

Human tissue transglutaminase antibody screening by immunochromatographic line immunoassay for early diagnosis of celiac disease in Turkish children

Türk çocuklarında çölyak hastalığının erken tanısı için immünokromatografik çizgi yöntemiyle insan doku transglutaminazına karşı antikor taraması

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Background/aims: Celiac disease has a large prevalence worldwide. There are a limited number of comparable epidemiological data for celiac disease in Turkey. The aim of this preliminary study was to determine the prevalence of celiac disease in a sample of 1000 Turkish children by a novel, simple, and visual one-step immunoassay screening test. **Methods:** This prospective study consisted of 1000 serum samples from apparently healthy children and children with disorders other than celiac disease aged between 2-18 years who presented as outpatients at Ankara University, Faculty of Medicine, Department of Pediatrics. Sera were tested for IgA-class antibodies against human tissue transglutaminase and gliadin by rapid immunochromatographic line immunoassay. Endomysial antibody IgA against human tissue transglutaminase and AGA IgA/IgG were also tested by ELISA as a second step when the result of the screening test was positive. Small bowel biopsy was recommended to all the children with positive anti-tissue transglutaminase and/or endomysial antibody results. **Results:** Ten of the 1000 individuals (1%) had positive antibody screening test to human tissue transglutaminase. All tissue transglutaminase-positive samples revealed good correlation with endomysial antibody by ELISA method. Subsequently small bowel biopsy was performed in all serology-positive cases. Biopsy results confirmed a diagnosis of celiac disease in nine cases. The prevalence of biopsy-proven celiac disease was 1:111 (0.9%). **Conclusions:** Determination of anti-tissue transglutaminase antibodies by simple visual system for celiac disease appeared to be as reliable as the ELISA system. It is easy to perform and interpret, cost-effective, and rapid, as pointed out in other previous studies, as a screening test in large population-based studies. The prevalence of celiac disease in the overall sample of Turkish children (1:111 or 0.9%) in this preliminary study is similar to that reported in European and Middle Eastern countries and the United States.

Key words: Celiac disease screening, immunochromatographic assay, line immunoassay, prevalence, tissue transglutaminase

Amaç: Çölyak hastalığı tüm dünyada yaygın sıklıkta görülmektedir. Türkiye’de dünya ile karşılaştırılabilecek epidemiyolojik veriler sınırlı sayıdadır. Bu ön hazırlayıcı çalışmanın amacı, Türk çocuklarında çölyak hastalığı sıklığını 1000 olguda yeni, kolay ve görsel tek adımda yapılabilen bir immünolojik tarama yöntemiyle belirlemektir. **Yöntem:** Bu prospektif çalışma, Ankara Üniversitesi Tıp Fakültesi Çocuk Polikliniğine başvuran, sağlıklı görünen veya çölyak hastalığı dışında bir rahatsızlığı olan, 2-18 yaş arası 1000 çocuk ve ergenden bilgilendirilmiş onam formu ile gönüllü olarak alınan serum örneklerinde yapıldı. Serumlar insan doku transglutaminazına ve gliadine karşı oluşmuş IgA sınıfı antikorlar için hızlı, görsel immünokromatografik çölyak hastalığı çizgisel immün yöntemiyle çalışıldı. Tarama testi sonuçları pozitif olan serumlar ikinci basamak doğrulama aşaması için ELISA yöntemi ile insan doku transglutaminazına karşı oluşmuş endomisyal antikor IgA ve anti gliadin antikorlar IgA/IgG için ayrıca test edildiler. Anti doku transglutaminaz ve/veya endomisyal antikor pozitif olan bütün çocuklara ince barsak biyopsisi önerildi. **Bulgular:** 1000 olgunun 10’unda (%1) insan doku transglutaminazına karşı pozitif antikor tarama sonucu saptandı. Bütün doku transglutaminaz antikor pozitif örnekler ELISA yöntemiyle endomisyal antikor çalışıldığında uyumlu görüldüler. Takiben yapılan ince barsak biyopsisinde de biri hariç dokuz olguda çölyak hastalığı tanısı doğrulandı. Biyopsi ile kanıtlanmış çölyak hastalığı prevalansı 1/111 (%0,9) olarak saptandı. **Sonuçlar:** Çölyak hastalığı için anti-doku transglutaminaz antikorlarının kolay görsel bir yöntemle belirlenmesi ELISA yöntemi kadar güvenilirirdir. Yapması ve yorumlaması kolay bu testin, ucuz ve hızlı bir tarama yöntemi olarak geniş toplum tabanlı çalışmalarda uygulanabileceği düşünüldü. Bu ön çalışmada Türk çocukları örnek grubunda saptanan çölyak hastalığı sıklığı (1:111 veya %0.9) Avrupa, Orta Doğu ülkeleri ve ABD verileri ile benzer bulundu.

