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Self transglutaminase-based rapid coeliac disease antibody detection by a lateral flow method

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SUMMARY

Background
The conventional coeliac disease antibody tests require patient's sera, and are laborious and time-consuming.

Aim
To evaluate a newly developed rapid whole blood test in coeliac disease antibody detection, and its suitability for office use.

Methods
Endogenous tissue transglutaminase found in red blood cells in a whole blood fingertip or venous sample is liberated upon haemolysis and complexes with tissue transglutaminase antibodies, if present. The complexes, captured by a lateral flow system, are visualized within 5 min. Stored samples from 121 untreated, 106 treated coeliac disease patients and 107 controls were evaluated and compared with serum endomysium and tissue transglutaminase antibody tests and histology; 150 patients were prospectively tested on site in the doctor's office.

Results
The rapid test showed sensitivity (96.7%) comparable with the serum endomysium and tissue transglutaminase antibody tests from stored samples; specificity was slightly lower (93.5%). When tested on site the results were concordant in 96.7% of cases compared with endomysium and tissue transglutaminase antibody results. The test recognized the disappearance of tissue transglutaminase antibodies on a gluten-free diet.

Conclusions
The self tissue transglutaminase-based rapid test can be easily carried out from a fingertip blood sample on site in the physician's office for both coeliac disease case finding and dietary monitoring purposes.

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INTRODUCTION

In untreated coeliac disease the clinical picture can range from the classic abdominal symptoms to extra-intestinal manifestations, or the disease may even be clinically silent.[1] The prevalence of the disease is as high as 1 in 100,[2, 3] but because of its protean picture, it frequently remains undiagnosed. General practitioners are in a crucial position in detecting the condition and therefore a non-invasive test which is also easy to use in a general practitioner's office would be helpful in selecting patients to undergo diagnostic small-intestinal biopsy.[4]

The conventional immunoglobulin (Ig) A-class endomysial antibody (EMA) test based on an indirect immunofluorescent (IIF) method is highly specific (97–100%) and sensitive (90–100%) in coeliac disease case finding,[5] but is subjective in interpretation.[6] Since the identification of tissue transglutaminase (tTG) as the endomysial autoantigen in coeliac disease,[7] it has been possible to develop easier and less expensive enzyme-linked immunosorbent assay (ELISA)-based screening tests.[8, 9] Both of these conventional screening tests require patient's sera and special laboratory facilities and test results are available only after a time lag.

The coeliac disease autoantigen, tTG, is an intracellular enzyme found for example in fibroblasts, endothelial, mononuclear and also red blood cells.[10] We recently established that the patient's own tTG can be used in coeliac disease antibody detection by haemolysing the whole blood sample and thus liberating the enzyme from the red blood cells.[11] The liberated tTG complexes with circulating coeliac-specific autoantibodies, if present. In this method there is thus no need for purified or recombinant tTG or for serum separation. We also showed the rapid point-of-care test, based on this new innovation, to have a sensitivity of 97% and a specificity of 98% in detecting untreated coeliac disease.[12] As the proof-of-concept test proved to be highly predictive for the disease, a more user-friendly rapid whole blood coeliac disease test utilizing a lateral flow method and the patient's self tTG was developed. The test can be performed from a finger tip or venous whole blood sample in a few minutes and interpreted visually on site.

Our aim was to evaluate the new self tTG-based rapid whole blood test in detecting coeliac disease and in monitoring treatment. We first assessed stored samples from coeliac disease patients and non-coeliac controls in a laboratory setting and secondly, sought to establish whether the new test works on site in the doctor's office in selecting patients for confirmatory small-bowel biopsy. The results of the rapid whole blood test were compared with those in conventional serum EMA and tissue transglutaminase antibody (tTG-ab) tests and to small-bowel mucosal histology.

PATIENTS AND METHODS

Subjects

The patients were investigated at the Department of Gastroenterology-Nephrology, Heim Pál Children's Hospital, Budapest, Hungary, at the Department of Paediatrics, University of Debrecen, Hungary and at the Department Gastroenterology and Alimentary Tract Surgery in Tampere University Hospital, Finland.

