First Salivary Screening of Celiac Disease by Detection of Anti-transglutaminase Autoantibody Radioimmunoassay in 5000 Italian Primary Schoolchildren

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ABSTRACT

Objective: The high prevalence of celiac disease (CD) prompted us to evaluate a new, noninvasive disease screening strategy. The aim was to identify CD in 6- to 8-year-old children for a timely diagnosis, start gluten-free diet (GFD) in compliant subjects, achieve the growth target, and prevent CD complications.

Methods: Five thousand subjects were invited to participate in the study. Four thousand forty-eight saliva samples were tested for anti-tissue transglutaminase (tTG) immunoglobulin (Ig)A using a fluid-phase radioimmunoprecipitation method. Positive children were tested for serum radioimmunoassay tTG IgA, enzyme-linked immunosorbent assay tTG IgA, and anti-endomysium IgA. Children confirmed as positive by serum assays underwent endoscopy with duodenal biopsies and, at the diagnosis of CD, were suggested to start GFD.

Results: Consent was obtained from 4242 parents (84.8%) for the screening to be performed, and adequate saliva samples were collected from 4048 children (95.4%). Thirty-two children were found to be salivary tTG IgA positive and 9 with borderline autoantibody levels. Thirty-one of the 32 and 3 of the 9 subjects were also serum positive. Twenty-eight children showed villous atrophy when undergoing intestinal biopsy, whereas 1 had Marsh 1 lesions; 3 children were suggested to start GFD without performing endoscopy. CD prevalence in the population investigated (including 19 CD known cases) was 1.16%. The ratio between screening-detected patients and those diagnosed before the screening was 3:2. The ratio between symptomatic and asymptomatic patients was 1:1.6.

Conclusions: We demonstrated that it is possible to perform a powerful, simple, well-accepted, and sensitive CD screening using saliva. Until now, the compliance with GFD in children with CD has been optimal.

Key Words: celiac disease, RIA anti-transglutaminase autoantibodies, saliva, schoolchildren, screening

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Celiac disease (CD) is an autoimmune enteropathy caused by the ingestion of wheat gluten and related prolamine of barley and rye in genetically susceptible individuals, characterized by villous atrophy and crypt hyperplasia of small bowel mucosa. CD may appear in a classical presentation, with gastrointestinal complaints and growth failure or with extraintestinal manifestations, that is, ''atypical forms'' (1) characterized by symptoms such as anemia (2) and short stature (3), or with a silent form, more frequent in first-degree relatives of patients with CD (4).

The high prevalence of CD worldwide (0.5%–1% in the United States and other developed countries) (5) largely in an asymptomatic form, and the complications of a long-lasting CD, make the early diagnosis of this disease, possibly in childhood, crucial. CD-related serum autoantibody detection, particularly anti-tissue transglutaminase (tTG IgA), contributes to the identification of candidates for intestinal biopsy (6–9).

We demonstrated that tTG IgA can be detected by a radioimmunoassay (RIA) in human saliva, a body fluid that can be easily obtained by noninvasive techniques. Furthermore, it is particularly helpful in children for CD screening purposes (10,11) bypassing the unpleasant blood sample collection.

The aim of our study was to identify CD in primary schoolchildren to perform a timely diagnosis of the disease, start gluten-free diet (GFD) in compliant children, achieve the growth target, and prevent CD complications.

MATERIALS AND METHODS

During the period from March to October 2007, 5000 primary schoolchildren attending 27 schools in 7 of 20 municipalities of Rome were invited to participate in the study. Children's parents and pediatricians were informed of the study by explanatory forms. Data concerning sex, age (Table 1), and the presence of CD in children were collected. Informed consent was obtained from parents and children using an illustrated form.

Children, fasting for at least 3 hours, were asked to collect a salivary sample by spitting into a plastic tube. Subjects found to be salivary tTG IgA positive were tested for the presence of serum RIA tTG IgA, anti-endomysium IgA (EMA), and enzyme-linked immunosorbent assay (ELISA) tTG IgA. For children confirmed as antibody positive by 1 or more serum assays performing upper endoscopy with multiple biopsies was suggested and, after CD diagnosis, starting gluten-free diet (GFD). The ethics committee of Policlinico Umberto I, ''La Sapienza'' University of Rome, approved the study.

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Collection and Treatment of Saliva Samples

Unstimulated whole saliva samples from all of the subjects participating in the study were collected and treated as previously described (10,11). The saliva supernatant, used for the tTG IgA detection, was aliquoted and stored at −80°C until analysis.

