


Simultaneous detection of celiac disease-specific IgA antibodies and total IgA

Kai Grossmann¹ · Nadja Röber² · Rico Hiemann³ · Stefan Rödiger³ · Peter Schierack³ · Dirk Reinhold⁴ · Martin W. Laass⁵ · Karsten Conrad² · Dirk Roggenbuck^{1,3} 

Received: 6 October 2015 / Accepted: 9 December 2015 / Published online: 30 January 2016
© The Author(s) 2016

Abstract

Purpose Celiac disease (CD) serology requires analysis of tissue transglutaminase type-2 (TG2autoAbs), deamidated gliadin (DGABs), and as reference endomysial autoantibodies (EmA). Total IgA assessment helps to determine IgA-deficient CD patients. The novel multiplex indirect immunofluorescence (IIF) technique CytoBead was used to develop the first quantitative one-step serological CD assay comprising both simultaneous IgA autoAb and total IgA testing.

Methods CytoBead CeliAK detecting TG2autoAb, DGAB, EmA, and simultaneously total IgA uses fluorescent microparticles for antigen and antibody immobilization along with monkey-esophagus tissue sections on glass slides. The assay was interpreted visually by classical fluorescent microscopy and digital IIF using AKLIDES[®]. Overall, 380 samples (155 CD patients, 5 with IgA deficiency, 68 with cystic fibrosis, 59 with eye disease, 93

blood donors) were run for performance analysis. Data were compared with classical IgA autoAb analysis by ELISA and IIF.

Results Comparing CD-specific IgA autoAb testing by CytoBead with classical IIF and ELISA, very good agreements for EmA, TG2autoAb, and DGAB were determined (Cohen's $\kappa = 0.98, 0.96, 0.85$, respectively). The difference between multiplex and single testing revealed a significant difference for TG2autoAb testing only (McNemar, $p = 0.0078$). Four CD patients and 4 controls demonstrated TG2autoAb positivity by ELISA but were negative by CytoBead. Further, 140/155 (90.9 %) CD patients demonstrated TG2autoAb levels above ten times the upper normal and all five IgA-deficient samples IgA levels <0.2 g/L by CytoBead.

Conclusions The novel multiplex CytoBead CeliAK enables simultaneous CD-specific autoAb and IgA deficiency analyses comparable with classical testing by single-parameter assays. Thus, comprehensive CD serology by CytoBead can alleviate the workload in routine laboratories.

Electronic supplementary material The online version of this article (doi:10.1007/s13317-016-0073-2) contains supplementary material, which is available to authorized users.

✉ Dirk Roggenbuck
dirk.roggenbuck@b-tu.de

- ¹ GA Generic Assays GmbH, Dahlewitz, Germany
- ² Institute of Immunology, Medical Faculty, Technical University Dresden, Dresden, Germany
- ³ Faculty of Natural Sciences, Brandenburg University of Technology Cottbus-Senftenberg, Großenhainer Str. 57, 01968 Senftenberg, Germany
- ⁴ Institute of Molecular and Clinical Immunology, Otto-von-Guericke-University, Magdeburg, Germany
- ⁵ Children's Hospital, Medical Faculty Carl Gustav Carus, Technical University Dresden, Dresden, Germany

Keywords Celiac disease · Tissue transglutaminase · Endomysial antibody · Deamidated gliadin · Digital fluorescence

Introduction

Celiac disease (CD) is a gluten-related and immune-mediated small intestinal disease which causes chronic inflammatory lesions leading to villous atrophy and hyperplasia of intestinal crypts [1, 2]. The destructive mucosal changes cause malabsorption in severe cases [3]. The disease demonstrates a strong human leukocyte

antigen (HLA) association and manifests mainly in HLA-DQA1*05-DQB1*02 (DQ2) or DQA1*03-DQB1*0302 (DQ8)-haplotype-positive patients [4, 5]. In general, the inflammatory process in CD is characterized by an increased intraepithelial lymphocyte count with values greater than 25/100 cells [6]. Celiac disease can occur from the second half of the first life year throughout life time with asymptomatic intermediate stages particularly in adult patients and differing clinical symptoms [7, 8]. High prevalences of CD have been reported in Caucasians: 1 in 99 in Finland, 1 in 122 in Northern Ireland and 1 in 175 in Italy [9–12].

The diagnosis of CD comprises clinical, serological, and histological findings and is confirmed by normalization of pathological findings under a gluten-free diet in the majority of patients [8, 13]. Serology is paramount for the diagnosis of CD requiring the detection of autoantibodies (autoAbs) to endomysium (EmA), deamidated gliadin (DGAb), and tissue transglutaminase type 2 (TG2autoAb) of the IgA isotype [14–16]. Due to the high specificity of EmA results obtained by indirect immunofluorescence (IIF) testing, this autoAb is still regarded as the reference standard for CD-specific Ab detection [8]. However, classical IIF reading may be subject to interobserver and substrate-related variability which favors the testing of IgA autoAb to TG2 by immunometric solid-phase assays such as enzyme-linked immunosorbent assay (ELISA) instead [8, 17]. Of note, 2.6 % of CD patients suffer from IgA deficiency and may present non-classical CD complicating the diagnosis of CD [18, 19]. In contrast, up to 7.7 % of children with IgA deficiency may have CD [20]. Noteworthy, selective IgA deficiency being the most common primary immunodeficiency disorder with approximately 1 case in 400 humans is associated with DQ2, too. Thus, analysis of CD-specific IgG has been recommended in the case of IgA-deficient subjects with CD (total serum IgA <0.2 g/L) [21, 22].

Novel diagnostic criteria have been developed by the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) recently strengthening the role of CD serology [8]. Accordingly, the diagnosis of CD can be confirmed without histology in case of TG2autoAb IgA levels 10 times higher than the upper limit of normal (ULN) in patients positive for HLA-DQ2 or DQ8 and a positive response to gluten-free diet.

Consequently, taking into account the number of tests required for an appropriate CD serology, there is a need for new strategies to reduce the workload and laboratory costs in routine laboratories [23–26]. Thus, the aim of this study was to evaluate a multiplex strategy detecting CD-specific IgA autoAbs with the novel CytoBead technique which has been already employed successfully for multiplex autoAb analysis [27, 28]. The assay allows the simultaneous

analysis of EmA, TG2autoAb, and DGAb as well as the determination of total IgA in one reaction environment by IIF for the first time.