In the first part of the study the rapid test was performed in the laboratory on stored whole blood samples. The study group comprised 121 consecutive untreated coeliac disease patients and 107 non-coeliac disease controls. The diagnosis of coeliac disease was based on severe partial or subtotal villous atrophy with crypt hyperplasia in the small-bowel and on the clinical or histological response to a gluten-free diet.[13] Patients evincing normal villous morphology served as non-coeliac controls. Demographic data on the patients and controls and the main indication for serological coeliac disease testing are shown in Table 1. None of the patients suffered from IgA deficiency. Follow-up results were available in 15 of the above-mentioned newly detected coeliac disease patients (median age 34 years, range 9–68 years) after 1 year on a gluten-free diet. Moreover, samples from 91 long-term treated (median duration of a strict gluten-free diet 9 years, range 1–24 years) coeliac disease patients (61 female; median age 58 years; range 23–82 years) were tested in laboratory. Small-bowel mucosal biopsy, serum and whole blood samples with ethylenediaminetetraacetic acid (EDTA) or sodium citrate were obtained from all patients before and after the gluten-free diet and stored at −20 °C until tested.

To assess the rapid whole blood testing onsite, 150 patients with suspicion of coeliac disease were studied prospectively in a tertiary gastroenterology centre (Table 1). Altogether 78 of these patients were referred to special health care due to symptoms suggestive of coeliac disease and the remaining 72 were first-degree family members of coeliac disease patients. The rapid test was performed from a fresh fingertip sample and

Table 1. Demographic data on untreated coeliac disease patients, non-coeliac disease controls and prospectively tested patients with coeliac disease suspicion

	Laboratory testing		On site testing
	Untreated coeliac disease ($n = 121$)	Non-coeliac disease controls ($n = 107$)	Prospectively tested patients ($n = 150$)
Age: median (range), years	12 (1.6–68)	15 (0.9–72)	9 (0.9–72)
Patients under 16 years, *n* (%)	81 (67)	59 (55)	95 (63)
Female, *n* (%)	85 (70)	46 (43)	86 (57)
Indication for coeliac disease antibody testing, *n* (%)			
Gastrointestinal complaints	85 (70)*	93 (87)†	58 (37)*
Anaemia or malabsorption	12 (10)	8 (7)	6 (4)
Screening of associated diseases or extraintestinal manifestations known to carry an increased risk of coeliac disease	24 (20)‡	6 (6)§	86 (57)¶

* Diarrhoea, abdominal distension and pain.
† Dyspepsia ($n = 60$), gastro-oesophageal reflux disease ($n = 15$), inflammatory bowel disease ($n = 14$), irritable bowel syndrome ($n = 2$), recurrent abdominal pain ($n = 2$).
‡ Insulin-dependent diabetes mellitus ($n = 2$), family history of coeliac disease ($n = 11$), retarded growth ($n = 5$), eating disorder ($n = 3$), arthritis ($n = 2$), rash ($n = 1$).
§ Familial adenomatosus polyposis ($n = 4$), intestinal lymphangiectasia ($n = 1$), rash ($n = 1$).
¶ Family history of coeliac disease ($n = 72$), rash ($n = 6$), retarded growth ($n = 6$), Sjögren's syndrome ($n = 1$), autism ($n = 1$).

the test result was interpreted immediately on site in the doctor's office and venous samples for serological EMA and tTG-ab testing were collected simultaneously. When patients yielded positive coeliac disease antibody test results they were also invited to undergo diagnostic small-intestinal endoscopy and biopsy.

