BRIEF REPORT

One-step immunochromatographic visual assay for anti-transglutaminase detection in organ culture system: An easy and prompt method to simplify the in vitro diagnosis of celiac disease

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Background: Anti-tissue transglutaminase (anti-tTG) and endomysium antibodies (EMA) are detectable in duodenal culture media of celiac disease (CD) patients. To improve the management of this organ culture system, we evaluated the anti-tTG occurrence by immunochromatographic assay (ICA).

Methods: A total of 103 CD patients and 41 disease controls underwent duodenal biopsy for the organ culture. In culture supernatants, IgA anti-tTG were tested by both enzyme-linked immunosorbent assay (ELISA) and ICA, IgA EMA were searched by indirect immunofluorescence analysis (iIFA).

Results: Endomysium antibodies and anti-tTG measured by ELISA were positive in culture media of all CD patients, while anti-tTG detected by ICA were positive in culture media of 87/103 CD patients. Anti-tTG ICA scores significantly correlated with anti-tTG ELISA values ($r=.71$, $P<.0001$). Sensitivity, specificity and diagnostic accuracy of anti-tTG detected by ICA were 84.5%, 100% and 88.9%, respectively.

Conclusions: Using ICA, anti-tTG are detectable in duodenal culture media of most CD patients and the intensity of indicative lines depends on the anti-tTG concentration. Sensitivity and diagnostic accuracy achieved with ICA are lower than those obtained with ELISA but, given that the first is a more easy and prompt method, data suggest the possibility of utilizing it in the in vitro diagnosis of CD.

KEYWORDS

anti-transglutaminase, celiac disease, diagnosis, immunochromatographic assay, organ culture system

1 | INTRODUCTION

Celiac disease (CD) is a systemic autoimmune disorder induced by wheat gluten and related proteins from rye and barley in genetically susceptible individuals.¹ In adults, the diagnosis of CD is still based

on the histological evidence of villous atrophy, crypt hyperplasia and increased intraepithelial lymphocyte (IEL) count on duodenal biopsy samples.² The presence of circulating anti-tissue transglutaminase (anti-tTG) and endomysium antibodies (EMA) and their disappearance after a gluten-free diet (GFD) support the diagnosis.³

Previous studies have shown that EMA and anti-tTG can also be found in culture supernatants of duodenal biopsies from untreated CD patients.^{4,5} This organ culture system has been proposed in the diagnostic procedure of CD, especially for cases with patchy atrophy or

Abbreviations: A_{450 nm}, absorbance 450 nm; anti-tTG, anti-tissue transglutaminase; CD, celiac disease; ELISA, enzyme-linked immunosorbent assay; EMA, endomysium antibodies; GFD, gluten-free diet; HE, hematoxylin and eosin; ICA, immunochromatographic assay; IECs, intestinal epithelial cells; IELs, intraepithelial lymphocytes; iIFA, indirect immunofluorescence analysis.

potential CD where, using only the conventional histology, the risk of missing the diagnosis is very high.⁶⁻⁸ However, some conflicting opinions and a number of technical difficulties have limited its widespread use in the diagnosis of CD.⁹⁻¹¹

In order to simplify the use of the organ culture system in the diagnostic procedure of CD, we evaluated the reliability and usefulness of anti-tTG detection in culture supernatants by one-step immunochromatographic visual assay (ICA), a more easy and prompt method than the standard immunoenzymatic technique.^{12,13}

2 | MATERIALS AND METHODS

2.1 | Patients

A total of 103 consecutive patients (23 males and 80 females, median age 34, range 18-64 years) with a clinical picture suggestive of CD as well as serum anti-tTG and EMA positive results on a gluten-containing diet were included in the study.

Forty-one consecutive patients (9 males and 32 females, median age 38, range 18-65 years) subjected to upper endoscopy for gastrointestinal disorders other than CD (25 gastroesophageal reflux disease, 7 *Helicobacter pylori* infection, 4 peptic ulcer disease, 3 gastritis, 1 diffuse large B-cell lymphoma, 1 small bowel carcinoma) were evaluated as disease controls.

All patients underwent upper endoscopy with duodenal biopsy sampling to perform the histological analysis and organ culture system. All procedures followed in this study were in agreement with the ethical standards of the institutional committee responsible for human experimentation. An informed consent was also obtained from each participant.

2.2 | Upper endoscopy with biopsy sampling

Patients underwent upper endoscopy after fasting for at least 8 hours. In most cases, conscious sedation was obtained using pharyngeal anesthesia with lidocaine spray 1% and midazolam 2 mg i.v. In few cases, deep sedation using propofol had to be carried out. Three biopsy samples were collected from the second portion of the duodenum, distally from the Vater's papilla of each patient using a standard 5-mm biopsy forceps. Two biopsies oriented on acetate cellulose filters (Bio-Optica, Milan, Italy) and embedded in 10% formaldehyde were subjected to histological analysis, while the other one sample was used for the organ culture system.

