Development of a novel rapid non-invasive screening test for coeliac disease

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Abstract

Background—Coeliac disease is one of the commonest underdiagnosed diseases in general practice. The autoantigen recognised by the sera of patients with coeliac disease has recently been identified as tissue transglutaminase.

Aims—We evaluated a simple non-invasive immunological dot blot assay for coeliac disease, suitable for use by the general physician in the ambulatory setting. The sensitivity and specificity of this dot blot assay based on recognition of recombinant human transglutaminase were compared with those of antiendomysial antibodies and an enzyme linked immunosorbent assay.

Methods—Serum samples were analysed from 64 healthy controls, 58 first degree relatives of coeliacs, 74 diseased controls, and 70 biopsy confirmed untreated patients with coeliac disease. Dot blot assay and enzyme linked immunosorbent assay were performed using recombinant human transglutaminase as antigen.

Results—The dot blot assay, which can be performed in 20 minutes, was positive in all 70 untreated coeliacs (sensitivity 100%). Among the three control groups, there were three false positive tests by dot blot (specificity 98%), all belonging to the group of healthy subjects. The anti-endomysial antibodies test missed five untreated coeliac patients (sensitivity 93%) and was negative in all three control groups (specificity 100%). The specificity of the immunosorbent assay was 99% for IgA and 98% for IgG, while sensitivity was 93% for IgA, 47% for IgG, and 100% for IgA and IgG combined.

Conclusions—The dot blot assay is highly accurate in detecting untreated subjects with coeliac disease and can be performed in the general physician's medical office during the course of a routine examination. This innovative test is a practical, reliable alternative to both the immunofluorescent based antiendomysial test and immunosorbent assay for detection of transglutaminase antibodies for the diagnosis of coeliac disease.

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Coeliac disease (CD) is one of the commonest underdiagnosed diseases in general practice,

estimated to occur in 0.4% of the general population.^{1,2} This is mainly due to its often atypical presentation, which in recent surveys³ have ranged from vague tiredness associated with anaemia to the consequences of associated autoimmune diseases (for example, thyroid disease or insulin dependent diabetes mellitus). Although determination of IgA antiendomysium antibodies (AEA) and IgA and IgG anti-transglutaminase antibodies have been suggested as valid alternatives to duodenal biopsy,⁴ everyday clinical practice is beginning to consider reducing its dependency on biopsy as the gold standard for the definitive diagnosis of CD. The easy availability of such tests has expanded the range of subjects tested and has revealed that CD is far more common than previously suspected.^{3,4} Recently, the antigen recognised by AEA has been identified as tissue transglutaminase (tTG),⁵ a finding which has been exploited to develop a number of enzyme linked immunosorbent assay (ELISA) tests based on guinea pig tTG,⁶⁻⁸ but also on human tTG (h-tTG).⁹ The use of the human recombinant transglutaminase antigen has proved to be more sensitive than the AEA test and just as specific.^{10,11} Although far faster and easier to perform than intestinal biopsy, these tests have remained within the realm of the specialised diagnostic laboratory. Given the high prevalence of the disease and its protean nature, a simple diagnostic test which could be used in the general practitioner's office would represent a great step forward in the rapid diagnosis of CD. Here we present a dot blot test based on detection of anti-h-tTG antibodies in serum or in one drop of whole blood that can be carried out in 20 minutes. We evaluated the sensitivity and specificity of our new test in comparison with AEA and a h-tTG ELISA technique for the diagnosis of CD.

Material and methods

PATIENTS AND HEALTHY CONTROLS

We examined serum samples from 70 untreated CD patients (30 males and 40 females, median age 12 years (range 2-60)) diagnosed between June 1997 and October 1999, following the revised ESPGHAN criteria for CD.¹² Controls comprised 58 first degree relatives of CD patients and 74 subjects suffering from

Abbreviations used in this paper: AEA, antiendomysium antibodies; CD, coeliac disease; ELISA, enzyme linked immunosorbent assay; h-tTG, human tissue transglutaminase; ELISA, enzyme linked immunosorbent assay; TBS, Tris buffered saline; TTBS, Tween 20 Tris buffered saline; PBS, phosphate buffered saline.