Anahtar kelimeler: Çölyak hastalığı taraması, immünokromatografik yöntem, çizgisel immün yöntem, prevalans, doku transglutaminazı

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INTRODUCTION

Celiac disease (CD) is an immune-mediated malabsorption disorder of the small intestine that develops following the ingestion of wheat gluten or related proteins from other cereals as rye and barley in genetically susceptible individuals. It is one of the most common chronic, autoimmune diseases at any age worldwide. Currently, the treatment of CD is lifelong gluten-free diet (GFD) (1-6).

The spectrum of clinical presentations is wide. It has two forms: symptomatic, characterized by gastrointestinal (GI) and extra-GI manifestations as dental enamel defects, short stature, osteopenic bone disease, pubertal delay, lactose intolerance, hypertransaminasemia, different liver disorders, and dermatitis herpetiformis; or asymptomatic, also named as silent and potential forms of CD. Many cases remain undiagnosed or the diagnosis may be significantly delayed, increasing the risk of long-term complications such as other autoimmune diseases, untreatable anemia, osteoporosis, and even infertility or intestinal malignancies. Appropriate screening tests may ease the diagnosis and prevent long-term serious complications of undiagnosed and untreated CD (1, 2).

Tissue transglutaminase (tTG) has recently been identified as the major autoantigen recognized by the endomysial antibody (EMA), and it has led to development of CD screening protocols (7). tTG is a ubiquitous cytoplasmic enzyme found mainly in respiratory and gut epithelial cells. Although tTG prevents tissue damage by catalyzing protein cross-linkage, it is also responsible for gliadin toxicity through its selective deamidation of glutamine residues of gliadin to glutamic acid in the presence of certain HLA-related DR and DQ molecules (8, 9). Increased tTG activity in the small bowel mucosa and the presence of antibodies against tTG strongly suggest CD (10,11).

Celiac disease has a large prevalence in the general population, but shows geographical variation. A number of studies in Europe and the United States found similar prevalence of approximately 1:300 to 1:80 of CD in childhood (1, 2, 5). There has not been a well-designed epidemiological, large population-based study for CD in Turkey, although it is frequently diagnosed in our medical practice. Previous studies from Turkey suggest a high prevalence of CD in the Turkish population (12-14).

The aim of this preliminary study was to determine the prevalence of CD among 1000 children aged

2-18 years using a screening test which detects human tTG antibodies with a simple visual system.

MATERIALS AND METHODS

Subjects

This prospective study was designed to screen 1000 children for the presence of IgA antibodies to tTG and gliadin by rapid immunochromatographic CD-line immunoassay (LIA). For this purpose, serum samples of apparently healthy children or children with different disorders other than CD aged between 2 to 18 years who presented as outpatients to Ankara University Faculty of Medicine, Department of Pediatrics were obtained over a one-year period between September 2002 and September 2003. The specific symptoms of CD such as diarrhea, constipation, abdominal pain, failure to thrive, and other symptoms were recorded as well. Patients who had been diagnosed as CD previously or had family history of CD were excluded. This study was approved by the Ethics Committee of Ankara University and was supported by the Ankara University Research Center. Parental written informed consent was obtained from all participants.

Serum samples were centrifuged and stored at -30°C until analysis. They were first detected by visual CD-LIA for IgA antibodies to human tTG and gliadin. Then, positive samples were tested again for EMA known as anti-tTG antibody synonymously against transglutaminase and anti-gliadin antibodies (AGA) by commercially available enzyme-linked immunosorbent assay (ELISA; Euroimmune, Germany) as a second step. Thus, confirmation of all positive anti-tTG and anti-gliadin tests by conventional ELISA tests before recommending a biopsy was done. Third step was small bowel biopsy (SBB) for definite diagnosis. Biopsy specimens were examined by a single pathologist blinded to the serology results and evaluated according to the Marsh criteria (15) (Figure 1).