2.3 | Histological analysis

Duodenal biopsy samples were fixed overnight in 4% formalin. After orientation, inclusion in paraffin and cutting, morphological analysis was performed by hematoxylin and eosin (HE) staining technique. Villous height/crypt depth ratio was measured and values <3:1 were considered indicative of morphological alteration. The number of IELs per 100 intestinal epithelial cells (IECs) was assessed by immunohistochemical staining for CD3 lymphocyte surface marker, with

25 IELs/100 IECs as upper limit of normal. The histological pattern was blindly evaluated, according to Marsh-Oberhuber classification,¹⁴ by a pathologist unaware if the biopsies belonged to suspected CD patients or disease controls. All type III lesions were considered diagnostic for CD.

2.4 | Organ culture system

Duodenal biopsy samples (minimal weight required: 5 mg/sample) were washed in physiological solution (NaCl, 9 g/L), placed in sterile tubes containing 500 µL of medium prepared as previously described⁸ and cultured for 48 hours at 37°C. Thereafter, culture supernatants were collected and stored at -20°C until used.

2.5 | Anti-transglutaminase antibody determinations

IgA anti-tTG antibodies were measured in culture supernatants diluted 1:5 by enzyme-linked immunosorbent assay (ELISA) on microtiter-plate wells coated with recombinant human tTG (INOVA Diagnostics, San Diego, CA, USA; distributed by Instrumentation Laboratory, Milan, Italy). Results were expressed in absorbance measured by an ELISA plate reader at 450 nm ($A_{450\text{ nm}}$). As previously reported,⁵ 0.300 $A_{450\text{ nm}}$ was used as cut-off to identify anti-tTG positive results.

IgA anti-tTG antibodies were also determined by an ICA commercial kit (Eurospital, Trieste, Italy). In brief, a stick containing latex particles conjugated to tTG was placed in each undiluted culture supernatant and incubated for 5 minutes. The antigen-specific antibodies eventually present in culture supernatants bind the tTG conjugated to latex particles and, by a chromatographic process, the particle-anti-tTG complexes move up to the reaction area. This area is rich of tTG that, reacting with the particle-anti-tTG complexes, determines the appearance of a pink-red indicative line. A blue control line indicates that the reaction took place. Results were expressed as positive/negative and, in relation to the signal intensity of the pink-red indicative lines, the following scores were

FIGURE 1 Anti-tTG visual results detected by ICA method. Negative (score 0) (A), mild positive (score 1) (B), positive (score 2) (C), and strong positive (score 3) (D) anti-tTG results detected by ICA in duodenal culture media. Anti-tTG, anti-tissue transglutaminase; ICA, immunochromatographic assay

assigned: 0=negative, 1=mild positive, 2=positive, 3=strong positive (Figure 1).

2.6 | Endomysium antibody detection

IgA EMA were searched in undiluted culture supernatants by indirect immunofluorescence analysis (iIFA) on cryostat sections of monkey esophagus (Eurospital). Results, blindly evaluated by two trained observers (agreement rate 99.6%), were expressed as positive/negative and, in relation to the fluorescence signal intensity, the following scores were assigned: 0=negative, 1=mild positive, 2=positive, 3=strong positive.

2.7 | Statistical analysis

Sensitivity, specificity and diagnostic accuracy of EMA as well as anti-tTG ELISA and ICA tests were calculated. The relationship among values and/or scores of the antibody tests was assessed by the Bravais-Pearson linear correlation, in which P values $<.05$ were considered significant. All statistical evaluations were performed using the GraphPad Prism package version 5.2 (GraphPad Software Inc., San Diego, CA, USA).

3 | RESULTS

One hundred out of 103 suspected CD patients showed type III duodenal lesions (23 IIIa, 31 IIIb and 46 IIIc). The remaining three patients presented type I duodenal lesion and, accordingly, were classified as having potential CD. All 41 disease controls had a normal duodenal histology (type 0 according to Marsh-Oberhuber classification).

Both EMA and anti-tTG measured by ELISA were positive in culture media of duodenal biopsies from all CD patients. Anti-tTG detected by ICA were positive in duodenal culture media of 87 out of 103 (84.5%)

TABLE 1 Diagnostic performance of EMA detected by iIFA and anti-tTG determined by ELISA or ICA in organ culture system

	EMA (iIFA)	Anti-tTG (ELISA)	Anti-tTG (ICA)
Sensitivity (%)			
Value	100 (103/103)	100 (103/103)	84.5 (87/103)
95% CI	96.5-100	96.5-100	76.0-90.9
Specificity (%)			
Value	100 (41/41)	100 (41/41)	100 (41/41)
95% CI	91.4-100	91.4-100	91.4-100
Diagnostic accuracy (%)			
Value	100 (144/144)	100 (144/144)	88.9 (128/144)
95% CI	97.5-100	97.5-100	82.6-93.5

95% CI, 95% confidence interval; anti-tTG, anti-tissue transglutaminase; ELISA, enzyme-linked immunosorbent assay; EMA, endomysium antibodies; ICA, immunochromatographic assay; iIFA, indirect immunofluorescence analysis.

Data in parentheses are the numbers used to calculate the percentages.

CD patients. None of these antibodies was found in culture media of duodenal biopsies from disease controls.