Salivary RIA tTG Antibody Detection

Salivary tTG IgA were detected as previously described (10,11), with minor modifications. Briefly, radiolabeled [^{35}S]-methionine tTG was incubated overnight at 4°C with 150 μL of saliva sample. After addition of 25 μL of goat anti-human IgA-agarose (Sigma, St Louis, MO), the solution was incubated for 3 hours at 4°C in a rotating platform. After exhaustive washings, a phosphate-buffered solution was added to each tube to resuspend the pellet, which was then transferred into a scintillation vial. This last step was repeated a second time. Each vial was then counted in a beta counter after the addition of scintillation liquid solution (Perkin Elmer Italia, Monza, Italy). Salivary autoantibody levels were expressed as an antibody index calculated as follows: 100 × (sample cpm−negative standard sample cpm)/(positive standard control cpm−negative standard control cpm).

Limit of Positivity of the Salivary RIA Assay

The positive autoantibody index of the salivary assay, defined as the value greater than the 99th percentile of the first 500 saliva samples (236 girls, 264 boys) collected in the study, was 44.9. From these subjects, 6 children previously diagnosed as having CD were excluded. All of the subjects having an antibody index >97.5th (37.5) were considered borderline.

Serum RIA tTG IgA Detection

Serum tTG IgA were detected using a previously described method (8,9) in which a receiver operator characteristic plot analysis was used to identify the optimal threshold value for assay sensitivity and specificity. Serum tTG IgA levels were calculated and expressed as in the salivary RIA. Serum samples were considered tTG IgA positive when the autoantibody index was >0.050.

Serum ELISA tTG IgA Detection

tTG IgA detection was performed by a commercial sandwich-type ELISA (Eurospital, Trieste, Italy).

EMA Method

EMA IgA, tested in sera diluted 1:5, were detected by an indirect immunofluorescence method using sections from the distal portion of monkey esophagus as substrate (Eurospital, Trieste, Italy).

Upper Endoscopy

All of the patients, fasting overnight, underwent upper endoscopy with multiple biopsies after narcosis. An Olympus (Olympus, Tokyo, Japan) XP 10 gastroscope was used in children younger than 6 years, whereas Olympus GIF E was used in older children and adolescents. During endoscopy, 2 samples from bulbous mucosa and 3 or 4 samples from the distal duodenum were taken using FB-19K-1 and FB-24Q forceps (12,13). To obtain a well-oriented sample, each biopsy was laid on filter paper and oriented so that the luminal surface was uppermost. Histological lesions have been classified according to Marsh classification, as modified by Oberhuber et al (14).

Each biopsy was fixed in 10% formalin and separately embedded in paraffin blocks. Sections were serially cut, stained with hematoxylin and eosin, and assessed under light microscopy.

Statistical Analysis

Sample size was computed as previously described (15): $N = (z\alpha/2/\varepsilon)2.\ p.\ (1\text{-}p)$, where $z\alpha/2 = 1.96$ is the value of the normal deviate associated with a 95% confidence interval; ε = desired approximation, that is, maximum distance from prevalence to be considered; p = prevalence of the disease. For $\varepsilon = 0.00275$ and $P = 0.01$ N = 5029; for $\varepsilon = 0.00300$ and $P = 0.01$ N = 4225. So a sample size of 5000 children allowed a good precision of the estimate ($0.725\% < P < 1.275$).

Our sample was selected with a multistage sampling procedure: in the first stage 5 of 20 Rome municipalities were randomly selected; in the second stage, from the 7 municipalities, 26 public primary schools were randomly selected and then all of the children of the first and second classes were included in the sample. From quantitative variables means, medians, and standard deviations were computed, and Student *t* test, when possible, was used to compare group means (16).

A 2-tailed $P < 0.05$ was considered significant. Correlation between the results obtained with the serum RIA tTG IgA and the saliva RIA tTG IgA was examined with linear regression analysis.

RESULTS

Consent for the study was obtained from parents of 4242 of 5000 children (84.8%). On the day of saliva collection 144 children were absent, and for 50, it was not possible to collect enough saliva to be tested. Therefore, adequate saliva samples of 4048 (95.4%) children were collected and tested for presence of RIA tTG IgA (Table 1). Among the subjects investigated, 19 (0.5%) were declared to be affected by CD.

A total of 32 of the 4048 children in the study (group 1) were found salivary tTG IgA positive (Table 1). All of them were tested for serum tTG IgA (RIA and ELISA) and EMA; 31 children gave positive resultes, whereas only in 1 girl, who was weakly positive on salivary detection, the suspicion was not confirmed (Table 2). In 9 subjects (group 2), borderline salivary tTG IgA levels were found (Table 1). Serum tests were performed in 8 of these children showing positive values in 3 (Table 2). A significant correlation ($r = 0.7216$) between RIA salivary and serum autoantibody titers was found ($P < 0.0001$).