CD-LIA/AGA and Anti-tTG Antibody Determinations

The test (Imtec-Celiac Disease-LIA, Germany) is based on the principle of the immunochromatographic LIA. Native gliadin of wheat and purified tTG are applied as lines dotted onto a nitrocellulose membrane. The nitrocellulose membrane is blocked to prevent unspecific reactions. During incubation of a blotting strip with diluted patient serum, antibodies present in the sample will bind to the antigens on the strip. For the detection of the

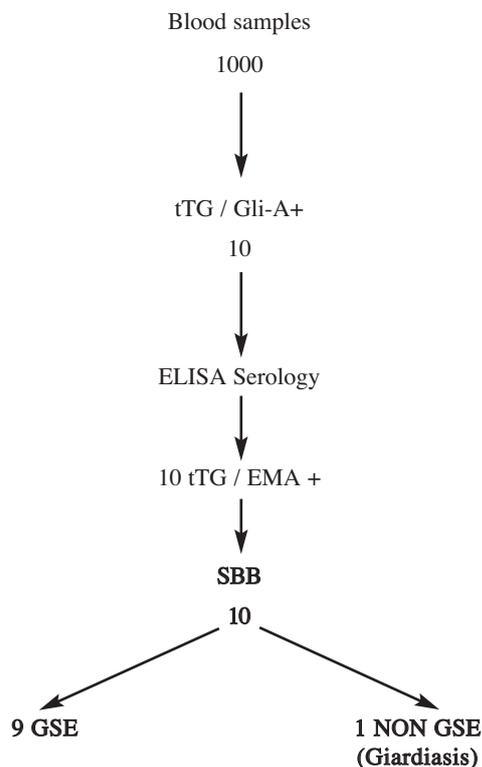


Figure 1. Flow chart of the different steps in the study. tTG: Tissue transglutaminase. Gli-A: Gliadin-IgA. EMA: Endomysial antibody. SBB: Small bowel biopsy. GSE: Gluten sensitive enteropathy.

antibodies bound to the strip an enzyme-labelled secondary antibody is used that is directed against human IgA (hIgA) and is coupled with the enzyme horseradish peroxidase. After addition of a substrate solution, the bound antibodies are visualized as blue lines (brown after stopping).

The test kits were stored at a temperature between +2 and +8°C and brought to room temperature before use. Serum samples were dissolved at room temperature first and then the test procedure was applied. The sticks were incubated with the diluted patient serum samples for 1 hour at room temperature with gentle agitation. Strips were then washed three times. Then, horseradish peroxidase conjugate was added and incubated 30 minutes at room temperature with gentle agitation as a second step. Horseradish peroxidase conjugates were sucked off again and strips were washed for 5 minutes with gentle agitation. Washing buffer was sucked off after all three washings. One ml of substrate solution was added and incubated for 10 minutes as a third step. It was sucked off again and washed 5 minutes with distilled water with gentle agitation. Distilled water was sucked off.

As a last step, 1 ml of stop solution was pipetted and colored strips were incubated for 5 minutes with gentle agitation to terminate the reaction. The strips were air dried between filter paper and interpreted by comparing with the evaluation template. A positive result, indicating the presence of antibodies to tTG and gliadin in the sample, is seen as two lines: one test line and one control line on the strip with a bluish purple color. A negative assay shows only a control line, no staining more intensive than the cutoff control (Figure 2).

The immunochromatographic tests were performed and interpreted by three operators (DÖ, EP, AÖ) who were blinded to the subjects' clinical and laboratory findings.

EMA IgA and AGA IgA/IgG Determinations

IgA-class anti-endomysium antibodies and IgA/IgG anti-gliadin antibodies were also tested by commercially available ELISA (Euroimmune, Germany) kit following the manufacturer's instructions as a second step when the result of the screening test was positive. Results are presented in relative units (RU); cutoff values are 20 RU/ml for EMA-IgA for children older than 2 years old, 25 RU/ml for AGA-IgA/IgG for children less than 4 years old, and 50 RU/ml for AGA-IgA/IgG for children older than 4 years old.

Serum immunoglobulin A (IgA) levels were not measured routinely, but were measured in cases with presumed serology negative CD or isolated AGA IgG positivity.

