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Comparative Evaluation of Serologic Tests for Celiac Disease: A European Initiative Toward Standardization

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ABSTRACT

Background: Serologic methods have been used widely to test for celiac disease and have gained importance in diagnostic definition and in new epidemiologic findings. However, there is no standardization, and there are no reference protocols and materials.

Methods: The European working group on Serological Screening for Celiac Disease has defined robust noncommercial test protocols for immunoglobulin (Ig)G and IgA gliadin antibodies and for IgA autoantibodies against endomysium and tissue transglutaminase. Standard curves were linear in the decisive range, and intra-assay variation coefficients were less than 5% to 10%. Calibration was performed with a group reference serum. Joint cutoff limits were used. Seven laboratories took part in the final collaborative study on 252 randomized sera classified by histology (103 pediatric and adult patients with active celiac disease, 89 disease control subjects, and 60 blood donors).

Results: IgA autoantibodies against endomysium and tissue transglutaminase rendered superior sensitivity (90% and 93%, respectively) and specificity (99% and 95%, respectively) over IgA and IgG gliadin antibodies. Tissue transglutaminase antibody testing showed superior receiver operating characteristic performance compared with gliadin antibodies. The κ values for interlaboratory reproducibility showed superiority for IgA endomysium (0.93) in comparison with tissue transglutaminase antibodies (0.83) and gliadin antibodies (0.82 for IgG, 0.62 for IgA).

Conclusions: Basic criteria of standardization and quality assessment must be fulfilled by any given test protocol proposed for serologic investigation of celiac disease. The working group has produced robust test protocols and reference materials available for standardization to further improve reliability of serologic testing for celiac disease. ***JPGN 31:513–519, 2000.*** **Key Words:** Celiac disease—Endomysium antibodies—Gliadin antibodies—Reproducibility—Standardization—Tissue transglutaminase antibodies.

Serologic test methods have gained high priority in screening for, in diagnostic work-up for, and in follow-up of celiac disease (1,2). Gliadin antibodies (3), endomysium antibodies (EMA) (4), and, recently, tissue transglutaminase (tTG) antibodies (5) have been used successfully in this context. However, methods described for antibody determination are manifold, and there is no standardization. Regarding the diagnostic standards in celiac disease set by the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) (6) and because of new epidemiologic findings in celiac disease extending across the diagnostic spectrum (7), standardization of methods and materials is of utmost importance. Previous studies involving comparative evaluation of different serology methods (8) and interlaboratory reproducibility (9) have produced conflicting data.

From 1993 through 1996 a combined international group set up by the European Medical Research Council (EMRC) and ESPGHAN has been working on serologic tests for celiac disease (10,11). This working group has produced new evidence on comparative evaluation of tests for celiac disease, and it has produced reference protocols and materials on a noncommercial basis.

The goals of the working group were

- establishment of robust test protocols for gliadin, endomysium, and tTG antibodies;
- calibration of antibody data according to a positive reference serum provided by the group; and
- internal and external quality control.

This article is the final document produced by the group.

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MATERIALS AND METHODS

Collaborative Study Design

Seven European laboratories were involved in the final evaluation (Bologna, Italy; Brussels, Belgium; Helsinki, Finland; Liestal, Switzerland; Linköping, Sweden; Tampere, Finland; and Tübingen, Germany). The definitive collaborative (ring) study was performed according to current guidelines (12). Sera were coded in a randomized manner. All antibody determinations were performed blindly in duplicate by all the centers. Independent statistical evaluation was conducted (RL, Institute of Medical Information Processing, University of Tübingen, Germany). The study was approved by the Ethics Committee of the University of Tübingen.

Patients

Patients' sera were collected by the participating centers based on clear-cut clinical and histologic definitions fulfilling revised ESPGHAN criteria (6). Fifty-two children with active celiac disease showed severe mucosal damage while consuming a gluten-containing diet and a positive clinical response to the introduction of a gluten-free diet (active infant celiac disease [AIC group], Fig. 1).

Fifty-one adults (more than 18 years of age) fulfilled the same criteria for diagnosis of celiac disease (active adult celiac disease [AAC group]). Thirty-four control children (failure to thrive, chronic diarrhea) exhibited either normal small intestinal biopsy results or minimal changes unrelated to celiac disease (disease controls children [DCC]) and 55 biopsy-screened adult disease control subjects fulfilled the same criteria (disease controls adults [DCA group]). Sixty apparently normal adult blood donors served as additional controls (normal adult controls [NAC group]). Immunoglobulin (Ig)A deficiency was shown exclusively in two pediatric patients with celiac disease.