Materials and methods

Patients and controls

In total, 380 patients and controls including 155 patients with de novo CD, 5 with IgA deficiency, 68 with eye diseases (ED), 59 with cystic fibrosis (CF), and 93 blood donors (BD) were included. Pediatric patients with CD, ED, and CF were diagnosed in the Children's Hospital of the Medical Faculty of the Technical University Dresden. Correctness of CD diagnosis was confirmed in all patients by two investigators (ML and CK). Patient characteristics are summarized in Table 1. Serum samples were stored at -20°C until use.

The study was approved by the Local Ethics Committee (EZ151052010) and conducted in accordance with the Helsinki declaration.

CD-specific Ab testing by classical IIF and ELISA

Anti-endomysium IgA was analyzed by IIF employing cryostat sections of monkey esophagus according the recommendations of the manufacturer (GA Generic Assays GmbH, Dahlewitz, Germany) and reported elsewhere [24]. Processed slides were read either visually by fluorescent microscopy or with the automated interpretation system AKLIDES[®] (Medipan GmbH, Berlin/Dahlewitz, Germany) [24, 29].

IgA TG2autoAb and DGAb were determined by commercially available enzyme-linked immunosorbent assays (ELISAs) according to the recommendations of the manufacturer (GA Generic Assays GmbH) as described elsewhere [24]. Absorbance was read in a microplate reader at 450 nm and results were expressed in arbitrary units per milliliter (U/mL) according to the standards provided.

CD-specific Ab and IgA deficiency testing by CytoBead immunoassay

The CytoBead CeliAK immunoassay (GA Generic Assays GmbH) uses a combination of monkey-esophagus cryostat tissue sections and autoantigen-coated fluorescent microbeads (Red 550, excitation 610 nm and emission 690 nm; sizes 9, 15 μm ; PolyAn GmbH, Berlin, Germany) on slides with compartmented wells for simultaneous autoAb analysis (Fig. 1). The 9- μm beads were covalently coated with recombinant TG2 (DiaRect AG, Freiburg, Germany) and the 15- μm beads with recombinant DG

Table 1 Characteristics of patients and control cohorts

Cohort	<i>n</i>	Male	Female	Male median age (IQR)	Female median age (IQR)
Celiac disease	155	56	99	6 (8)	9 (13)
Age <18	131	50	81	6 (8)	6 (9)
Age ≥18	24	6	18	31 (24)	32 (17)
IgA deficiency	5	3	2	17 (13)	7 (4)
Cystic fibrosis	59	26	33	13 (22)	11 (23)
Eye diseases	68	43	25	6 (4)	6 (3)
Blood donors	93	44	49	23 (2)	23 (4)

IQR interquartile range

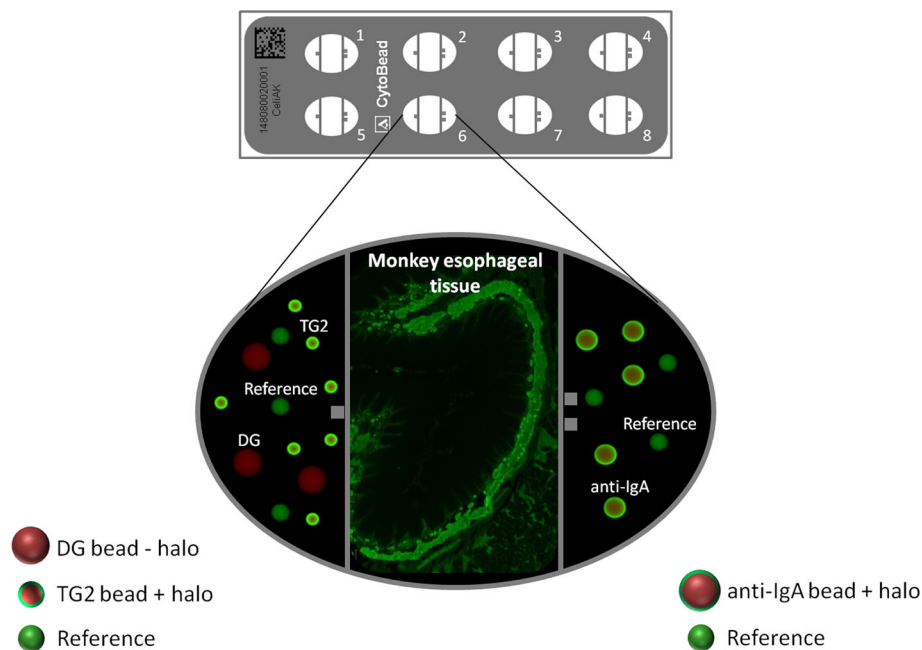


Fig. 1 Multiplex reaction environment of the CytoBead CeliAk for the simultaneous analysis of celiac disease (CD)-specific IgA autoantibodies and IgA deficiency. Employing compartmented wells on classical indirect immunofluorescence (IIF) glass slides, tissue transglutaminase type 2 (TG2)- and deamidated Gliadin (DG)-coated fluorescent beads as well as reference beads were immobilized in the *left-hand* well section. Further, cryopreserved tissue sections of monkey esophagus were fixed in the *middle* section for classical endomysial antibody (EmA) analysis by IIF as well as antihuman IgA

antibody-coated and reference beads in the *right-hand* well section. Exemplary, the well demonstrates a typical finding of a serum from a patient with CD by showing the classical EmA pattern on monkey esophagus in the *middle* section and a positive fluorescent halo of TG2-coated beads in the *left-hand* as well as of anti-IgA beads in the *right-hand* sections. Reference beads aid in distinguishing TG2-coated beads from DG-coated ones and orientation by visual evaluation with fluorescence microscope

(DiaRect) or sheep antihuman IgA antibody (Seramun Diagnostica GmbH, Heidsee, Germany). To create one common reaction environment with the immobilized cryopreserved esophagus tissue in the middle compartment, antigen- and Ab-coated beads were immobilized in the peripheral compartments together with reference beads (12 μ m, dye rhodamine, excitation at 526 nm and emission at 555 nm) supporting the following beneficial assay characteristics: (1) focusing and orientation in the left and right parts of the well, (2) discrimination of 9- μ m TG2-

coated beads from 15- μ m DG-coated beads in case of a positive autoAb reaction to TG2 and/or DG, and (3) providing focal point to check for IgA deficiency. Thus, TG2- and DG-coated fluorescent beads as well as reference beads were immobilized in the left-hand and antihuman IgA Ab-coated and reference beads in the right-hand well sections. In the case of a non-sufficient number of beads, automated evaluation by AKLIDES[®] does not analyze the result.