Self tissue transglutaminase-based rapid coeliac antibody detection

The self tTG-based coeliac antibody detection was based on our innovation utilizing endogenous tTG found in the red blood cells.[11] The basic concept is to liberate the patient's own tTG from the red blood cells by haemolysing an anticoagulated citrated or EDTA whole blood sample. When tTG-specific antibodies are present in the sera they recognize and form complexes with the liberated self tTG. The complexes can be detected by binding tTG to a solid surface coated with tTG-capturing proteins. The bound antigen-antibody complexes can be seen in colour reaction with the help of labelled anti-human IgA solution.[12]

In the present study we evaluated a commercial application (Biocard Celiac disease™, AniBiotech, Vantaa, Finland) based on the above-mentioned innovation. This test utilizes lateral flow immunochromatographic strip system and colloidal gold-labelled mouse antibodies to human IgA as signal generator. In short, the testing was performed from thawed venous or fresh fingertip whole blood samples. Using a capillary supplied with the test, 10 μL of EDTA, citrate or capillary whole blood is added to the tube containing 0.5 mL of haemolysing sample buffer, thus achieving a sample dilution of 1:50. Three drops of the haemolysed sample dilution are then added to the round application field of the test card. In the card the diluted blood sample migrates by capillary diffusion through the conjugate pad, redydrating the gold conjugate.[14] If tTG-ab are present in the sample, they complex with tTG. These complexes bind with colloidal gold-labelled anti-IgA antibodies and are captured by tTG binding protein[12] linked to the nitrocellulose test membrane, forming a visible red test line (Figure 1). In addition, an integrated control system ensures the proper function of the test. The reaction in

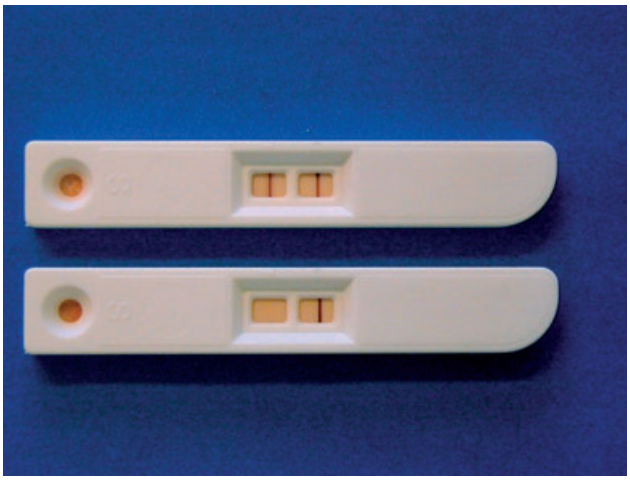

Figure 1. The rapid whole blood test card for coeliac-specific immunoglobulin A class tissue transglutaminase antibody detection. The haemolysed blood dilution is dropped onto the round application field. In a positive test result both the control line (right) and the line in the test field (left) can be seen (upper test card). When the result is negative (lower test card), only the control line is seen.

the control line happens between the colloidal gold-labelled anti-IgA mouse antibodies which passed the test line without binding and anti-mouse IgG antibodies, and shows that both the sample and reagents had moved over the test line and reached the control point. The manufacturer has suggested to interpret the results after 5 min but not later than 10 min. However, a positive test result may appear already within 1–2 min. The test result is positive when both the control line and the line in the test field can be seen; in negative cases only the control line forms.

Interobserver variability in the rapid test was assessed with 20 randomly selected EDTA or sodium citrate whole blood samples from the study cohort between two investigators in blinded fashion. Furthermore, intraobserver variation was evaluated blindly with the corresponding samples at different time points.

Serology

Serum IgA-class EMA was determined by an IIF method as previously described.[9, 15] Serum IgA-class tTG-ab were determined by ELISA using the Celikey™ test (Pharmacia Diagnostics, GmbH, Freiburg, Germany) according to the manufacturer's instructions.

Small-bowel mucosal morphology

Small-bowel mucosal biopsies were taken either by upper gastrointestinal endoscopy from the distal part of the duodenum or by Watson capsule from the proximal jejunum. Haematoxylin-eosin-stained biopsy specimens were studied under light microscopy and the villous height/crypt depth ratio calculated as previously described;[16] a ratio of <2 was considered to be abnormal and indicative of untreated coeliac disease.