Thus, 192 sera from children and adults with clear-cut diagnoses based on small intestinal biopsy and 60 sera from healthy blood donors entered the definitive collaborative study.

Working Group Reference Sera

A negative reference was created by a pool of 100 healthy blood donors. A positive reference was created by pooling 13 sera from patients with known celiac disease and diluting the resultant serum 1:5 with sera from six healthy blood donors. The positive reference pool was subjected to sterile filtration, recovery was 200 mL of reference serum (ES, Helsinki). This standard for gliadin, endomysium, and tTG antibodies was prepared to make precise calibration possible, particularly in the low and intermediate antibody range.

Test Protocols

After intensive preliminary work (group protocols 1993–1998 available upon request) (10,11) the following methods and test protocols were chosen for final evaluation. Similar reagents from other suppliers and similar technical equipment (enzyme-linked immunosorbent assay [ELISA] readers, microscopes) are acceptable for further work.

Enzyme-Linked Immunosorbent Assay for Gliadin Antibodies

The assay was modified according to a method by Granditsch (13).

- Coating: Microtiter plates, high binding capacity, Maxisorp (Nunc, Roskilde, Denmark), 10 μg/mL gliadin (Sigma, St. Louis, MO, U.S.A.) in 70% ethanol; 30 minutes incubation; 37°C, 100 μL/well, wash three times (200 μL phosphate-buffered saline [PBS; pH 7.4], 0.05% Tween 20).
- Incubation: Patient's serum 1:100 IgA, 1:500 IgG, positive and negative controls, blanks (PBS-Tween, pH 7.4) 100 μL/well, 1 hour, 37°C moist chamber, wash three times.
- Conjugate: Peroxidase, rabbit anti-human IgG, IgA, 1:1000 (Dako, Glostrup, Denmark) 50 μL/well, 1 hour incubation, 37°C, wash three times.
- Substrate: 5-Aminosalicylic acid (Sigma) (100 mg/100 mL) in phosphate buffer (47.17 mL 0.01 mol/L $NaH_2PO_4 \cdot H_2O$, 51.89 mL 0.01 mol/L $Na_2HPO_4 \cdot 2H_2O$, 943 μL 0.01 mol/L ethylenediaminetetraacetic acid [EDTA]; [pH 5.9–6.0]), 0.1 mL 0.5% H_2O_2/10 mL, 45 minutes incubation at room temperature, 100 μL/well).
- Reading: Microplate reader 490 nm (MRX; Dynatech, Guernsey, UK).
- Standard curve: Positive reference pool diluted 1 to 400, 800, 1,600, 3,200, and 6,400 for IgG and 1 to 200, 400, 800, 1,600, and 3,200 for IgA.
- Expression of results: Results were expressed in arbitrary units (AU). One hundred arbitrary units equaled the optical density (OD) of the positive reference pool: 1:100 for IgA, 1:500 for IgG. Cutoff values were established by receiver operating characteristic (ROC) curves (see Results). Cutoff for IgG gliadin antibodies was 16 AU and for IgA gliadin antibodies, 5 AU.

Immunofluorescence for Endomysium Antibodies

Assay performed according to methods by Mäki et al. (14) and Chorzelski et al. (15), as modified by Ladinser et al. (16).

- Substrate: Human umbilical cord sections (5-μm full transverse cryostat sections).
- Incubation: Patient's serum 1:5 and 1:50, 30 minutes at room temperature, wash three times in PBS (pH 7.4). For screening purposes, 1:5 dilution was sufficient.
- Positive sera: Titration, 1 to 5, 50, 100, 200, 400, 800, 1,600, 3,200, and 6,400.
- Conjugate: Fluorescein isothiocyanate (FITC), goat anti-human IgA (Kallestad, Austin, TX, U.S.A.), optimal working dilution 1:120 (chessboard titration), 30 minutes incubation at room temperature, wash three times.
- Mounting: Ten milliliters 1 mol/L Tris (pH 9.1), 0.2 mL 0.5% phenol red, 150 mL H_2O, warm to boiling point, and add slowly 10 g polyvinyl alcohol, 50 mL glycerol 87%, 0.2 g chlorobutanol, check pH 8.2. Add a drop of mounting medium on slides.
- Reading: Fluorescence microscope (Leitz, Wetzlar, Germany), cutoff at titer 1:5, positivity defined as fluorescence of:
  - reticulin fiber in vessel walls, and
  - external borders of vessels and/or structures in Wharton's jelly.