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various diseases (10 Crohn's disease, four ulcerative colitis, one lactose intolerance, one Zollinger-Elison syndrome, two epilepsy, 12 anaemia, 30 failure to thrive, 10 diarrhoea, three arthralgia, and one gastritis). These groups consisted of 79 males and 53 females (median age 7 years (range 1–47)). In addition, healthy control sera were taken from a group of 64 blood donors (35 males and 29 females, median age 30 years (range 19–54)).

From January 1999 onwards, as well as testing sera, we also consecutively collected and examined whole blood samples from 17 untreated coeliacs (11 males and six females, median age 12 years (range 2–60)), 13 CD patients (five males and eight females, median age 9 years (range 3–35)) who had been on a gluten free diet for at least two years, and 22 patients tested as patient controls (seven Crohn's disease, five anaemia, three failure to thrive, two diarrhoea, two ulcerative colitis, two epilepsy, one arthralgia; 10 males and 12 females, median age 27 years (range 25–47)).

ANTIENDOMYSIUM ANTIBODY ASSAY

Serum IgA antiendomysium antibodies were measured by means of indirect immunofluorescence using cryostat sections of human umbilical cord, as previously described.¹³ Briefly, the sections were incubated with the subject's serum, diluted 1:5, for 30 minutes. After washing, sections were incubated with fluorescein labelled goat antihuman IgA antibodies for 30 minutes. The slides were washed, mounted in aqueous mounting medium, and examined by fluorescent microscopy.

HUMAN RECOMBINANT TRANSGLUTAMINASE

We cloned h-tTG cDNA from the intestinal biopsy of a patient with active CD using primers specific for the coding regions of the gene.¹⁴ cDNA was cloned into an expression vector (pET28b-Novagen, Madison, Wisconsin, USA), expressed, and purified under non-denaturing conditions using IMAC (Qiagen, Valencia, California, USA).

ELISA

Serum IgA and IgG anti-h-tTG antibodies were determined, as reported recently.⁹ Microtitre plates (EIA/RIA 2580, Costar, Cambridge, Massachusetts, USA) were coated with 1 mg of h-tTG in 100 ml of 0.05 M Tris buffered saline (TBS) with 5 mM CaCl₂, pH 7.5. The plates were washed three times with 0.05 M TBS, 0.01 M EDTA, and 0.1% Tween 20 (TTBS). Serum samples diluted 1:100 in TTBS were incubated for one hour at room temperature. The plates were washed and incubated for one hour at room temperature with either 1:3000 phosphatase conjugated antihuman IgA (Sigma A-3062, St Louis, Missouri, USA) or 1:7000 antihuman IgG (Sigma A-8542) diluted in TTBS. The immune reaction was developed by adding substrate solution and the absorbance read in a microplate reader at 405 nm until the positive control serum reached an optical density value of 2 for IgA and 1.5 for IgG. Results were expressed as a percentage of the positive control serum.

Normal values were taken as <13% for IgA and <30% for IgG, which represented a value >2 SD above the mean of 100 healthy subjects (60 males, 40 females, aged 1–16 years).