Statistics

The sensitivities were calculated from the equation $a/(a + c) \times 100$, specificities $d/(b + d) \times 100$, positive predictive values $a/(a + b) \times 100$, negative predictive values $d/(d + c) \times 100$ and efficiencies of the tests $(a + d)/(a + d + c + b) \times 100$ respectively. In the equations, a stands for the number of untreated biopsy-proven coeliac disease patients recognized by the test; b for number of biopsy-proven non-coeliac disease controls with a positive test result; c for the number of untreated patients misclassified by the test and d for non-coeliac disease controls negative for the test.

Ethical considerations

The study protocol was approved by the local ethical committees in Hungary and Finland. All subjects gave informed consent.

RESULTS

In stored samples analysed in the laboratory the rapid whole blood test gave sensitivity results comparable with those of the serum EMA and tTG-ab tests (Table 2). The specificity of the rapid test was lower compared to the conventional serum tests. The test recognized untreated coeliac disease in children aged <16 years (sensitivity 99%, specificity 97%) better than patients aged over 16 years (sensitivity 93%, specificity 90%, respectively) (Table 3). The rapid test results were concordant with serum EMA test results in 215 of 228 cases (94.3%) and with serum tTG-ab test results in 216 (94.7%) respectively. In the laboratory, both the interobserver agreement between two investigators and the intraobserver agreement for the rapid whole blood test was 100%.

After adherence to a strict gluten-free diet for 1 year the rapid test result converted from positive to negat-

Table 2. Sensitivity, specificity, positive and negative predictive value and efficiency of the IgA-class rapid whole blood test, serum tissue transglutaminase antibody (tTG-ab) and serum endomysium (EMA) tests on stored samples in the laboratory. The sensitivities and specificities have been calculated from the untreated biopsy-proven coeliac disease patients and biopsied non-coeliac disease controls

	Rapid whole blood test		Serum EMA		Serum tTG-ab	
	Positive	Negative	Positive	Negative	Positive	Negative
Untreated coeliac disease, $n = 121$	117	4	117	4	120	1
Controls, $n = 107$	7	100	0	107	0	107
Sensitivity (%)	96.7		96.7		99.2	
Specificity (%)	93.5		100.0		100.0	
Positive predictive value (%)	94.4		100.0		100.0	
Negative predictive value (%)	96.2		96.4		99.1	
Efficiency of the test (%)	95.2		98.2		99.6	

Table 3. Demographic data and the results of the immunoglobulin A-class rapid whole blood test, serum tissue transglutaminase antibody (tTG-ab) and serum endomysium (EMA) tests on patients under and over 16 years when tested on stored samples in the laboratory

	Patients under 16 years ($n = 140$)	Patients over 16 years ($n = 88$)
Female, *n* (%)	77 (55)	54 (61)
Indication for coeliac disease antibody testing, *n* (%)		
Gastrointestinal complaints	110 (79)	68 (77)
Anaemia or malabsorption	9 (6)	11 (13)
Screening of associated diseases or extra intestinal manifestations	21 (15)	9 (10)
Untreated coeliac disease patients, $n = 121$ (%)	81 (67)	40 (33)
Rapid test positive, *n* (%)	80 (99)	37 (93)
EMA positive, *n* (%)	81 (100)	36 (90)
tTG-ab positive, *n* (%)	81 (100)	39 (98)
Controls, $n = 107$ (%)	59 (55)	48 (45)
Rapid test positive, *n* (%)	2 (2)	5 (5)
EMA positive, *n* (%)	0 (0)	0 (0)
tTG-ab positive, *n* (%)	0 (0)	0 (0)

ive in 13 (87%) coeliac disease patients and the test result remained positive in two (Figure 2). Initially the two rapid test-positive treated patients had highly positive serum tTG-ab values before starting a gluten-free diet and they also had borderline serum tTG-ab results (4.2 and 4.6 U/mL) while adhering to the diet. In addition, from the 91 long-term treated coeliac disease patients 88 (96.7%) were negative in the rapid test, 88 (96.7%) in the serum EMA test and 90 (98.9%) in the serum tTG-ab test. Three of the 91 treated patients had small-bowel mucosal villous atrophy with crypt hyperplasia, in the rest villous mucosal morphology was normal. The rapid and serum EMA tests recognized two of the three patients with abnormal mucosa and the serum tTG-ab test one, respectively.