Enzyme-Linked Immunosorbent Assay for Tissue Transglutaminase Antibodies

Assay was performed according to methods by Dietrich et al. (5), with slight modifications from Sulkanen et al. (17).

- Coating: Microtiter plates, high binding capacity, Maxisorp (Nunc), tTG (Sigma), 1 μg/well in 100 μL coating buffer (0.05 mol/L Tris-HCl, 0.15 mol/L NaCl, 5 mmol/L $CaCl_2$ [pH 7.5]), 2 hours incubation at 37°C.
- Wash and block: Blocking solution 0.05 mol/L Tris-HCl, 0.15 mol/L NaCl, 0.01 mol/L EDTA, 0.1% Tween-20 (pH 7.4), overnight incubation at 4°C.
- Incubation: Serum dilution 1:100, 1 hour at room temperature, wash three times.
- Conjugate: Peroxidase, rabbit antihuman IgA, 1:1,000 (Dako, Glostrup, Denmark), 1 hour incubation at room temperature, wash three times.
- Substrate: 1 mg/mL O-phenylene diamine hydrochloride (OPD) (Sigma) in 0.1 mol/L citrate buffer, 200 μL/well 0.06% H_20_2 (pH 4.2), 30 minutes incubation at room temperature, darkness.
- Reading: Microplate reader 450 nm (MRX; Dynatech).
- Standard curve: Positive reference pool diluted 50, 100, 200, 400, 800, 1,600, and 3,200.
- Expression of results: Results were given in arbitrary units. One hundred arbitrary units equals the optical density of the reference pool 1:100. Cutoff values were established by ROC curves (see Results). Cutoff for IgA tTG antibodies was 8 AU.

Statistical Evaluation

Sensitivity, specificity, and negative and positive predictive values were determined, and 95% confidence intervals were calculated (8,11,18). Receiver operating characteristic curves were used to establish clinical cutoff values (8,19,20). Intralaboratory day-to-day reproducibility was determined by calculating intralaboratory coefficients of variation. Interlaboratory reliability was determined by calculating Cohen's κ values according to Dunn (21) and Shrout and Fleiss (22). Cohen's κ is a measure of intraclass correlation between different laboratories for binary scales. Values higher than 0.75 indicate excellent agreement, and 1.0 indicates complete agreement.

RESULTS

Intralaboratory Variation Coefficients

Mean intralaboratory variation coefficients were 3.4% for IgG gliadin antibodies, 3.2% for IgA gliadin antibodies, and 8.1% for tTG antibodies (data available from Brussels, Tampere, and Tübingen). For IgA tTG antibodies, there were some differences using different lots of the enzyme (Sigma 37 H 9564, 18 H 7820, and 38 H

