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A multiplex assay combining insulin, GAD, 1A-2 and transglutaminase autoantibodies to facilitate screening for pre-type 1 diabetes and celiac disease

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Abstract

At the current time, multiple candidate interventions are being proposed to abrogate or slow progression to type 1 diabetes (T1D) among islet autoantibody (iAb) positive subjects, but mass screening for eligible subjects and the general population remains a laborious and inefficient process. We have recently developed and extensively validated nonradioactive iAb assays using electrochemiluminescence (ECL) detection with an excellent sensitivity and specificity compared to the gold-standard radioassays. Using ECL detection on a platform from MesoScale Discovery (MSD) allows the measurement of four antibodies in a single well using a small blood volume (6 µl). In the present study using a MSD QuickPlex 4-Spot plate, we successfully combined three iAb to insulin (IAA), GAD65 (GADA), and IA-2 (IA-2A) with tissue transglutaminase autoantibodies (TGA) in a single well of a 96 well plate. We tested 40 new onset T1D patients, all positive for at least one iAb and a half of them positive for TGA by radioassay, as well as 50 healthy controls. The multiplex assay retained 100% sensitivity and 100% specificity for all four autoantibodies in terms of positivity identified in patients versus normal controls compared to the corresponding standard radioassays and our single ECL assays. The multiplex ECL assay was able to identify more positivity than current radioassays for IAA and TGA. The development of this multiplex assay will facilitate high-throughput screening for T1D and celiac disease risk in the general population.

Keywords

Autoantibodies; Assay; Diabetes; Celiac disease

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Author contributions

L.Y. wrote manuscript, contributed to discussion. D.M. and Z.Z. researched data. A.M., A. S. and M.R. contributed to discussion, reviewed/edited manuscript. L.Y. takes full responsibility for the contents of the article.

1. Introduction

The incidence of type 1 diabetes (T1D), the immune-mediated form of diabetes (Atkinson et al., 2014), has doubled in the last 20 years (Anon, 2008), especially in young children. T1D affects an estimated 1.4 million people in the U.S. alone with an equal number of people with preclinical disease characterized by multiple islet autoantibodies (iAbs), but still normal glucose homeostasis. The presence of iAbs, their number (Bingley, 2010; Yu et al., 2012a; Steck et al., 2011) and levels (Sosenko et al., 2011, 2013; Vehik et al., 2011), are currently used to stage diabetes risk and as inclusion criteria into prevention trials (Schlosser et al., 2010). Nearly all children positive for two or more iAbs, against insulin (IAA), glutamic acid decarboxylase (GADA), islet antigen 2 (IA-2A), or zinc transporter 8 (ZnT8A), develop clinical T1D (Ziegler et al., 2013). When identified prior to the onset of symptoms, these children can avoid life-threatening diabetic ketoacidosis and hospitalization as well as participate in trials to prevent T1D or studies to define the causes of T1D.

The prevalence of celiac disease is ~1:100 in Europe (Lohi et al., 2007) and North America (Fasano et al., 2003); however, most patients are undiagnosed or diagnosed with significant delay. Gluten-free diet is an effective treatment and early detection by measuring transglutaminase autoantibodies (TGA) has been widely recommended (Husby et al., 2012). Celiac disease and T1D share HLA Class II and non-HLA genetic susceptibility and co-occur in up to 10% of the patients (Bao et al., 1999). The American Diabetes Association recommends routine screening for celiac disease at diagnosis of T1D (Chiang et al., 2014). Combined population screening for pre-clinical T1D and celiac disease would be a rational pairing.

Preventive trials for T1D are underway and likely to expand to multiple candidate interventions; however, mass screening for eligible subjects remains a laborious bottleneck. The goal of this study was to multiplex previously extensively validated (Yu et al., 2012b, 2013; Miao et al., 2013, 2014) IAA, GADA, IA-2A and TGA assays using electrochemiluminescence (ECL) detection to simultaneously screen for risk of T1D and celiac disease, which would be more efficient for mass screening of large population.

2. Materials and methods

2.1. Subjects

Serum samples from 40 newly diagnosed T1D patients were randomly selected from the Barbara Davis Center for Childhood Diabetes (mean age of 11.2 years with median age of 10.8 and male/female: 23/17) and 50 healthy controls (mean age of 11.9 years with median age of 11.2 and male/female: 28/22). All T1D patients were positive for at least one iAb by a standard radioassay and 19/40 were TGA positive by radioassay. The healthy controls were negative for all iAbs and TGA by radioassay. Signed written informed consents were obtained from participants and the study was approved by the Colorado Multiple Institutional Review Board.

