten that has been digested by an in vitro process that replicates in vivo digestion. Therefore, the antigen in the neoAGA assay is likely to consist of a variety of peptides with a varying degree of deamination. Agardh investigated the utility of DGP assays for coeliac screening in children, and reported that a very high percentage of, but not all, coeliac children are positive for anti-DGP or anti-TTG antibodies (19). However, any assay that utilized a combined DGP–TTG antigen detected 100% of coeliac children. Therefore, Agardh concluded that an assay that detects both IgA and IgG antibodies to DGP–TTG antigen is optimal for frontline CD screening in children (19). Nevertheless, an updated neoAGA assay may still have a role in monitoring CD disease progress. As mentioned previously, AGA antibodies may appear earlier and resolve sooner compared with TTG antibodies (15). Therefore, neoAGA assays may have utility as a tool for monitoring adherence to a gluten-free diet.

In conclusion, this study indicates that AGA detection is improved with refined gliadin assays and, at present, neoAGA assays may still have a limited role in CD

testing and monitoring; however, further improvements to CD screening may be achieved with an assay directed toward both AGA and TTG antibodies. Further, this study has indicated that TTG may be a suitable screening tool for CD in children under 5 years of age, although confirmation with specific investigation of children less than 18 months of age is required.

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