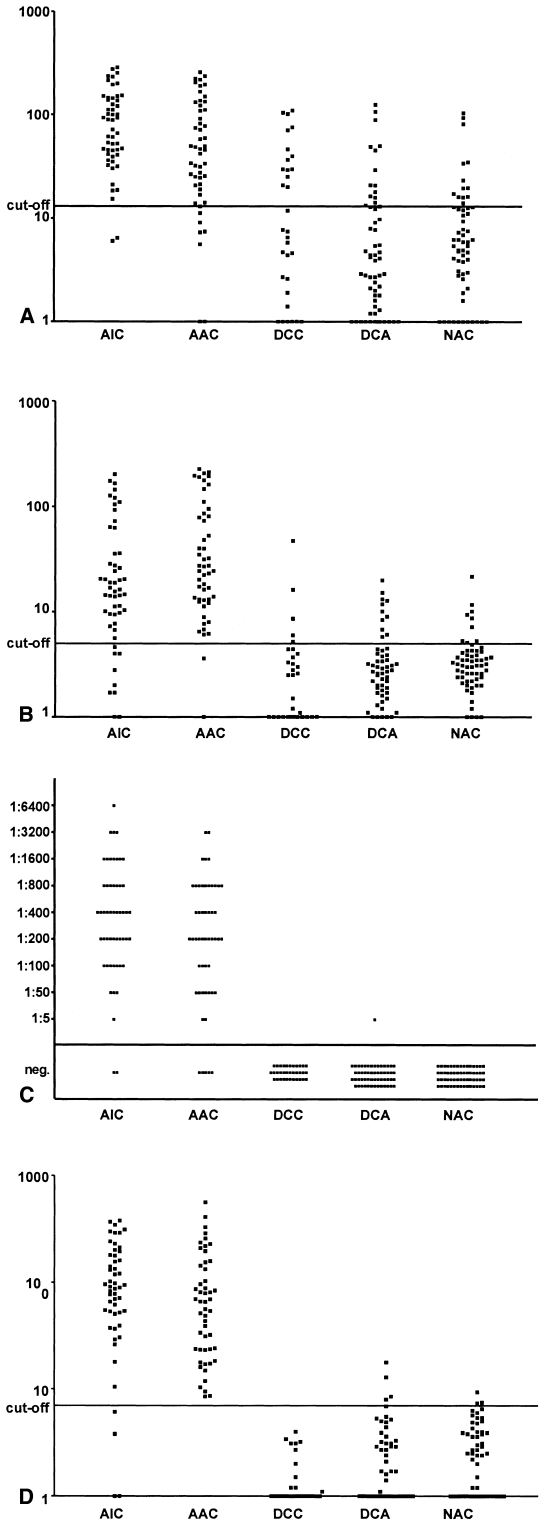

FIG. 1. Serologic results in 252 sera tested in the collaborative study. Results are shown in arbitrary units (AU) calibrated by group reference pool for enzyme-linked immunosorbent assay (ELISA; **A**, **B**, and **D**) and in titers for immunofluorescence (**C**). Cutoff values are indicated by horizontal lines. AAC, active adult celiac disease; AIC, active infant celiac disease; DCA, disease controls adults; DCC, disease controls children; NAC, normal adult controls. (**A**) Immunoglobulin (Ig)G gliadin antibodies; (**B**) IgA gliadin antibodies; (**C**) IgA endomysium antibodies (human umbilical cord); and (**D**) IgA tissue transglutaminase antibodies.

7820). The intralaboratory variation coefficient was zero for IgA EMA positivity or negativity at a dilution of 1:5 (Tübingen, $n = 10$). For positive sera, EMA titers varied up to one or two steps, when repeated and compared day to day by one laboratory (Tübingen, $n = 10$).

For IgG and IgA gliadin antibodies and for IgA tTG antibodies, standard curves exhibited linearity between 5 and 100 AU (data not shown).

Serologic Data in Clinical Groups

In Figure 1, A through D, serologic data of all 252 sera tested are shown (for clarity, only the Tübingen data are reported herein; interlaboratory reproducibility is shown later). The respective cutoff values were determined by ROC curves, taking into account that, for screening purposes only, very high sensitivities (>90%) were acceptable.

Two pediatric patients with celiac disease with IgA deficiency (group AIC) were included. For gliadin antibodies (Fig. 1A and B), there were a considerable number of false-negative and, in particular, false-positive results. For IgA EMA (Fig. 1C, false-negative results were restricted to the two IgA-deficient patients in group AIC, but there were additional false-negative results in adults (group AAC) with normal serum IgA values. One individual control patient (group DCA) showed a false-positive result for IgA EMA. However, it cannot be ruled out that this patient had latent celiac disease. Figure 1D again shows some false-negative results for IgA tTG antibodies, although results gave a good distinction, particularly in group AAC (no false-negatives). Some false-positive results in adult control subjects (DCA, NAC) overlapped AAC data slightly.

Sensitivity, Specificity, and Receiver Operating Characteristic Curves

Sensitivity, specificity, positive predictive values, and negative predictive values are shown in Table 1 with 95% confidence intervals. These data are based on values produced by all participating laboratories applying joint cutoff limits as defined earlier. It is clear from the figures that gliadin antibodies were inferior to endomysium and tTG antibodies in clinical sensitivity, specificity, and positive–negative predictability. Predictive values are based on the prevalence of celiac disease (53.6%) according to the patients' group classification. They are not applicable to any screening situation (8,18).

The ROC curves were drawn where applicable (IgG/IgA gliadin antibodies, IgA tTG antibodies) and are shown in Fig. 2. For logical reasons, the ROC curve could not be drawn for EMA. Clearly, the ROC curve for IgA tTG antibodies was superior—also indicated by the very high area under the curve (AUC) of 99.8%.

For reasons given in the following paragraph, tTG antibody results from only five laboratories could be included in the final evaluation.