Sensitivity, specificity and diagnostic accuracy of EMA and anti-tTG measured by ELISA were 100% while, for anti-tTG detected by ICA, these parameters were 84.5%, 100% and 88.9%, respectively (Table 1).

Anti-tTG ICA scores significantly correlated with both anti-tTG ELISA values ($r=.71$, $P<.0001$) (Figure 2A) and EMA iIFA scores ($r=.56$, $P<.0001$). Consistently, anti-tTG ELISA values and EMA iIFA scores

FIGURE 2 Correlation among anti-tTG ICA scores, anti-tTG ELISA values, and EMA iIFA scores. Data were determined in duodenal culture media of CD patients ($n=103$). The r and P values refer to Bravais-Pearson linear correlation. The best-fit line (solid line) and its 95% confidence bands (dashed lines) are plotted in each graph. The correlation between anti-tTG ICA and EMA iIFA scores ($r=.56$, $P<.0001$) is not reported because all data are expressed as integers and, therefore, overlap. $A_{450\text{ nm}}$, absorbance 450 nm; anti-tTG, anti-tissue transglutaminase; CD, celiac disease; ELISA, enzyme-linked immunosorbent assay; EMA, endomysium antibodies; ICA, immunochromatographic assay; iIFA, indirect immunofluorescence analysis

correlated between them ($r=.42$, $P<.0001$) (Figure 2B), even if with a lower correlation index.

4 | DISCUSSION

Although CD diagnosis based on clinical case identification, serological screening and duodenal histology is well defined and standardized,² each of these diagnostic tools has its own weaknesses.¹⁵ As a consequence, some in vitro methods have been proposed as supporting tools to diagnose CD.¹⁶ Among these, the organ culture system has been proposed as a supporting tool for duodenal histology, especially in cases with patchy atrophy or potential CD where, using only the conventional histology, the risk of missing the diagnosis is very high.⁶⁻⁸ This system is further able to evaluate the production/release of antibodies pathognomonic of CD directly from duodenal mucosa, adding advantages in terms of both sensitivity and specificity with respect to detection of the same antibodies in serum. Indeed, it has been demonstrated that the organ culture system can usefully assist the histology also in diagnosing seronegative CD patients^{5,8} and, rationally, could be useful to discriminate the anti-tTG false-positive results occurring in serum of patients with illnesses not involving the small intestine, such as arthritic diseases,¹⁷ inflammatory bowel disease,¹⁸ and cardiovascular disorders.¹⁹ Some conflicting opinions have, however, limited its widespread use in the diagnosis of CD.^{9,10} Moreover, the organ culture system has been described as a labor intensive and time consuming tool that can be used only by specialist centers.¹¹

Some years ago, it has been developed an ICA method able to detect circulating anti-tTG antibodies easily and quickly.¹² This serological tool seems to be a useful alternative way to screen for CD, especially in patients with few or atypical symptoms, at-risk groups such as first-degree relatives and subclinical patients.^{12,13,20}

On the basis of above, we assessed the reliability and usefulness of anti-tTG detection in culture supernatants by ICA in order to simplify the use of the organ culture system in the diagnostic procedure of CD. For this purpose, 103 suspected CD patients and 41 gastrointestinal disease controls have been evaluated. A limit of our study is that the histological examination has been performed on 2 rather than 4-5 duodenal biopsy samples, as suggested by guidelines,² with negative implications on the assessment of patchy abnormalities, especially in the three patients diagnosed as having potential CD since presenting type I duodenal lesion according to Marsh-Oberhuber classification.

The presence of EMA and anti-tTG measured by ELISA in duodenal culture media of all CD patients but not of disease controls firstly confirm the reliability of the organ culture system in identifying CD.⁶⁻⁸ Using ICA, anti-tTG antibodies are detectable in duodenal culture media of most CD patients but not of disease controls, highlighting the reliability and usefulness of this method in identifying the organ cultures as positive, while negative results need to be confirmed by the standard ELISA technique. The correlation of anti-tTG ICA scores with anti-tTG ELISA values ($r=.71$, $P<.0001$) suggests that the signal intensity of indicative lines depends on the anti-tTG antibody concentration. This correlation, together with that found between anti-tTG

ICA and EMA iIFA scores ($r=.56$, $P<.0001$), also confirms the reliability of ICA method in organ culture system.

Sensitivity and diagnostic accuracy achieved with ICA are lower than those obtained with ELISA but, given that the first is a more easy and prompt method compared with the second, our data suggest the possibility of utilizing it as a routine procedure in the in vitro diagnosis of CD. On the other hand, since the occurrence of ICA false-negative results is probably due to low anti-tTG antibody concentrations, the development of an ICA method specific for the organ culture system, for example, at high sensitivity, could improve its performance in diagnosing CD. Another advantage of ICA method is that requires a one-strip for detection without adding reagents, differently from ELISA and iIFA techniques that require, respectively, an eight-wells strip and a four-wells slide even for a single determination, as well as the addition of reagents. This could result in a reduction of both costs and turnaround time, especially in the evaluation of a single or few suspected CD patients.

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