Small Bowel Biopsy and Histopathologic Evaluation

Small bowel biopsy (SBB) was recommended to all children with positive anti-tTG and EMA/AGA results as a third step. SBB was performed with pediatric upper GI endoscope (Olympus GIF type P30®, Japan). Intestinal biopsy specimens were obtained from the second part of the duodenum. Biopsy specimens were fixed in formalin, embedded in paraffin, treated with hematoxylin-eosin stain, and evaluated according to the Marsh criteria by a single expert pathologist who was blinded to the serology results and clinical information.

Statistical Analyses

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 11.5 for Windows (SPSS Inc., Chicago, IL) software computer program. Chi-square and Student's *t* tests were used to compare results. A *p* value less than 0.05 was considered significant.

RESULTS

A total of 1000 children aged between 2-18 years (mean age 9.1±4.2 years) were screened for the presence of tTG-IgA and gliadin-IgA class antibodies in this study. The study group included 526 females (52.6%) and 474 males (47.4%). Participants were classified into three groups according to their age as follows: 229 (22.9%) were aged 2-5 years, 455 (45.5%) were aged 6-11 years, and 316 (31.6%) were aged 12-18 years. Of the participants, 200 (20%) were completely healthy and the remainder had diseases other than CD and associated disorders.

Of the 1000 participants, 10 (1%) had positive antibody screening test to human tTG, and 21 had positive antibody screening test to gliadin (2.1%) by immunochromatographic assay, CD-LIA. Of these 10 anti-tTG antibody-positive subjects, six also revealed antibody positivity to gliadin (60% of all the tTG seropositive subjects). Anti-tTG positivity was significantly higher in females than males (8/2) and showed an increase by age (p<0.05). Gliadin-IgA antibody positivity was also higher in females with 66.6% (14/7).

The 10 subjects with anti-tTG antibody positivity by LIA also had positive EMA by ELISA. We also measured AGA-IgA and AGA-IgG in the 25 subjects with both tTG and gliadin IgA positivity and found them as 77% and 78%, respectively. Both the screening and the ELISA tests revealed a statistical significance with AGA-IgA positivity and increasing age (p<0.05). In addition, there was a good correlation between anti-tTG antibody by CD-LIA and EMA positivity by ELISA. Subsequently, SBB was performed in all 10 cases with anti-tTG antibody and EMA positivity. Biopsy results confirmed a diagnosis of CD in nine cases and giardiasis in one whose autoantibody results revealed weak positivity (Table 1). Six patients (60%) had type 3 (destructive), two (20%) had type 2 (hyperplastic), and one (10%) had type 1 (infiltrative) gluten sensitive enteropathy according to Marsh's criteria.

Asymptomatic or atypical CD based on tTG seropositivity was found to be higher in the older age groups: 0.8%, 0.6%, and 1.6% for tTG-IgA antibodies in age groups 2-5, 6-11, and 12-18 years, and 0%, 2.2%, and 3.5% for gliadin-IgA antibodies in the same age groups, respectively (p<0.05).

In this preliminary study, the prevalence of CD in a large sample of the Turkish pediatric population in Ankara, Turkey, based only on cases with proven enteropathy was 1:111 (0.9%). All biopsy-proven CD subjects and their results of comparative tTG, gliadin, and EMA autoantibody screening are summarized in Table 1. The nine patients diagnosed as silent or atypical CD were prescribed a GFD and followed regularly. The siblings of these patients were offered a screening test, and one sibling was even diagnosed with silent CD.

DISCUSSION

Celiac disease is a serious lifelong GI disorder that results in malabsorption. Therefore, early diagnosis is important and reduces the risk of complica-

A

Patient	21	24	25	23	22	26	27	28	29	30	31	32	33	34	35	36
Bezugslinie / Reference Line	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Streifen / Strips	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Gliadin	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
tTG	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-

B

Patient	689	689	690	692	693	694	695	696	697	698	699	700	701	702	703	704
Bezugslinie / Reference Line	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Streifen / Strips	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Gliadin	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
tTG	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-

Figure 2. Positive tTG and Gli-A results by CD-LIA in samples 21,22* (A), and 700 (B). tTG: Tissue transglutaminase. Gli-A: Gliadin-IgA. LIA: Line immunoassay.

*Negative result of Gli-A in sample 22.