DOT IMMUNOBINDING ASSAY

Squares of Immobilon-P membrane (Millipore, IPVH00010) were wetted in 100% methanol for 15 seconds. They were washed with distilled water for two minutes and h-tTG was absorbed as a spot (0.1 µg/spot) onto the squares. After 15 minutes, the Immobilon-P free binding sites were blocked with phosphate buffered saline (PBS)-3% Tween 20 for 30 minutes and the squares were washed, allowed to dry, and stored at 4°C. The coated membranes were found to remain stable for at least one year. Serum samples or one drop (30 µl) of whole blood samples were diluted 1:50 in PBS-0.3% Tween 20 and incubated for five minutes at room temperature. The squares were washed three times with PBS-0.3% Tween 20 and incubated for five minutes at room temperature with phosphatase conjugated antihuman IgA and IgG immunoglobulins (Sigma A-3062, A-8542) diluted 1:2500 in PBS-0.3% Tween 20. The squares were then washed and the immunocomplexes were revealed by substrate solution. The strips were dried and examined, and the results expressed as a colorimetric reaction.

The immunological tests were performed by four operators (VB, AT, CT, IB) who were unaware of the clinical and laboratory findings of the subjects tested.

HLA-DQ TYPING

The known susceptibility alleles for CD were determined by polymerase chain reaction with allele specific primers identifying DQ2 and DQ8, carried out with a Dynal Classic SSP DQ kit (Dynal AS, Oslo, Norway).

Results

An example of the colorimetric reaction using our dot blot assay is shown in fig 1. Serum and whole blood samples from both CD patients and controls showed similar results with a clear discrimination between positives and negatives.

All 70 CD patients tested positive to both the dot blot test and the ELISA (taking into account the results of specific responses to IgG and IgA, sensitivity was 100%, sensitivity for ELISA IgA was 92.8%, and for ELISA IgG 47%). In contrast, the antiendomysial IgA test missed five patients (sensitivity 92.8%) either because of IgA deficiency (two patients) or the presence of IgG anti-h-tTG antibodies alone, which the IgA AEA test was unable to detect (three patients). Among the 64 healthy blood donors, 58 first degree relatives of CD patients and 74 diseased controls, three healthy blood donors tested positive by dot blot (specificity 98.4%) and ELISA (specificity for IgA 99.4%, for IgG 98.9%, for IgA and IgG combined 98.4%), predominantly due to the presence of anti-h-tTG IgG antibodies. In contrast, all controls were negative by AEA (table 1).

None of the three subjects who tested positive for anti-h-tTG antibodies by both dot

Figure 1 Dot blot assay using either serum or whole blood. This assay identified typical coeliac disease (CD) patients (first lane), CD patients with IgA deficiency (second line), and CD patients that tested negative by AEA assay (third line). No signal was detected in controls (fourth and fifth lines).

blot and ELISA had a clinical history or symptoms associated with CD (such as gastroenterological complaints, unexplained anaemia, dental enamel defects, autoimmune disorders, or central nervous system involvement). We analysed HLA DQ2 and DQ8 of three healthy controls and only one had the DQA1 *0501 and DQB *0201 haplotypes. The blood donor positive for the CD related allele refused intestinal biopsy to confirm the diagnosis of CD, even after being informed of the possible risks involved in not treating the disease.

We extended our investigations to the small subset of subjects who had provided samples of whole blood as well as serum (table 2). Similar results were obtained: of 17 biopsy confirmed CD patients, all tested positive by h-tTG dot blot, while eight healthy blood donors, 22 disease controls, and 13 CD patients on a gluten free diet all tested negative. The dot blot test using whole blood performed better than the AEA test. Of the 17 patients with CD, the AEA assay identified only 14 subjects as two were IgA deficient and one tested positive only for anti-h-tTG IgG antibodies. Furthermore, one CD patient on a gluten free diet also tested positive by AEA.

Table 1 Dot blot results on serum samples

Study group			Human tTG ELISA					
	Human tTG DOT		IgA		IgG		IgA AEA	
	+	-	+	-	+	-	+	-
70 Patients with CD	70	0	65	2 ¹ , 3 ²	2 ¹ , 3 ² , 28	37	65	2 ¹ , 3 ²
64 Healthy controls	3	61	1	63	2	62	0	64
58 First degree CD relatives	0	58	0	58	0	58	0	58
64 Disease controls	0	64	0	64	0	64	0	64
10 Patients with Crohn's	0	10	0	10	0	10	0	10

¹Subjects with IgA serum deficiency.