Briefly, diluted patient samples at a dilution of 1:10 were incubated for 30 min at room temperature. Unbound

serum components were removed by a subsequent wash cycle. The second incubation of sheep antihuman IgA conjugated to fluorescein isothiocyanate (FITC) (Seramun Diagnostica) for 30 min at room temperature was followed by another wash cycle to remove excess secondary Ab-conjugate molecules.

After mounting, slides were subjected to visual interpretation under a fluorescence microscope equipped with a FITC filter (excitation 495 nm; emission 519 nm, EUROStar, Euroimmun AG, Lübeck, Germany) at a magnification of 100 \times and to automated analysis using the digital imaging platform AKLIDES[®]. For confirmation of EmA positivity, a higher magnification of 400 \times was deployed. A serum is considered positive for EmA if a clearly discernible fluorescent pattern in the *Muscularis mucosae* (staining of the endomysium around the smooth muscle fibers) has been detected.

A positive reaction of CD-specific autoAb or serum IgA at the concentration of 0.2 g/L or higher revealed a green-emitting halo on the microbeads which can be readily discriminated from the homogeneous fluorescence of the reference beads (Fig. 1).

Fluorescent signals of EmA patterns and TG2-, DG- or anti-IgA-coated beads were categorized semi-quantitatively as negative (-), borderline (\mp), weak positive (1+), medium positive (2+), or strong positive (3+).

For automated evaluation, digital image taking was performed on tissue sections of monkey esophagus first using diamidino phosphatidyl inositol (DAPI) added to the conjugate buffer for image focusing on cell nuclei as

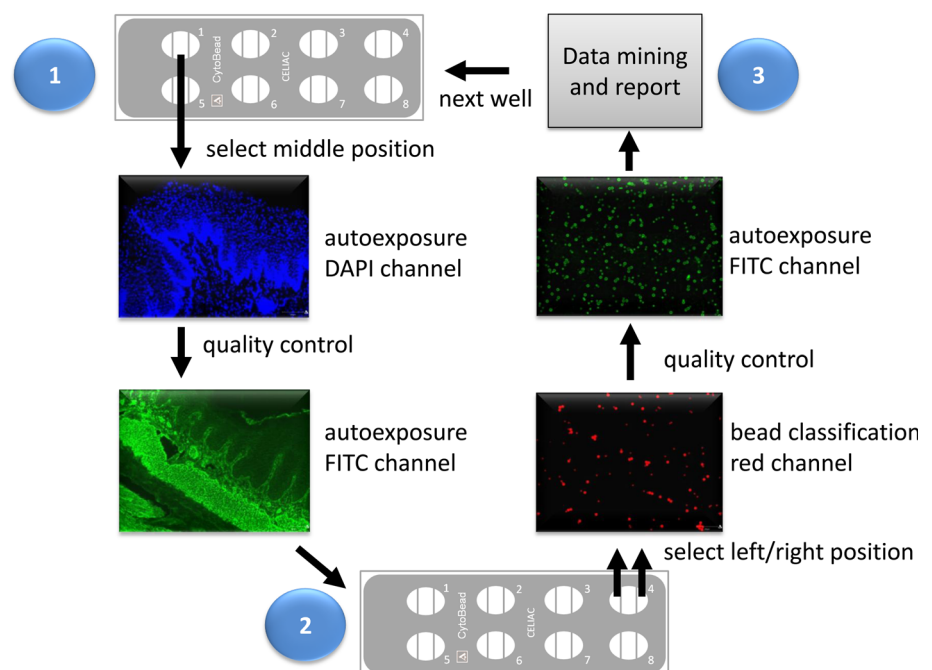
reported elsewhere [29, 30]. After switching into the FITC channel, nine images were taken and merged into an overview picture by the AKLIDES[®] software which was used for EmA analysis by the observer afterwards.

Subsequently, focusing and classification of the microbeads according to size and fluorescence intensity was run including the measurement of the green fluorescence (halo) intensity of positive signals. The latter intensity is directly proportional to the amount of autoAb or serum IgA bound. Results were read quantitatively in arbitrary U/mL by employing master standard curves for each parameter. Of note, the reference beads are not necessary for automated evaluation but only for bead-size differentiation during visual reading. Obtained fluorescence images and corresponding quantitative data were stored in lossless compressed tagged image file (TIF) format (Fig. 2).

Statistical analysis

The D'Agostino–Pearson test was employed to check for normal distribution. The Kruskal–Wallis test was used for analyzing the difference among patient and control groups. Inter-rater agreement statistics (Cohen's kappa, κ) and McNemar's test were employed for testing the concordance of different techniques. p values below 0.05 were considered significant. Receiver operating characteristics (ROC) curve analysis was performed like all other tests using MedCalc[®] software (MedCalc[®], Mariakerke, Belgium; Version 12.4.0).

Fig. 2 Workflow for automated reading of CytoBead CeliAK tests with the AKLIDES system. 1 Focusing of monkey-esophagus tissue sections in the DAPI channel and subsequent image taking in the FITC channel in the central compartment. 2 Focusing and classification of the microbeads according to size and fluorescence intensity and subsequent analysis of the green fluorescence of positive signals in both peripheral compartments. 3 Report generation



Results

Cutoff determination of CD-specific autoAb testing

To determine the cutoffs of the CytoBead CeliAK for IgA CD-specific autoAb, CD patients ($n = 155$) and control sera ($n = 220$) were run on the automated IIF interpretation system Aklides[®]. ROC curve analysis revealed the following optimal cutoffs for TG2autoAb and DGAAb testing: 3.91 U/mL and 2.89, respectively. For practical DGAAb analysis, a higher cutoff of 5.0 was used which corresponds to a specificity of 97.2 % instead of 93.5 %. Sensitivity dropped from 87.6 to 75.2 % accordingly.