The rapid test, performed on site prospectively in the doctor's office, yielded concordant results with serologic EMA and tTG-ab tests in 145 of the 150 patients (96.7%) (Table 4). The rapid test achieved a sensitivity of 95.5% and a specificity of 97.1% in relation to serum EMA and tTG-ab results. Altogether 47 of the 150 patients (36 symptomatic patients and 11 first-degree relatives) were rapid test-positive. Forty-four of them agreed to undergo intestinal biopsy and they all had small-bowel mucosal lesion typical of coeliac disease (positive predictive value 100%). This high positivity rate was because of the fact that the setting of the testing was a tertiary centre with frequent referral of patients having a high probability of coeliac disease. The rapid test was negative in 103

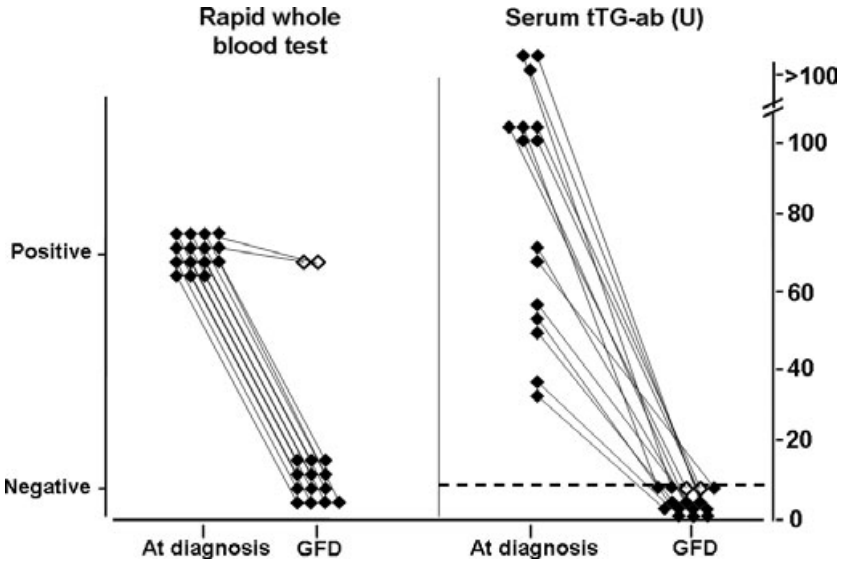

Figure 2. The rapid whole blood test results and serum tissue transglutaminase antibody (tTG-ab) results in 15 coeliac disease patients at the time of diagnosis and after a one-year gluten-free diet. Diamonds connected with a line represent the values of the same patient before and after a gluten-free diet. Two coeliac disease patients (open diamonds) were still positive in the rapid test after a gluten-free diet and had also borderline tTG-ab values. The cut-off level for serum tTG-ab positivity (5 U/mL) is shown in the horizontal dotted line. GFD, gluten-free diet.

Table 4. Comparison of on site rapid whole blood test results and serum endomysial antibody (EMA) and tissue transglutaminase antibody (tTG-ab) test results when patients under coeliac disease suspicion were investigated prospectively

	Serum EMA		Serum tTG-ab	
	Positive	Negative	Positive	Negative
Rapid test positive	44	3	44	3
Rapid test negative	2*	101	2*	101

* One patient was positive in serum EMA and negative in tTG-ab test, the other negative in EMA and positive in tTG-ab test.

patients. Three of them had either positive serum EMA or tTG-ab test result and the small-bowel mucosal morphology showed villous atrophy with crypt hyperplasia.