²Subjects with IgA antiendomysium negative tests but with positive human tTG IgG ELISA and dot blot.

CD, coeliac disease; human tTG, human tissue transglutaminase.

Table 2 Dot blot results on whole blood samples

Study group	Human tTG dot		IgA AEA	
	+	-	+	-
17 Patients with CD	17	0	14	2 ¹ , 1 ²
13 Patients on GFD	0	13	1	12
22 Disease controls	0	22	0	22
8 Healthy controls	0	8	0	8

¹Subjects with IgA serum deficiency.

²Subjects with IgA antiendomysium negative tests but with positive human tTG IgG ELISA and dot blot.

CD, coeliac disease; human tTG, human tissue transglutaminase; GFD, gluten free diet.

Discussion

This simple dot blot test diagnosed all CD patients as effectively as the tTG ELISA and with greater sensitivity than the commonly used AEA test. This is likely to be due to the fact that while both tests detect tTG specific antibodies, AEA can only detect IgA, whereas the dot blot detects both IgA and IgG. As shown here and previously by others,¹⁵ some CD patients with normal levels of serum IgA class immunoglobulin lack IgA antibodies to tTG but have anti-tTG IgG antibodies. It is noteworthy that our rapid test identified those patients with serum IgA deficiency which is found more frequently in CD patients.¹⁶

The test described here is quick and requires minimal handling; its cost is low (1 EURO per sample) and in view of its high sensitivity and specificity could easily be introduced into the general physician's armoury of medical office tests for patients complaining of vague symptoms of tiredness, as well as those with more classic gastroenterological symptoms. This is in line with a recent report³ that chronic tiredness, and not gastrointestinal symptoms, were the commonest presentation of CD, when this was considered as a possible diagnosis in a large number of patients tested using AEA serology.

In view of the risks accompanying prolonged untreated CD, which include autoimmune diseases,¹⁷ depression,¹⁸ multiple miscarriage,¹⁹ cerebellar ataxia,²⁰ drug resistant epilepsy with occipital calcification,²¹ and intestinal lymphoma,²² and given the increased prevalence of CD among close relatives of CD patients²³ and sufferers from autoimmune diseases,²⁴⁻²⁷ testing for CD should become a routine part of many clinical workups; the quick and easy test described here makes this a practical reality.

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- Catassi C, Ratsch I, Fabiani E, et al. High prevalence of undiagnosed coeliac disease in 5280 Italian students screened by anti-gluten antibodies. *Acta Paediatr* 1995;84:672-6.
- Not T, Horvath K, Hill I, et al. Celiac disease risk in the USA: high prevalence of antiendomysium antibodies in healthy blood donors. *Scand J Gastroenterol* 1998;33:494-8.
- Hin H, Bird G, Fisher P, et al. Coeliac disease in primary care: case finding study. *BMJ* 1999;318:164-7.
- Valdimarsson T, Franzen L, Grodzinsky E, et al. Is small bowel biopsy necessary in adults with suspected celiac disease and IgA anti-endomysium antibodies? 100% positive predictive value for celiac disease in adults. *Dig Dis Sci* 1996;41:83-7.