In line with the new CD diagnosis criteria, $10 \times$ ULN of IgA TG2autoAb can be used for the diagnosis of CD avoiding duodenal biopsy. The ULN represents the mean autoAb value of non-diseased subjects plus two standard deviations. Thus, we determined the ULN for two different age groups of CD patients younger ($n = 130$) and 18 years and older ($n = 24$) (Table 2). Since both control groups (pediatric patients with ED and BD, respectively) did not show normality, the corresponding 97.5 % percentiles were determined instead. Altogether, the obtained values were compared to the cutoffs obtained by ROC curve analysis. The IgA TG2autoAb cutoffs for the two different CD age groups and all CD patients revealed an optimal area under the curve of 1.0, respectively, indicating 100 % specificity and sensitivity regarding the cohorts used for ROC curve calculations. Remarkably, ULN, 97.5 % percentiles, and cutoffs obtained by ROC curve analysis demonstrated different values whereas the latter were at least 1.5 times higher than the respective ULN (Table 2).

Intra-assay and inter-assay coefficient of variation analysis

The intra-assay and inter-assay coefficients of variation (CV) were analyzed using reference sera with the

corresponding autoAb specificities. Intra-assay CV was determined by eight measurements for each serum while inter-assay CV was assessed by analyzing eight determinations for each serum on five different days in accordance with the CLSI protocol EP15-A2.

The intra-assay CV of the CytoBead CeliAK assay for CD-specific autoAb to TG2 and DG ranged from 1.5 to 9.5 % and inter-assay CV from 1.4 to 11.5 % (Table 3).

Analysis of IgA deficiency by CytoBead CeliAK

The presence of IgA deficiency was evaluated visually by the lack of a fluorescent signal (halo) on antihuman IgA-coated microbeads in the CytoBead CeliAK. All 5 IgA-deficient samples (IgA <0.2 g/L) were scored negative by visual evaluation. Out of the remaining 375 patient and control samples 374 revealed a positive fluorescence on the antihuman IgA microbeads. The patient suffering from ED and Dandy–Walker syndrome with negative fluorescence was confirmed by nephelometry as IgA deficient.

Quantitative IgA testing by CytoBead CeliAK demonstrated for all five IgA-deficient samples IgA levels below 0.2 g/L. Like visual examination, automated IgA analysis scored 374/375 patient and control samples as IgA sufficient with IgA levels above 0.2 g/L (Suppl. Fig. 1).

CD-specific autoAb analysis by CytoBead CeliAK

In total, 380 serum samples were analyzed with the CytoBead CeliAK assay for the detection of EmA on monkey-esophagus tissue sections by visual examination of an overview image created by the software and specific autoAb to TG2 and DG (Table 4). The latter autoAb testing was performed visually to obtain qualitative results and automatically for quantitative assessment. Thus, detection of EmA, TG2autoAb, and DGAAb revealed significantly different levels in the cohorts investigated whereas, as expected, patients with CD demonstrated significantly

Table 2 Upper limit of normal (ULN), 97.5 % percentiles and cutoffs for autoantibody (autoAb) testing to tissue transglutaminase 2 (TG2)

	ULN (U/mL)	97.5 % percentile (U/mL)	Cutoff (U/mL)
ED ($n = 68$)	1.65	1.94	3.91 ^a
BD ($n = 91$)	1.15	1.42	1.71 ^b
ED + BD ($n = 159$)	1.41	1.54	3.91 ^c

TG2autoAb were determined by CytoBead CeliAK in 130 pediatric/adolescent patients with celiac disease (CD) (<18 years) and 24 adult patients (≥ 18 years) as well as pediatric patients with eye disease (ED) and blood donors (BD) and subjected to receiver operating characteristics curve analysis. ULN (mean + 2 standard deviations), 97.5 % percentiles, and cutoffs were calculated. Total sample numbers can differ due to lack of human material or assay failure

^a ROC: CD <18 years vs ED

^b ROC: CD ≥ 18 years vs BD

^c ROC: CD vs ED + BD

Table 3 Assay performance of the CytoBead CeliAK assay

Day	TG2autoAb (U/mL)					DG Ab (U/mL)					Total IgA (g/L)				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Intra-assay variance															
	68.6	69.5	67.6	70.2	69.0	49.2	35.6	41.7	44.0	41.6	0.32	0.29	0.28	0.29	0.27
SD	4.3	1.8	2.6	3.2	1.1	3.4	3.4	2.6	1.8	2.9	0.02	0.02	0.02	0.02	0.02
CV %	6.3	2.6	3.8	4.5	1.5	6.9	9.5	6.2	4.0	6.9	6.2	6.9	7.1	6.8	7.4
Inter-assay variance days a–e															
	68.9					42.4					0.29				
SD	0.9					4.9					0.01				
CV %	1.4					11.5					6.4				

Autoantibodies (autoAb) to tissue transglutaminase type 2 (TG2) and deamidated gliadin (DG) as well total serum IgA were determined by CytoBead CeliAK in reference sera diluted 1–10. Intra-assay and inter-assay (8 times per run over 5 days) coefficients of variation (CV) were determined in accordance with the CLSI protocol EP15-A2

AU arbitrary units, SD standard deviation

Table 4 Prevalence of celiac disease (CD)-specific IgA autoantibodies (autoAb) by classical indirect immunofluorescence (IIF), enzyme-linked immunosorbent assay (ELISA) and multiplex CytoBead CeliAK analysis in 380 patients and controls