In addition, we found 2 patients, already diagnosed and studied in other pediatric gastroenterological centers in Rome, showing positive values of tTG salivary antibodies who were not compliant with a GFD, and other 17 patients with CD negative for salivary tTG antibodies.

A total of 26 group 1 and 3 group 2 scholars positive for serum antibodies were allowed by their parents to undergo endoscopic intestinal biopsies. On performing histological examination, all of the patients except 1 showed type 3 a, b, or c lesions (according to the Oberhuber et al (14) classification), sometimes with a patchy distribution. The remaining group 2 patients showed only type 1 lesions (Table 3). Parents of 3 children, 1 with both the mother and the sister affected by CD, denied permission to perform an intestinal biopsy, but started GFD autonomously. Furthermore, 2 children maintained the gluten-containing diet.

TABLE 1. Number, sex, age range, median age, and salivary tTG IgA mean levels of the children participating in the study subdivided into groups according to their tTG IgA pattern

No.	All children N = 4048	Group 1 N = 32	Group 2 N = 9	Ab-negative children N = 4007
Sex	1759 M/2289 F	11 M/21 F	3 M/6 F	1745 M/2262 F
Age range, y	5.3–9.8	5.8–8.7	6.1–7.9	5.3–9.8
Median age	7.1	7.4	6.9	6.8
Salivary RIA tTG IgA (mean ± SD)	8.8 ± 11.7	104.7 ± 45.6	43.1 ± 4.5	8.1 ± 7.8

Group 1 represents all of the subjects found with salivary tTG IgA levels >99th. Group 2 represents all of the subjects found with salivary tTG IgA levels comprised between >97.5th and 99.0th. Ab = antibody; RIA = radioimmunoassay; tTG = tissue transglutaminase.

In our series, CD prevalence was 1.16%, including the 19 children diagnosed before the screening, and the 28 saliva- and serum-positive children, who were confirmed by histological examination. One group 2 girl, asymptomatic, showing only type 1 lesions (14) and positive to the serology, was classified as having a potential form of CD and thus was not included in the count. However, if we also consider the saliva- and serum-positive children who did not undergo intestinal biopsy (5 group 2 children), the prevalence of CD in our cohort increases to 1.28%.

All of the patients with CD detected in the present study showed a silent form of the disease, except for the 3 group 1 subjects who were symptomatic: one 8.8-year-old girl from Venezuela, who complained of abdominal pain and headache on consuming the Italian diet, which was rich in bread and pasta; one 6.3-year-old girl with constipation with a mild iron-deficient anemia; and one 7.6-year-old boy with chronic abdominal pain. In our series 18 patients were symptomatic (3 screening detected and 15 previously diagnosed as children with CD) and 29 asymptomatic, with a symptomatic:asymptomatic ratio of 1:1.6.

The 12-month follow-up demonstrates an optimal compliance with a GFD (100%) and a satisfactory weight and height increase.

DISCUSSION

A population screening strategy implies the presence of a common disease that causes a serious health problem; the availability of a valid therapy, which should prevent the complications of the disease; a gap between the preclinical stage and complications; and the availability of an accurate, sensitive, specific, less-invasive and possibly low-cost screening test.

CD is frequent in Europe and the United States (17), with an increased risk of osteoporosis (18), autoimmune diseases (19), and a high mortality related to untreated diseases (20). A valid CD therapy is available: GFD. Thus, a screening strategy could be advisable. There is a consensus for CD screening in risk groups, such as relatives of patients with CD (4,17), whereas the high cost and the low compliance with a GFD in asymptomatic subjects make CD screening in adults or adolescents debatable (21). As shown by Fabiani et al (22), only a small percentage of screening-detected adolescents (5 of 25) maintained a GFD 5 years after the diagnosis, whereas the majority of subjects (15 of 22) diagnosed in early childhood were compliant.

We previously demonstrated that it is possible to detect salivary tTG IgA with high sensitivity using a simple, reproducible fluid-phase RIA method (10,11). Screening of saliva, a specimen that can be collected easily by noninvasive procedures, may be reliable, easy to perform, and a powerful tool for the screening of CD, bypassing the unpleasant blood sample collection. There are no technical aspects limiting the applicability of this new method, neither in saliva collection nor in the inexpensive assay procedure, which uses instruments commonly found in clinical research laboratories (9).