Interlaboratory Reproducibility

Gliadin and endomysium antibody results were available from all seven participating laboratories, whereas one laboratory was not able to participate in the tTG antibody determination. The correlation between IgA EMA results and IgA tTG antibody results was relatively high, corroborating data from the literature (17). This, however, was true for only five of the six laboratories involved in both antibody determinations (Fig. 3). Laboratory number 5 produced data in good agreement with those of the other laboratories in EMA findings but not in tTG antibody findings. In fact, this laboratory fulfilled the Cochran criteria for outlier data, exclusively for the tTG antibody part (12). Thus, this laboratory had to be excluded, and final data for interlaboratory reproducibility were calculated after the exclusion.

For simplicity, interlaboratory reliability was based only on the binary decision for positivity and negativity, using cutoff limits as shown earlier (Table 2). The κ values higher than 0.75 represented excellent agreement beyond chance between laboratories. Obviously, interlaboratory reliability was superior for the EMA determination, based on the protocol described. The inferior values for gliadin antibody (particularly IgA) determinations were not totally expected because the main effort of the group's work had been directed toward standardization of every single step in the gliadin antibody protocol.

TABLE 1. *Sensitivity, specificity, predictive values*

	IgA-AGA		IgG-AGA		IgA-EMA/HUC		IgA-tTG	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Sensitivity	83	73.8–89.3	86	78.2–92.4	90	82.7–95.2	93	86.5–97.2
Specificity	82	72.5–89.3	76	66.2–84.8	99	93.9–99.9	95	87.4–98.2
Positive predictive value	76	66.4–84.0	71	61.1–79.6	98	93.0–99.8	93	86.1–97.1
Negative predictive value	87	78.8–92.9	89	81.2–94.4	93	86.1–97.1	95	88.7–98.4

AGA, gliadin antibodies; EMA, endomysium antibodies; HUC, human umbilical cord; Ig, immunoglobulin; tTG, tissue transglutaminase antibodies.

Percents shown in brackets: 95% confidence interval.

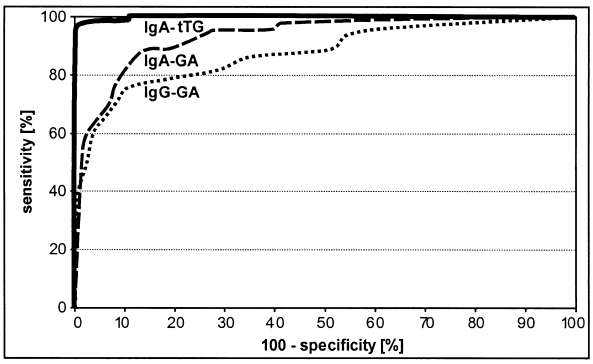

FIG. 2. Receiver operating characteristic (ROC) curve for gliadin and tissue transglutaminase antibodies. Data are shown for immunoglobulin (Ig)G gliadin antibodies (AUC 87.4%), for IgA gliadin antibodies (AUC 92.9%) and IgA tissue transglutaminase antibodies (AUC 99.8%).

DISCUSSION

Numerous comparative studies have shown the value of gliadin antibodies and autoantibodies against endomysium or tTG as serologic indicators of celiac disease in children and adults (23–28). Clinical sensitivity and specificity of various test systems indicate high potential. However, actual figures have varied widely, because of the limited biologic significance of gliadin antibodies in celiac disease and because of standardized test protocols and reference sera have not been available. In several comparative studies, investigators have tried to establish practical procedures useful for screening and for helping to diagnose celiac disease (8,18,29). However, interlaboratory reproducibility of serologic test results for celiac disease has been reported only rarely (9), with disappointing results in particular for gliadin antibodies.

Therefore, the joint EMRC/ESGPHAN initiative was formed to find ways of standardizing technical procedures that would allow comparison of sensitivity, specificity, and predictability of robust noncommercial test protocols for gliadin antibodies, endomysium, and tTG antibodies on a solid basis (10). Even more important,

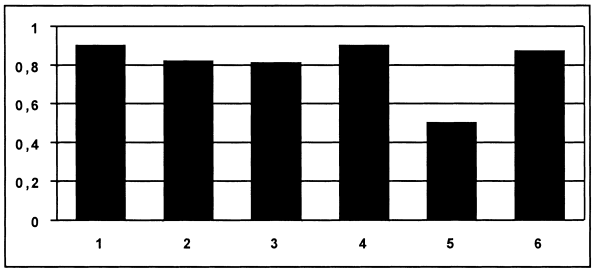

FIG. 3. Correlation coefficients: Immunoglobulin (Ig)A endomysium antibodies versus IgA tissue transglutaminase (tTG) antibodies (positivity and negativity at the cutoff level for laboratories 1–6). Data from laboratory 5 were classified as outliers for tTG antibody and were excluded from the evaluation.