	CD <18 years <i>n</i> = 131	CD ≥18 years <i>n</i> = 24	CD <i>n</i> = 155	CF <i>n</i> = 59	ED <i>n</i> = 68	IgA deficiency <i>n</i> = 5	BD <i>n</i> = 93
IIF EmA	130/131 (99.2)	23/23 (100.0)	153/154 (99.4)	0/59 (0.0)	0/68 (0.0)	0/5 (0.0)	2/93 (2.2)
ELISA							
TG2autoAb	129/129 (100.0)	23/24 (95.8)	152/153 (99.3)	2/59 (3.4)	0/68 (0.0)	0/5 (0.0)	4/93 (4.3)
DGAb	103/130 (79.2)	16/24 (66.7)	119/154 (77.3)	2/59 (3.4)	1/68 (1.5)	0/5 (0.0)	6/93 (6.5)
At least 1 autoAb positive	129/129 (100.0)	23/23 (100.0)	152/153 (99.3)	2/59 (3.4)	1/68 (1.5)	0/5 (0.0)	6/93 (6.5)
CytoBead CeliAK visual							
IIF EmA	128/131 (97.7)	24/24 (100.0)	152/155 (98.1)	1/59 (1.7)	0/68 (0.0)	0/5 (0.0)	1/93 (1.1)
TG2autoAb	130/131 (99.2)	24/24 (100.0)	154/155 (99.4)	3/59 (5.1)	0/68 (0.0)	0/5 (0.0)	2/93 (2.2)
DGAb	102/131 (77.9)	22/24 (91.7)	124/155 (80.0)	3/59 (5.1)	0/68 (0.0)	0/5 (0.0)	4/93 (4.3)
At least 1 autoAb positive	130/131 (99.2)	24/24 (100.0)	154/155 (99.4)	3/59 (5.1)	0/68 (0.0)	0/5 (0.0)	4/93 (4.3)
Automated							
TG2autoAb	128/130 (98.5)	22/24 (91.7)	150/154 (97.4)	0/59 (0.0)	0/68 (0.0)	0/5 (0.0)	0/93 (0.0)
DGAb	97/129 (75.2)	18/24 (75.0)	115/153 (75.2)	3/58 (5.2)	0/67 (0.0)	0/5 (0.0)	3/93 (3.2)
At least 1 autoAb positive	128/130 (98.5)	24/24 (100.0)	152/155 (98.1)	3/58 (5.2)	0/67 (0.0)	0/5 (0.0)	3/93 (3.2)

Endomysial antibodies (EmA) as well as autoAb to transglutaminase type 2 (TG2) and deamidated gliadin (DG) were determined by classical IIF and ELISA, respectively. For comparison, these CD-specific autoAbs were analyzed by multiplex CytoBead testing including simultaneous EmA analysis on monkey-esophagus tissue sections as well as TG2 and DG autoAb determination using microbeads by either visual qualitative or automated quantitative AKLIDES[®] interpretation. Total sample numbers can differ due to lack of human material or assay failure

BD blood donors, CF cystic fibrosis, ED eye disease

higher levels than patients with ED and CF as well as BD (Kruskal–Wallis test, $p < 0.001$, respectively; see Suppl. Fig. 2).

Regarding TG2autoAb and DGAb prevalence, there was no statistical difference between visual qualitative and automated quantitative analysis by AKLIDES[®],

respectively. Furthermore, there was no statistical difference in EmA prevalences detected by visual classical and CytoBead reading. However, visual examination of fluorescence halos on the antigen-coated beads by visual observation revealed more positives for TG2autoAb (154/155 vs 150/154) and DGAb (124/155 vs 115/153) testing in

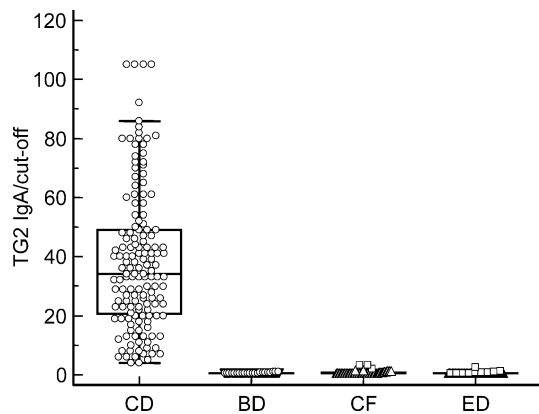


Fig. 3 Ratio of tissue transglutaminase type 2 (TG2) IgA autoantibody levels to their cutoff as upper limit of normal (ULN) obtained by CytoBead CeliAK in 379 patients and controls. *BD* blood donors, *CD* celiac disease, *CF* cystic fibrosis, *ED* eye disease

patients with CD. Of note, in control cohorts like patients with CF, visual examination of corresponding beads also revealed more positives (TG2autoAb 3/59 vs 0/59).

Employing the cutoff of 3.91 U/mL instead of ULN in accordance with the recommendations of the ESPGHAN, 101 (65.6 %) out of 154 patients with CD demonstrated a TG2autoAb cutoff ratio above 10 (Fig. 3). Of note, all controls revealed ratios below 10 for this cutoff value. In contrast, the highest ULN obtained for children with ED as controls was only 1.65 U/mL (Table 2). In this case, 134/154 (87.0 %) of CD patients revealed TG2autoAb levels $>10\times$ ULN whereas 13/220 (5.9 %) controls also did. At the lowest ULN of 1.15 U/mL obtained for BD as controls only, 140/154 (90.9 %) of CD patients demonstrated values $>10\times$ ULN. Both, the numbers of patients and controls with TG2autoAb levels $>10\times$ ULN compared to those with $>10\times$ cutoff, respectively, were significantly different using the cutoff obtained by ROC curve analysis and highest ULN ($p < 0.0002$; respectively).

Comparison of CytoBead CeliAK with classical IIF and ELISA

Comparing automated CD-specific autoAb testing by CytoBead CeliAK with classical IIF and ELISA analysis, a very good agreement was established for EmA, TG2autoAb, and DGAb determination (Cohen's $\kappa > 0.8$) (Table 5). There was a significant difference between CytoBead and classical testing for TG2autoAb only according to McNemar's statistics ($p = 0.0078$). Eight out of 372 (2.2 %) patients and controls revealed different TG2autoAb findings. All eight sera demonstrated negative TG2autoAb levels by CytoBead CeliAK and positive ones by classical ELISA. Out of these eight sera four belonged to patients with CD, two to patients with CF and two were

from BD. Three out of the four CD patients also demonstrated positive EmA findings both by CytoBead CeliAK and classical IIF.