- 5 Dietrich W, Ehnis T, Bauer M, *et al.* Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nat Med* 1997;**3**:797–801.
- 6 Sulkanen S, Haltunen T, Laurila K, *et al.* Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease. *Gastroenterology* 1998;**115**:1322–8.
- 7 Troncone R, Maurano F, Rossi M, *et al.* IgA antibodies to tissue transglutaminase: an effective diagnostic test for celiac disease. *J Pediatr* 1999;**134**:166–71.
- 8 Dietrich W, Laag E, Schopper H, *et al.* Autoantibodies to tissue transglutaminase as predictors of celiac disease. *Gastroenterology* 1998;**115**:1317–21.
- 9 Sblattero D, Berti I, Trevisiol C, *et al.* Human recombinant tissue transglutaminase ELISA: an innovative diagnostic assay for celiac disease. *Am J Gastroenterol* 2000;**95**:1253–7.
- 10 Bazzigaluppi E, Lampasona V, Barera G, *et al.* Comparison of tissue transglutaminase-specific assays with established antibody measurements for coeliac disease. *J Autoimmunity* 1999;**12**:51–6.
- 11 Seissler J, Boms S, Wohlrab U, *et al.* Antibodies to human recombinant tissue transglutaminase measured by radioligand assay: evidence for high diagnostic sensitivity for celiac disease. *Horm Metab Res* 1999;**31**:375–9.
- 12 Walker-Smith J, Guandalini S, Schmitz J, *et al.* Revised criteria for diagnosis of coeliac disease. *Arch Dis Child* 1990;**65**:909–11.
- 13 Not T, Città A, Lucchesi A, *et al.* Anti-endomysium antibody on human umbilical cord vein tissue: an inexpensive diagnostic tool for the screening of coeliac disease. *Eur J Pediatr* 1997;**156**:616–18.
- 14 Gentile V, Saydak M, Chiocci F, *et al.* Isolation and characterization of cDNA clones to mouse macrophage and human endothelial cell tissue transglutaminase. *J Biol Chem* 1991;**266**:4789–80.
- 15 Sulkanen S, Collin P, Laurila K, *et al.* IgA and IgG class antihuman umbilical cord antibody tests in adult coeliac disease. *Scand J Gastroenterol* 1998;**33**:251–4.
- 16 Cataldo F, Marino V, Ventura A, *et al.* Prevalence and clinical features of selective immunoglobulin A deficiency in coeliac disease: an Italian multicentre study. *Gut* 1998;**42**:362–5.
- 17 Ventura A, Magazzu G, Greco L. Duration of exposure to gluten and risk for autoimmune disorders in celiac patients. *Gastroenterology* 1999;**117**:303–10.
- 18 Corvaglia L, Catamo R, Pepe G, *et al.* Depression in adult untreated celiac subjects: diagnosis by the pediatrician. *Am J Gastroenterol* 1999;**94**:839–43.
- 19 Ciacci C, Cirillo M, Auriemma G, *et al.* Celiac disease and pregnancy outcome. *Am J Gastroenterol* 1996;**91**:718–22.
- 20 Hadjivassiliou M, Gibson A, Davies-Jones G, *et al.* Does cryptic gluten sensitivity play a part in neurological illness? *Lancet* 1996;**347**:369–71.
- 21 Gobbi G, Bouquet F, Lambertini A, *et al.* Coeliac disease, epilepsy and cerebral calcifications. *Lancet* 1992;**340**:439–52.
- 22 Holmes G, Prior P, Lane M, *et al.* Malignancy in coeliac disease—effect of gluten free diet. *Gut* 1989;**30**:333–8.
- 23 Maki M, Holm K, Lipsanen V, *et al.* Serological markers and HLA genes among healthy first-degree relatives of patients with coeliac disease. *Lancet* 1991;**338**:1350–3.
- 24 Corazza G, Andreani M, Venturo N, *et al.* Celiac disease and alopecia areata: report of a new association. *Gastroenterology* 1995;**109**:1333–7.
- 25 Collin P, Salmi J, Hallstrom O, *et al.* Autoimmune thyroid disorders and coeliac disease. *Eur J Endocrinol* 1994;**130**:137–40.
- 26 Lepore L, Martellosi S, Pennesi M, *et al.* Prevalence of celiac disease in patients with juvenile chronic arthritis. *J Pediatr* 1996;**129**:311–13.
- 27 Cronin C, Fergus S. Insulin dependent diabetes mellitus and coeliac disease. *Lancet* 1997;**349**:1096–7.