We decided to perform the salivary screening test in 6- to 8-year-old schoolchildren because at that age children usually eat at home or at school; thus, it is easier to achieve a good compliance with the diet. In addition, we have time before adolescence arrives,

TABLE 2. Salivary (second sample) and serum autoantibody results of the subjects identified as positive (group 1) or borderline (group 2) in the course of the screening

	Group 1 (N = 32)		Group 2 (N = 8)	
	n	%	n	%
Salivary RIA tTG IgA positive (second sample)	31	97	3	37
Serum RIA tTG IgA positive	31	97	3	37
Serum ELISA tTG IgA positive	30	94	3	37
Serum EMA positive	30	94	3	37

Serum IgA were analyzed with 3 different methods. ELISA = enzyme-linked immunosorbent assay; EMA = anti-endomysium Ab; RIA = radioimmunoassay; tTG = tissue transglutaminase.

TABLE 3. Results of intestinal biopsy (according to the Marsh classification as modified by Oberhuber et al (14)) in patients confirmed as autoantibody positive and who permitted endoscopy

	Group 1 (N = 25)		Group 2 (N = 3)	
	n	%	n	%
Type 1	0	—	1	33.3
Type 3c	20	80	1	33.3
Type 3a, 3b, 3c (patchy distribution)	2	8	0	—
Type 3b, 3c (patchy distribution)	3	12	1	33.3

Type 1: intraepithelial lymphocytes >40/100 enterocytes; type 3a: slight atrophy of intestinal villi; type 3b: subtotal atrophy of intestinal villi; type 3c: total atrophy of intestinal villi.

at which age good compliance with the diet will become more difficult (22,23).

Until now, all CD screenings reported in children in the literature (17,24–26) have been performed by blood samples obtained with the unpleasant needle puncture. The noninvasive, sensitive, and reproducible test on saliva samples used in our study has been well accepted by both children and parents, whose consent was obtained in 84.8% of the cases. Children usually were able to collect enough saliva. The slight difference of numbers from consents obtained (4242) and samples collected (4048) is due to the absence of children from school on the day of saliva collection or due to the difficulties in spitting by children with disabilities.

In our study we obtained an optimal compliance of parents with the study. Moreover, the probability of getting permission to follow the subsequent steps was high (Fig. 2), not only for the second evaluation but also for endoscopy (85.3%). Indeed, 29 of 34 serum autoantibody–confirmed children fulfilled the diagnostic CD protocol.

Our salivary test proved to be sensitive and specific, having a good correlation with the relative serum samples. In fact, 31 of 32 children positive with saliva were confirmed by serum test, and 28 of 29 children who underwent intestinal biopsy showed villous atrophy. Only 1 girl, with borderline salivary tTG IgA levels, but positive on serum, showed Marsh type 1 lesions (14). Therefore, she was considered to have a potential form of CD, and thus was not taken into account in calculating CD prevalence.

Our previous report indicates that 99th percentile values of the salivary assay are able to detect a sensitivity of 94.5% (11). Of note, in the present study, the analysis of borderline patients increased the sensitivity of the assay (3/8 patients confirmed as patients with CD); thus, we can hypothesize that the patients with CD eventually missed are few if not absent. However, the prevalence of CD found (minimum 1.16%, maximum 1.28%) was concordant with the expected prevalence of the disease reported in other studies.

In the present pilot study only RIA tTG IgA (but not IgG) have been detected due to the costs involved in assaying 2 autoantibodies, because the possibility of finding an IgA-deficient subject in our small series of children with CD was low. Nevertheless, it may be useful to assay also IgA salivary levels, particularly when screening large series, to detect IgA-deficient patients with CD.

The high number of children (19) with a previous diagnosis of CD in our study, with a ratio of 6:4 between the patients detected by the screening and the patients diagnosed before, demonstrates that pediatricians take CD in primary schoolchildren into consideration. Probably also the difference in the ratio between symptomatic and asymptomatic CD of 1:1.6 in our series in comparison with the ratio of 1:7 in a previous multicenter Italian study (24) should be related to the improved knowledge of CD compared to 17 years ago.

In conclusion, the present study demonstrates that it is possible to perform a powerful, noninvasive, simple, well-accepted, inexpensive (€7.50), reproducible, and sensitive CD screening using saliva. The enthusiastic reaction of parents to screening and the amusement of children during salivary collection represent the goal of our screening strategy. Until now, compliance with GFD in screened children with CD has been optimal. Only a longer follow-up may show whether an early diagnosis in asymptomatic children with CD, who initiate primary school and a strict GFD, will permit proper growth and prevent complications.