Altogether, there was no significant difference in the diagnostic sensitivity (99.4 vs 98.1 %) and specificity (96.9 vs 97.3) taking into account positivity of at least one CD-specific autoAb by classical and CytoBead testing ($p > 0.05$, respectively).

Discussion

The new diagnostic guidelines presented by ESPGHAN recently recommend the detection of EmA, TG2autoAb, and DGAb for the serology of CD [8]. Accordingly, EmA and histology are still considered the reference methods for CD and EmA should be used as reference standard for the validation of CD-specific autoAb tests. Of note, for the first time, duodenal biopsies can be waived for the diagnosis of CD in children and adolescents provided TG2autoAb levels reach values $>10\times$ ULN, and patients demonstrate signs and symptoms suggestive for CD. Further laboratory testing including HLA typing of DQ2/DQ8 positivity or confirmation of autoAb positivity by EmA and response to gluten-free diet are requested. From a routine autoimmune laboratory perspective, this approach requires the detection of CD-specific autoAb by different techniques and, hence, creates constraints to run large numbers of samples cost-effectively. Furthermore, DGAb are recommended as additional CD-specific Ab in the serological workup of patients. Accumulating evidence indicates that in particular IgG DGAb appears to have an excellent positive predictive value and their combination with IgA TG2Ab can omit total IgA assessment [31]. Indeed, total IgA analysis is required by the guidelines since IgA deficiency is frequently found in patients with CD to prevent false-negative IgA autoAb findings.

Thus, there is obviously a demand for multiplex methods to analyze CD-specific autoAb and IgA deficiency simultaneously in one sample. To the best of our knowledge, the CytoBead technique based on digital fluorescence is the first assay technology available so far which provides the unique opportunity to combine autoAb testing employing tissue and purified antigenic targets in one reaction environment [28]. Thus, we developed the CytoBead CeliAK assay for the simultaneous detection of CD-specific IgA autoAb including the reference autoAb EmA and total IgA testing using this novel technology. The efficacy thereof has been demonstrated for similar laboratory demands of multiplex autoAb testing like antinuclear and antineutrophilic cytoplasmic Ab analyses recently [27, 28, 32]. Novel pattern recognition algorithms and digital fluorescence have paved the way for quantification and

Table 5 Comparison of celiac disease-specific IgA assessment by automated multiplex CytoBead CeliAK analysis and classical indirect immunofluorescence (IIF) as well as enzyme-linked immunosorbent assays (ELISA) in 376 patients and controls without IgA-deficient samples

CytoBead CeliAK	Classical IIF		Cohen's κ^a	95 % CI	Difference (%)	95 % CI	p^b
EmA	Pos	Neg	0.98	0.96–1.00	0.27	–0.65 to 0.79	1.0000
Pos	153	1					
Neg	2	217					
	ELISA						
TG2autoAb	Pos	Neg	0.96	0.92–0.99	2.15	0.56 to 2.15	0.0078
Pos	150	0					
Neg	8	214					
DGAb	Pos	Neg	0.85	0.79–0.91	0.55	–2.27 to 3.23	0.8388
Pos	108	13					
Neg	20	231					

Autoantibody, autoAb; DG, deamidated gliadin; EmA, endomysial antibody; Neg, negative; Pos, positive TG2 tissue transglutaminase type 2

^a Cohen's kappa ($\kappa \leq 0.2$ poor, 0.21–0.40 fair, 0.41–0.6 moderate, 0.61–0.8 good, 0.81–1.0 very good agreement)

^b McNemar's test

automation of autoAb testing through IIF interpretation systems [33–36]. This has ushered in a new era in the routine autoimmune laboratory for IIF testing with regard to improved standardization and modern data management [34, 36–38].

The comparison of CD-specific autoAb testing by CytoBead CeliAK with classical IIF and ELISA revealed a very good agreement for EmA and TG2autoAb (Cohen's $\kappa > 0.8$) as well as a good one for DGAb analysis. Thus, the serum dilution of 1 in 10 deployed for the CytoBead assay did not bring about an elevated rate of false-positive findings in the control groups and showed a satisfactory sensitivity for EmA. Other CytoBead reaction environments for the detection of autoAb deploying also lower dilutions than classical techniques like ELISA have demonstrated a similar performance [27]. As a fact, the difference between CytoBead and classical testing was only significant for TG2autoAb testing. The eight discrepant sera revealing TG2autoAb positivity by ELISA and negative results by CytoBead CeliAK belonged to four CD patients and four controls. Thus, IgA TG2autoAb analysis by CytoBead CeliAK seems to be an appropriate method to fulfill the requirements by the novel ESPGHAN diagnostic CD criteria to avoid duodenal biopsy. Indeed, a strong association of EmA with the presence of CD even irrespective of the EmA titre has been reported recently [39]. The majority of EmA-positive CD patients demonstrated IgA TG2autoAb levels above 10 \times ULN in our study. However, we found different values for ULN, corresponding 97.5 % percentiles in case of non-normal distribution, and cutoffs determined by ROC curve analysis. This resulted even in significantly different numbers of CD patients and controls with >10 \times ULN compared to those with >10 \times cutoff values, respectively. The novel

diagnostic criteria allow the use of ULN or cutoffs for this kind of evaluation which can, as shown in our study, lead to misinterpretations. The application of a common multiple of ULN in this respect has been criticized elsewhere calling into question the universal use of this approach [40]. Thus, we feel that the diagnostic criteria need a detailed definition thereof in a further update badly.

Instead of testing for IgA TG2autoAb preferentially as recommended by the new ESPGHAN guidelines, the simultaneous detection of EmA by CytoBead can be an aid in case of TG2autoAb findings leading to values below the respective 10 \times ULN or 10 \times cutoff. Of note, all controls demonstrated values below 10 \times cutoff in our study. Furthermore, IgA DGAb can further support the serological diagnosis of CD. The simultaneous detection of IgG DGAb, which was not demonstrated in this study, however, can be achieved by the use of antihuman IgG labeled to a different fluorescent dye and might even further strengthen this diagnosis as reported elsewhere [31].