TABLE 2. *Interlaboratory reliability: intraclass correlation coefficient kappa*

	Kappa value
IgA EMA/HUC	0.93
IgA tTG	0.83
IgG AGA	0.82
IgA AGA	0.62

AGA, gliadin antibodies; EMA, endomysium antibodies; HUC, human umbilical cord; Ig, immunoglobulin; tTG, tissue transglutaminase antibodies.

Seven laboratories for AGA and EMA, five laboratories for tTG antibodies.

internal and external quality control was introduced into test evaluation.

Robust test protocols were defined by the working group based on previous studies (5,13–17). Clinical sensitivity was high for all antibodies used (83%–93%). However, sensitivity of IgA and IgG gliadin antibodies was inferior to that of autoantibodies against endomysium and tTG. Moreover, specificity of gliadin antibodies (82% for IgA, 76% for IgG) did not reach the very high specificity of the autoantibodies (95% for tTG, 99% for EMA). However, the main purpose of this study was not clinical evaluation. There was a strong selection bias by the given high prevalence of celiac disease (53.6%), and for definitive clinical purposes the total number of 103 sera from pediatric and adult patients with celiac disease was not sufficient. (8,18).

Analysis by ROC (30) has added a new dimension to comparative evaluation of different test protocols. The ROC curves shown for gliadin and tTG antibodies demonstrated the superiority of IgA tTG antibody testing (AUC 99.8%). High values for sensitivity, specificity, and ROC testing (AUC) produced by either EMA or tTG antibody determinations were not exceeded by combined testing of gliadin antibodies plus endomysium or tTG antibody testing (11).

The problem of IgA deficiency (31–33), which was present exclusively in two of our pediatric patients with celiac disease, remains to be solved in celiac serology. In this context, there is an argument to test for IgG gliadin antibodies (33). Testing for serum IgA deficiency is simple and cheap and should be included in any serologic protocol for celiac disease.

Testing for EMA was performed effectively using human umbilical cord tissue as a substrate to immunofluorescence (16,34). The present study corroborated high clinical potential of EMA testing (35,36). However, EMA may not be appropriate for follow-up (37,38). Interlaboratory reliability of EMA testing provided data ($\kappa = 0.93$) superior to data for all other test protocols, even though the immunofluorescence reading was subjective. Consequently, there is the need for experienced and well-trained laboratory personnel to read the test results—in particular, to differentiate between endomysium and smooth muscle antibodies. The test protocol for EMA

immunofluorescence was robust and provided the best reproducibility data. Semiquantitative evaluation (titration) exhibited some interlaboratory variation. This was not considered crucial for screening or diagnostic purposes. Unfortunately, the immunofluorescence test protocol did not provide data appropriate for ROC analysis.

Testing for the tTG antibody has been introduced recently into the field and has already produced highly promising data (5,17,39–41). However, interlaboratory reproducibility of tTG antibody testing ($\kappa = 0.83$) did not reach that of EMA testing in our collaborative study. There were questions concerning variations in tTG enzyme preparations used as an antigen and concerning minor variations in test protocols that have been described to date that call for further improvement. The introduction of human recombinant tTG may be a significant further step.

After many serologic studies into celiac disease, including the present one, small intestinal biopsy is still considered the gold standard of diagnosis (42,43). This is true even in cases of celiac disease with mild enteropathy that does not reach the destructive level of subtotal villus atrophy (44). Comparison of clinical diagnosis based on ESPGHAN criteria (6) with serologic test results has produced diverging data, particularly in adults with mild enteropathy (45–47) indicating that small intestinal biopsy should not be left as the primary diagnostic procedure for celiac disease.

The use of serologic methods for celiac screening has been settled (48–53). The superiority of the EMA test protocol also applies in this context, because interlaboratory reproducibility is particularly important for screening. Serologic screening is an important tool, particularly to identify incidences of silent celiac disease. A cost–benefit analysis of screening strategies has not yet been substantiated (54).

In the present study, test protocols have been standardized, and reference sera have been created by the European working group. Reference sera are now available for calibration (upon request from the corresponding author). Besides calibration, the importance of internal and external quality control was demonstrated. Quality control based on comparison of ROC curves, intralaboratory coefficients of variation, and κ values for interlaboratory reliability must be included in any comparative trial. National training programs and quality assurance systems should help to extend the reliability and impact of serologic testing for celiac disease. Future ESPGHAN guidelines and diagnostic standards must include the potential and limits of celiac serology in more detail.

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