Table 1. Characteristics of children with serology- and biopsy-proven CD

Case no	Age/Gender (yr)	Presenting symptoms**	Anti-Gli-A	Anti-tTG	EMA-A (>20 RU/ml)	SBB (Marsh) (n=10)
1	11/F	Constipation	-	+	+	Type 1 GSE
2	14/M	Failure to thrive, diarrhea	+	+	++++	Type 3 GSE
3	12/F	Abdominal pain, diarrhea, failure to thrive	+	+	+++	Type 3 GSE
4	2/F	Failure to thrive, pica	+	+	+++	Type 2 GSE
5	14/F	Short stature	+	+	++++	Type 3 GSE
6	4/F	Apparently healthy	-	+	+	Type 3 GSE
7	15/M	Failure to thrive	-	+	+++	Type 3 GSE
8	16/F	Pallor, lassitude	+	+	+++	Type 3 GSE
9	15/F	Apparently healthy	+	+	+++	Type 2 GSE
10*	10/F	Obesity	+/-	+/-	+	Giardiasis

F: Female. M: Male. GSE: Gluten sensitive enteropathy.

*Anti-tTG weak positive, EMA positive. **When specifically asked and examined these symptoms were present.

tions (1-4). Up until the last decade, CD was considered to be relatively uncommon worldwide. However, the advancement of serologic screening tests has revealed that the prevalence of CD is actually higher (3-6). The diagnosis of the silent form, in particular, is challenging and CD seems to be underdiagnosed. (16-19). The most commonly used screening policies in large populations, which combine parallel or serial AGA plus EMA testing or parallel IgA and IgG AGA immunoassays, are both expensive and time-consuming. However, the recently suggested tTG-based population CD screening is cost-effective, quick, and requires minimal handling. Therefore, this screening test has allowed an easier diagnosis (20, 21). In our study, a similar method, LIA, for the detection of recombinant hIgA antibodies to both transglutaminase and gliadin in human serum was used for screening of CD. The results of SBB in 9 of 10 cases suggested that this screening test is reliable. Other previous studies also showed that sensitivity and specificity of visual systems are nearly identical to those of ELISA (22-25). Garrote et al. (22) reported that their visual system was simple, reliable, and highly reproducible. Baldas et al. (23) also reported that a rapid dot blot assay for the detection of antibodies to tTG was highly accurate. Sorell and Acevedo (24) developed the one-step immunochromatographic assay for the detection of both IgA and IgG antibodies to transglutaminase in human serum or plasma and also found it to be highly reliable. Ferre-Lopez et al. (25) obtained a sensitivity of 97.1% and a specificity of 99.0% with the tTG stick for pediatric patients and of 83.3% and 100% in adults, respectively. Nemeč et al. (26) was also convinced that this new way of testing for CD can be used on a large scale in daily practice. Results were comparable

with the corresponding ELISAs, and the visual assays are efficient for CD screening as an alternative to ELISAs (22-27). Our results demonstrated good correlation of tTG IgA antibodies by LIA method compared to EMA by ELISA in screening of CD as well.

EMA was used originally in diagnosis by indirect immunofluorescence test (IIFT), but there is need for experienced and well-trained laboratory personnel to interpret the test results and the higher cost is considered. Correlation between IIFT and ELISA using the human endomysium whole antigen reached 95.4%. With medium to high immunofluorescence titers, there was a correlation of 100%. The sensitivity and specificity of the newly developed EMA ELISA correlate highly with EMA IIFT. In such studies, sensitivity ranged from 85% to 100% and specificity from 90% to 95%. All the results were found reliable as reported by others in recent literature (28-32).

Furthermore, Mankai et al. (33) concluded that a similar dot blot assay could screen for CD in patients who did not have anti-tTG antibodies by ELISA. Recently, some studies were published investigating the detection of tTG antibodies in other body fluids like human saliva and stool as a non-invasive test. However, neither of these tests was reliable or suitable for CD screening in children (34-36).

While performing the multi-step screening approaches, serum IgA levels were not measured routinely as part of the screening regimen except in suspected cases. The prevalence of CD associated with IgA deficiency was found as only 1 in 8500 in one study (1, 37) and 0 in 1263 (12). Therefore, routine serum IgA level screening in asymptomatic individuals in the general population has not been

recommended in recent years. However, patients who were clinically suspected to have IgA deficiency with CD were evaluated for serum IgA and tTG IgG - AGA IgG levels (1).