Although there was no improvement of the already high sensitivity of single IgA CD-specific autoAb testing, the CytoBead technology appears to provide a unique cost-effective opportunity for multiplex parameter CD serology. Earlier attempts to multiplex CD-specific autoAb and total IgA analyses by line immunoassay did not provide quantitative autoAb testing [24]. To the best of our knowledge, the CytoBead CeliAK is the first multiplex quantitative IgA TG2autoAb and DGAb test which enables additional simultaneous EmA analysis as reference method and IgA deficiency testing. This comprehensive approach may improve the laboratory efficacy of CD serology. Of note, it can easily incorporate further CD-specific autoAb such as autoAbs to GP2, which can stratify CD patients [41–43].

Compliance with ethical standards

Conflicts of interest Dirk Roggenbuck has a management role and is a shareholder of GA Generic Assays GmbH and Medipan GmbH. Both companies are diagnostic manufacturers.

Ethical approval The study was approved by the Local Ethics Committee (EZ151052010) and conducted in accordance with the Helsinki declaration.

Human and animal rights All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent For this type of study formal consent is not required.

References

- Sollid LM (2002) Coeliac disease: dissecting a complex inflammatory disorder. *Nat Rev Immunol* 2:647–655
- Ludvigsson JF, Leffler DA, Bai JC, Biagi F, Fasano A, Green PHR, Hadjivassiliou M, Kaukinen K, Kelly CP, Leonard JN, Lundin KEA, Murray JA, Sanders DS, Walker MM, Zingone F, Ciacci C (2012) The Oslo definitions for coeliac disease and related terms. *Gut* 62:43–52
- Fasano A (2005) Clinical presentation of celiac disease in the pediatric population. *Gastroenterology* 128:S68–S73
- Sollid LM (2000) Molecular basis of celiac disease. *Annu Rev Immunol* 18:53–81
- Sollid LM, Jabri B (2013) Triggers and drivers of autoimmunity: lessons from coeliac disease. *Nat Rev Immunol* 13:294–302
- Walker MM, Murray JA, Ronkainen J, Aro P, Storskrubb T, D'Amato M, Lahr B, Talley NJ, Agreus L (2010) Detection of celiac disease and lymphocytic enteropathy by parallel serology and histopathology in a population-based study. *Gastroenterology* 139:112–119
- Porcelli B, Verdino V, Bossini L, Terzuoli L, Fagiolini A (2014) Celiac and non-celiac gluten sensitivity: a review on the association with schizophrenia and mood disorders. *Autoimmun Highlights* 5:55–61
- Husby S, Koletzko S, Korponay-Szabo IR, Mearin ML, Phillips A, Shamir R, Troncone R, Giersiepen K, Branski D, Catassi C, Leigeman M, Maki M, Ribes-Koninckx C, Ventura A, Zimmer KP (2012) European Society for Pediatric Gastroenterology, Hepatology, and Nutrition guidelines for the diagnosis of coeliac disease. *J Pediatr Gastroenterol Nutr* 54:136–160
- Mäki M, Mustalahti K, Kokkonen J, Kulmala P, Haapalahti M, Karttunen T, Ilonen J, Laurila K, Dahlbom I, Hanssom T, Höpfl P, Knip M (2003) Prevalence of celiac disease among children in Finland. *N Engl J Med* 25:2517–2524
- Johnston SD, Watson RG, McMillan SA, Sloan J, Love AH (1997) Prevalence of coeliac disease in Northern Ireland. *Lancet* 350:1370
- Volta U, Bellentani S, Bianchi FB, Brandi G, De FL, Miglioli L, Granito A, Balli F, Tiribelli C (2001) High prevalence of celiac disease in Italian general population. *Dig Dis Sci* 46:1500–1505
- Bizzaro N, Villalta D, Tonutti E, Tampoia M, Bassetti D, Tozzoli R (2003) Association of celiac disease with connective tissue diseases and autoimmune diseases of the digestive tract. *Autoimmun Rev* 2:358–363
- Tonutti E, Bizzaro N (2014) Diagnosis and classification of celiac disease and gluten sensitivity. *Autoimmun Rev* 13:472–476
- Chorzelski TP, Beutner EH, Sulej J, Tchorzewska H, Jablonska S, Kumar V, Kapuscinska A (1984) IgA anti-endomysium antibody. A new immunological marker of dermatitis herpetiformis and coeliac disease. *Br J Dermatol* 111:395–402
- Basso D, Guariso G, Fogar P, Meneghel A, Zambon CF, Navaglia F, Greco E, Schiavon S, Rugge M, Plebani M (2009) Antibodies against synthetic deamidated gliadin peptides for celiac disease diagnosis and follow-up in children. *Clin Chem* 55:150–157
- Dieterich W, Ehnis T, Bauer M, Donner P, Volta U, Riecken EO, Schuppan D (1997) Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nat Med* 3:797–801
- Dieterich W, Laag E, Schopper H, Volta U, Ferguson A, Gillett H, Riecken EO, Schuppan D (1998) Autoantibodies to tissue transglutaminase as predictors of celiac disease. *Gastroenterology* 115:1317–1321
- Cataldo F, Marino V, Ventura A, Bottaro G, Corazza GR (1998) Prevalence and clinical features of selective immunoglobulin A deficiency in coeliac disease: an Italian multicentre study. Italian Society of Paediatric Gastroenterology and Hepatology (SIGEP) and “Club del Tenue” Working Groups on Coeliac Disease. *Gut* 42:362–365
- Collin P, Maki M, Keyrilainen O, Hallstrom O, Reunala T, Pasternack A (1992) Selective IgA deficiency and coeliac disease. *Scand J Gastroenterol* 27:367–371
- Cunningham-Rundles C (2001) Physiology of IgA and IgA deficiency. *J Clin Immunol* 21:303–309
- Rittmeyer C, Rhoads JM (1996) IgA deficiency causes false-negative endomysial antibody results in celiac disease. *J Pediatr Gastroenterol Nutr* 23:504–506
- Lenhardt A, Plebani M, Marchetti F, Gerarduzzi T, Not T, Meini A, Villanacci V, Martelossi S, Ventura A (2004) Role of human-tissue transglutaminase IgG and anti-gliadin IgG antibodies in the diagnosis of coeliac disease in patients with selective immunoglobulin A deficiency. *Dig Liver Dis* 36:730–734
- Volta U, Villanacci V (2011) Celiac disease: diagnostic criteria in progress. *Cell Mol Immunol* 8:96–102
- Conrad K, Roggenbuck D, Ittenson A, Reinhold D, Buettner T, Laass MW (2012) A new dot immunoassay for simultaneous detection of celiac specific antibodies and IgA-deficiency. *Clin Chem Lab Med* 50:337–343
- Conrad K, Roggenbuck D, Reinhold D, Sack U (2012) Autoantibody diagnostics in clinical practice. *Autoimmun Rev* 11:207–211
- Porcelli B, Ferretti F, Vindigni C, Scapellato C, Terzuoli L (2011) Assessment of a combination screening assay for celiac disease. *Autoimmun Highlights* 2:67–71
- Sowa M, Grossmann K, Knutter I, Hiemann R, Rober N, Anderer U, Csernok E, Bogdanos DP, Borghi MO, Meroni PL, Schierack P, Reinhold D, Conrad K, Roggenbuck D (2014) Simultaneous automated screening and confirmatory testing for vasculitis-specific ANCA. *PLoS One* 9:e107743
- Sowa M, Großmann K, Scholz J, Röber N, Rödiger S, Schierack P, Conrad K, Roggenbuck D, Hiemann R (2014) Der CytoBead-assay—Eine neue Möglichkeit der multiparametrischen Autoantikörperanalytik bei systemischen Autoimmunerkrankungen. *J Lab Med* 38(6):309–317
- Willitzki A, Hiemann R, Peters V, Sack U, Schierack P, Rödiger S, Anderer U, Conrad K, Bogdanos D, Reinhold D, Roggenbuck D (2012) New platform technology for comprehensive serological diagnostics of autoimmune diseases. *Clin Dev Immunol* 2012:284740
- Hiemann R, Hilger N, Sack U, Weigert M (2006) Objective quality evaluation of fluorescence images to optimize automatic image acquisition. *Cytometry A* 69:182–184