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REFERENCES

1. Branski D, Troncone R. Celiac disease: a reappraisal. *J Pediatr* 1998; 133:181–7.
2. Bonamico M, Vania A, Monti S, et al. Iron deficiency in children with celiac disease. *J Pediatr Gastroenterol Nutr* 1987;6:702–6.
3. Bonamico M, Scirè G, Mariani P, et al. Short stature as the primary manifestation of monosymptomatic celiac disease. *J Pediatr Gastroenterol Nutr* 1992;14:12–6.
4. Bonamico M, Ferri M, Mariani P, et al. Serologic and genetic markers of celiac disease: a sequential study in the screening of first degree relatives. *J Pediatr Gastroenterol Nutr* 2006;42:150–4.
5. Catassi C, Fasano A. Celiac disease. *Curr Opin Gastroenterol* 2008; 24:687–91.
6. Dieterich W, Laag E, Schöpper H, et al. Autoantibodies to tissue transglutaminase as predictors of celiac disease. *Gastroenterology* 1998;115:1317–21.
7. Mearin ML, Koning F. Tissue transglutaminase: master regulator of coeliac disease? *J Pediatr Gastroenterol Nutr* 2003;36:9–11.
8. Bonamico M, Tiberti C, Picarelli A, et al. Radioimmunoassay to detect antitransglutaminase autoantibodies is the most sensitive and specific screening method for celiac disease. *Am J Gastroenterol* 2001;96:1536–40.
9. Li M, Yu L, Tiberti C, et al. A report on the international Transglutaminase autoantibody workshop for coeliac disease. *Am J Gastroenterol* 1009;104:154–63.
10. Bonamico M, Ferri M, Nenna R, et al. Tissue transglutaminase autoantibody detection in human saliva: a powerful method for celiac disease screening. *J Pediatr* 2004;9:69–76.
11. Bonamico M, Nenna R, Luparia RPL, et al. Radioimmunological detection of anti-transglutaminase autoantibodies in human saliva: a useful test to monitor celiac disease follow-up. *Aliment Pharmacol Ther* 2008;28:364–70.
12. Bonamico M, Mariani P, Thanasi E, et al. Patchy villous atrophy of the duodenum in childhood celiac disease. *J Pediatr Gastroenterol Nutr* 2004;38:204–7.
13. Bonamico M, Thanasi E, Mariani P, et al. Duodenal bulb biopsies in celiac disease: a multicenter study. *J Pediatr Gastroenterol Nutr* 2008;47:618–22.
14. Oberhuber G, Granditsch G, Vogelsang H. The histopathology of coeliac disease: time for a standardized report for pathologists. *Eur J Gastroenterol Hepatol* 1999;11:1185–94.
15. Rothman KJ. *Modern Epidemiology.* Boston: Little, Brown; 1994.
16. Armitage P, Berry G. Statistical Methods in Medical Research. Oxford: Blackwell Scientific; 1998.
17. Fasano A, Berti I, Gerrarduzzi T, et al. Prevalence of celiac disease in at-risk and not-at risk groups in the United States. A large multicentre study. *Arch Intern Med* 2003;163:286–92.
18. González D, Mazure R, Mautalen C, et al. Body composition and bone mineral density in untreated and treated patients with celiac disease. *Bone* 1995;16:231–4.
19. Toscano V, Conti FG, Anastasi E, et al. Importance of gluten in the induction of endocrine autoantibodies and organ dysfunction in adolescent celiac patients. *Am J Gastroenterol* 2000;95:1742–8.
20. Corrao G, Corazza GR, Bagnardi V, et al. Mortality in patients with coeliac disease and their relatives: a cohort sudy. *Lancet* 2991;558:354–61.
21. Collin P. Should adults be screened for celiac disease? What are the benefits and harms of screening? *Gastroenterology* 2005;128:104–8.
22. Fabiani E, Taccari LM, Rätsch IM, et al. Compliance with gluten-free diet in adolescents with screening-detected coeliac disease: a 5-year follow-up study. *J Pediatr* 2000;136:841–3.
23. Mariani P, Viti MG, Montuori M, et al. The gluten-free diet: a nutritional risk factor for adolescents with celiac disease? *J Pediatr Gastroenterol Nutr* 1998;27:519–23.
24. Catassi C, Fabiani E, Rätsch IM, et al. The coeliac iceberg in Italy. A multicentre antigliadin antibodies screening for coeliac disease in school-age subjects. *Acta Paediatr Suppl* 1996;412:29–35.
25. Maki M, Mustalahti K, Kokkonen J, et al. Prevalence of coeliac disease among children in Finland. *N Engl J Med* 2003;348:2517–24.
26. Tommasini A, Not V, Kiren V, et al. Mass screening for coeliac disease using antihuman transglutaminase antibody assay. *Arch Dis Child* 2004;89:512–5.