Based on a number of studies, the prevalences of CD in Europe and the United States is 3 to 13 per 1000 children, or approximately 1:300 to 1:80 in the general population (1-5, 38). The prevalences from the Netherlands, Hungary, Finland, Switzerland, Portugal and Spain were reported as 1:198, 1:85, 1:99, 1:132, 1:134, and 1:118, respectively (16-18,39-41). Limited studies from Turkey have revealed a similar prevalence. Ertekin et al. (12) reported that prevalence of CD based on positive anti-tTG was 1:115 (0.9%), while biopsy-proven CD was 1:158 (0.6%) in apparently healthy schoolchildren. Tatar et al. (13) presented the prevalence of serology positive CD as 1:77 (1.3%) and Karaaslan et al. (14) found anti-tTG positivity as 1:140 in healthy blood donors in Turkey. In this present preliminary study, the prevalence of CD was 1:111 (0.9%) in a large sample of Turkish children, similar to results of a limited number of previous studies from Turkey and comparable to rates found in other screening studies throughout the world, including some European countries and North America (12-14, 16-19, 39-42). CD has also recently been reported as being common in Iran, other Middle Eastern countries and North African populations, particularly Tunisians (43, 44). The prevalences of CD in non-at-risk and at-risk subjects in this area were reported as being even higher compared to those in western countries (43). The prevalence of CD in Tunisia was also reported as 1/157 serologically and 1/224 biopsy-proven (44). Furthermore, in Africa, the only screening study from Western Sahara demonstrated the highest prevalence of CD (1/18) in children (45). New epidemiological studies have provided evidence that this disorder is also common in many developing countries, showing that the "global village of CD" does indeed have a worldwide distribution. In a recent study, the selective serological screening of 198 symptomatic schoolchildren out of 4347 subjects in Punjab, North India, yielded a CD prevalence of at least 1 in 310 in the overall sample. This is clearly an underestimate, as many cases with atypical or silent CD remained undetected in the study (46). A genetic susceptibility together with environmental factors such as shorter exclusive breast-feeding and early gluten introduction seem to increase the prevalence in our country across the two continents and located in the 'Fertile Crescent' (41, 43, 46-48).

Our study and some others in the literature found a significant female preponderance in all antibody-positive participants (17, 39, 43, 44, 49, 50). This study showed that predominance of females with CD was significant (8 of the 10 patients). This may be explained by the susceptibility of female gender to autoimmune diseases.

Asymptomatic or atypical CD was found to be higher in the older age groups in our study, consistent with the literature. This may be due to prolonged exposure to gluten (51). Atypical symptoms of our patients were lassitude, failure to thrive or growth failure, short stature, constipation, and iron deficiency anemia. Therefore, it is important to include asymptomatic or atypical CD in the differential diagnosis in children with the above symptoms (3, 6). The availability of powerful and simple diagnostic tools, such as the quick anti-tTG determination, will doubtless facilitate the exploration of these more remote areas of the celiac iceberg (26, 27).

An important finding of this study is that most of the atypical cases had SBB revealing severe villous atrophy (60%) concluded malabsorption and other symptoms. This indicates the significance of early diagnosis. Histopathologically, Marsh 1-2 type mucosal lesions with anti-tTG and EMA positivity were also considered to be early changes of CD. The reality known today is that the mucosa may deteriorate later in spite of genetic susceptibility (1, 51).

In conclusion, determination of anti-tTG antibodies by simple visual assay, the immunochromatographic LIA method, appeared to be as reliable as the ELISA system for CD diagnosis. It is quick, easy to perform and interpret, and cost-effective for screening large populations at risk for CD (case-finding situations such as anemia, autoimmune diseases, and fatigue) and for selecting candidates for SBB (21-26). Although SBB is still the gold standard for definite diagnosis of CD, an appropriate strategy is to screen suspected individuals and larger populations for anti-tTG by immunochromatographic visual assay as a first step and for EMA by conventional ELISA as a second step to confirm the positive results prior to performing SBB. This preliminary study revealed that CD is not rare in Turkey. Large-scale, multicenter epidemiological studies should be performed to determine the precise prevalence of CD in Turkey and reliable quick screening tests could be used for this purpose. CD is not only treatable, but also carries the risk of serious, though preventable, long-term complications.

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