31. Wolf J, Hasenclever D, Petroff D, Richter T, Uhlig HH, Laabeta MW, Hauer A, Stern M, Bossuyt X, de Laffolie J, Flemming G, Villalta D, Schlumberger W, Mothes T (2014) Antibodies in the diagnosis of coeliac disease: a biopsy-controlled, international, multicentre study of 376 children with coeliac disease and 695 controls. *PLoS One* 9:e97853
32. Scholz J, Grossmann K, Knutter I, Hiemann R, Sowa M, Rober N, Rödiger S, Schierack P, Reinhold D, Bogdanos DP, Meroni PL, Radice A, Conrad K, Roggenbuck D (2015) Second generation analysis of antinuclear antibody (ANA) by combination of screening and confirmatory testing. *Clin Chem Lab Med* 53(12):1991–2002
33. Knutter I, Hiemann R, Brumma T, Buttner T, Grossmann K, Cusini M, Pregolato F, Borghi MO, Anderer U, Conrad K, Reinhold D, Roggenbuck D, Csernok E (2012) Automated interpretation of ANCA patterns—a new approach in the serology of ANCA-associated vasculitis. *Arthritis Res Ther* 14:R271
34. Mahler M, Meroni PL, Bossuyt X, Fritzler MJ (2014) Current concepts and future directions for the assessment of autoantibodies to cellular antigens referred to as anti-nuclear antibodies. *J Immunol Res* 2014:315179
35. Bizzaro N, Antico A, Platzgummer S, Tonutti E, Bassetti D, Pesente F, Tozzoli R, Tampoia M, Villalta D (2014) Automated antinuclear immunofluorescence antibody screening: a comparative study of six computer-aided diagnostic systems. *Autoimmun Rev* 13:292–298
36. Tozzoli R, Antico A, Porcelli B, Bassetti D (2012) Automation in indirect immunofluorescence testing: a new step in the evolution of the autoimmunology laboratory. *Autoimmun Highlights* 3:59–65
37. Roggenbuck D, Hiemann R, Bogdanos D, Reinhold D, Conrad K (2013) Standardization of automated interpretation of immunofluorescence tests. *Clin Chim Acta* 421:168–169
38. Roggenbuck D, Hiemann R, Schierack P, Reinhold D, Conrad K (2014) Digital immunofluorescence enables automated detection of antinuclear antibody endpoint titers avoiding serial dilution. *Clin Chem Lab Med* 52:e9–e11
39. Kurppa K, Rasanen T, Collin P, Iltanen S, Huhtala H, Ashorn M, Saavalainen P, Haimila K, Partanen J, Maki M, Kaukinen K (2012) Endomysial antibodies predict celiac disease irrespective of the titers or clinical presentation. *World J Gastroenterol* 18:2511–2516
40. Egner W, Shrimpton A, Sargur R, Patel D, Swallow K (2012) ESPGHAN guidance on coeliac disease 2012: multiples of ULN for decision making do not harmonise assay performance across centres. *J Pediatr Gastroenterol Nutr* 55:733–735
41. Roggenbuck D, Vermeire S, Hoffman I, Reinhold D, Schierack P, Goihl A, von Arnim U, De Hertogh G, Polymeros D, Bogdanos DP, Bossuyt X (2015) Evidence of Crohn's disease-related anti-glycoprotein 2 antibodies in patients with celiac disease. *Clin Chem Lab Med* 53:1349–1357
42. Laass MW, Rober N, Range U, Noss L, Roggenbuck D, Conrad K (2015) Loss and gain of tolerance to pancreatic glycoprotein 2 in celiac disease. *PLoS One* 10:e0128104
43. Gross S, Bakker SF, van Bodegraven AA, van Hoogstraten IM, Gelderman KA, Bouma G, Mulder CJ, von Blomberg ME, Bontkes HJ (2014) Increased IgA glycoprotein-2 specific antibody titres in refractory CD. *J Gastrointest Liver Dis* 23(2):1–7