DISCUSSION

The rapid self tTG-based whole blood test showed comparable sensitivity to detect untreated coeliac disease as the currently widely employed serological EMA and tTG-ab tests. The test result was easy to interpret visually on site and the test turned out to be highly repeatable and reproducible. This method speeds up and facilitates the diagnostic work-up of coeliac disease, as test-positive individuals can be sent for endoscopy without any time lag. The slightly lower specificity of the test in laboratory testing of stored samples is of no major importance, as the positive test results can be verified with serum EMA, tTG-ab or small-intestinal mucosal biopsy. Interestingly, three of the seven rapid test-positive controls without villous atrophy from the series tested in the laboratory showed signs of early developing coeliac disease upon further investigation beyond this study; they had coeliac-type HLA DQ2, an increased density of $\gamma\delta$+ intraepithelial lymphocytes or tTG-specific IgA-deposits in their small-bowel mucosa.[17, 18] Furthermore, when the rapid testing was performed on site from fingertip blood, the test results were more concordant with the serum EMA and tTG-ab test results and had 100% positive predictive value for a coeliac-type histology finding. These results suggest that the rapid test might be more specific when used with fresh blood samples.

Currently, the only effective treatment of coeliac disease is a strict gluten-free diet.[1] It is known that the

coeliac-specific autoantibodies disappear from the blood during the diet parallel with the recovery of the small-intestinal mucosal damage.[19, 20] Similarly to the conventional serum tests, the rapid test result also seroconverted from positive to negative in coeliac disease patients after 1 year on a gluten-free diet and the test result was negative in 97% of long-term treated coeliac disease patients. The test might be thus suitable, in addition to coeliac disease case finding, for the detection of tTG-ab seroconversion from positive to negative after adoption of a long-term gluten-free diet. Subsequently, the test can be used again in coeliac disease patient's dietary monitoring, as a test result reconverted from negative to positive indicates dietary lapses. As noted in our earlier study, a rapid coeliac disease antibody test done on site in the doctor's office enables immediate feedback to encourage coeliac disease patients to strengthen their diet.[12] The commercial rapid test might also become available to coeliac patients themselves for diet monitoring at home as the manufacturer has taken care of the quality control issues outside laboratory situations and the test documentation has been evaluated and accepted for home testing and CE-marking (Communautée européenne) by Notified Body (RWTÜV Systems GmbH, the manufacturer number CE 0044).

The serological tTG-ab tests and also the previously reported coeliac disease rapid tests utilize external tTG,[21–23] which is sensitive to storage problems.[10] In the rapid test fresh tTG is liberated from the red blood cells of a whole blood sample on site. Furthermore, all the equipment needed in testing comes with the test kit and the test result can be read visually immediately. For these reasons, the coeliac disease rapid whole blood test seems to be useful in a wide range of circumstances, for example in developing countries or in remote areas, where are no centralized laboratories and sample storing possibilities.

The current rapid test was developed to uncover IgA-class tTG antibodies as do the frequently used serum IgA-class EMA and tTG-ab tests. However, clinicians must be aware of the limitation of IgA-class antibody detection in coeliac disease case finding among patients with selective IgA deficiency, which is found more often in coeliac disease patients.[24, 25] Further research is needed to attain a coeliac disease rapid test which can uncover, in addition to IgA-class tTG antibodies, also IgG-class tTG antibodies or deficiency of IgA. In addition, clinicians should be also aware of the variable prevalence of coeliac disease in different populations. In the study the prevalence of coeliac disease was high because the testing was done among coeliac disease risk groups. In general population the prevalence of coeliac disease is no more than 1 in 100.[2, 3] Instead, among first-degree relatives of coeliac patients the prevalence is clearly higher, around 10%.[1] Further investigations are still needed to see how the rapid test functions on a population level.

In conclusion, the present study showed that the self tTG-based commercial rapid whole blood test is as sensitive in detecting untreated coeliac disease as the conventional serum-based tTG-ab and EMA tests and thus in pinpointing patients for confirmatory endoscopy. The test also showed visually the seroconversion to negative during a gluten-free diet and it was easy to carry out onsite without any need for laboratory facilities. The test can therefore offer a useful tool in the general practitioner's office in coeliac disease case finding and coeliac disease diet monitoring.

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