2.2. Four autoantibody multiplex ECL assay

Previously described individual ECL IAA (Yu et al., 2012b, 2013) and GADA (Miao et al., 2013) assays were adapted to measure four antibodies as illustrated in Fig. 1. Briefly, 12 μ l of patient serum (the amount sufficient for duplicate measurement) was mixed with 14.5 μ l of 500 mM of acetic acid which is necessary for IAA determination. After incubation for 45 min at room temperature, 25 μ l of the acid treated serum solution was transferred to a 96 well plate with freshly prepared antigen/neutralization solution consisting of 8.3 μ l of 1 M Tris-HCl (pH = 9.0) and 35 μ l of labeled antigen mixture (Sulfo-TAG and linker-A labeled proinsulin at concentration of 100 ng/ml; Sulfo-TAG and linker-B labeled IA-2 at the concentration of 125 and 250 ng/ml respectively; Sulfo-TAG and linker-C labeled GAD65 at the concentration of 125 and 500 ng/ml respectively; Sulfo-TAG and biotin labeled transglutaminase at the concentration of 100 and 400 ng/ml respectively) in PBS with 5% BSA (v/v). The mixture was incubated at room temperature for 2 h with agitation followed by incubation at 4°C overnight (> 16 h). On the same day, QuickPlex 4-Spot plates were blocked with 150 μ l of 3% Blocker A (MSD) per well overnight at 4°C. On the second day, the blocked 4-Spot plate was washed with PBST (PBS with 0.05% Tween-20) 3 times followed by addition of the overnight incubated serum mixture and the component from each well were divided into two wells, 30 μ l per well on a 4-Spot plate. After incubation at room temperature for 1 h followed by 3 washes with PBST to remove excess labeled antigens, 150 μ l/well of 2 $\times$ Read buffer (MSD) were added and the plate was counted on a MSD Sector Imager 2400 (MSD, Rockville, MD). A mouse monoclonal insulin antibody-125 (kindly provided by Dr. Tom Thomas of Vanderbilt), a mouse monoclonal GAD65 antibody (GAD-6, Abcam, Cambridge, MA), a standard internal IA-2A positive serum, and a standard internal TGA positive serum were used as the assay internal standard positive controls for the 4 autoantibodies, respectively, and the results for all 4 autoantibodies were expressed as an index ($\text{index} = [\text{Signal}_{\text{sample}} - \text{Signal}_{\text{NegativeControl}}] / [\text{Signal}_{\text{PositiveControl}} - \text{Signal}_{\text{NegativeControl}}]$). All 4 single ECL assay cutoffs were based on 99th percentile except for IA-2A (100th percentile) of 100 normal controls. The cutoffs for 4 single ECL assays are 0.006, 0.023, 0.010, and 0.015 for IAA, GADA, IA-2A, and TGA, respectively. In the multiplex assay with all 100th percentile of 50 normal controls, 4 antibody cutoffs were 0.006, 0.019, 0.009, and 0.010 for IAA, GADA, IA-2A, and TGA, respectively. The cutoff values from multiplex assay were either the same or little lower than their single assays, but very close in general. Considering inter-assay variation and fewer normal controls used in the multiplex assay, we, in a more conservative way, adopted the same cutoffs for the multiplex assay from their corresponding single assays. The sensitivity and specificity of our single ECL iAb assays for T1D in the 2015 Islet Autoantibody Standard Program (IASP) Workshop were among the top performers: 60% and 98% respectively for IAA; 78% and 96% respectively for GADA; 72% and 98% respectively for IA-2A.

2.3. Radioassay

The radioassays for mIAA, GADA, IA-2A, and TGA used in the present study were all performed in our laboratory as previously described (Bao et al., 1999; Yu et al., 2000; Bonifacio et al., 2010). The radioassay cutoffs were set at the 99th percentile of 500 normal

control samples for GADA and IA-2A and 106 controls for mIAA, respectively. The cutoff for the TGA radioassay was set at 100th percentile of 184 normal control samples.

2.4. Statistics

Statistical analyses were performed using correlation analysis, rank sum or Fisher's exact test in PRISM 6.0 software (GraphPad Software Inc., San Diego, CA). A p-value <0.05 was considered statistically significant.

3. Results

Of the 40 newly diagnosed patients with T1D, 19, 31, 24 and 19 were positive for, respectively, mIAA, GADA, IA-2A, and TGA. The ECL multiplex assay detected IAA, GADA, IA-2A, and TGA in, respectively, 26, 31, 26 and 31 subjects. Thus, the ECL multiplex assay detected mIAA, IA-2, and TGA (but not GADA) in more patients than the radioassay. The increase of positivity in patients was especially evident for IAA (26 versus 19; $p = 0.17$) and TGA (31 versus 19; $p = 0.01$). All control samples tested negative for all iAbs and TGA in the ECL multiplex assay.

The levels of autoantibodies in the ECL multiplex assay correlated well with the corresponding single-antibody radioassays (Fig. 2) even though ECL-IAA and ECL-TGA assays detected more positives.

To evaluate a potential signal interference from neighboring spots, we compared each antibody measurement in the multiplex ECL assay to an individual antibody ECL assay (Fig. 3). Only 3/160 paired comparisons showed discrepancies that could be attributed to signal interference: two for IAA (Panel A) and one for IA-2A (Panel B). All three positive results in the multiplex ECL assay corresponding to negative single ECL antibody assays were at a low level and were accompanying another high autoantibody signal in the same well. These three low level positives were very likely false-positives in the multiplex ECL assay caused by signal interference.

4. Discussion

The novel multiplex autoantibody assay reported in this communication represents a major progress towards simplification of a large-scale population screening for pre-T1D and celiac disease. The ECL-based multiplex assay has a number of attractive features, compared with a combination of the current "gold standard" single autoantibody assays used for TrialNet, The Environmental Determinants of Diabetes in the Young (TEDDY), Immune Tolerance Network (ITN), Type 1 Diabetes Genetic Consortium (T1DGC), the Diabetes Autoimmunity Study in the Young (DAISY) and other studies (Table 1). The assay condition of current multiplex ECL assay presented will need to be further optimized and large scale validation will be needed before this assay can be applied for mass screening.

Using a QuickPlex 4-Spot plate with separate linkers for each labeled antigen, four different autoantibody assays can be accommodated in a single well with a small amount of serum sample (6 μ l). The sensitivity and specificity of the multiplex ECL assay was comparable to

the gold standard radioassay measurements in terms of positivity in patients versus normal controls and more positives were identified in patients regarding IAA, which is similar to our previous individual results on ECL-IAA (Yu et al., 2012b, 2013). The single ECL assays for IAA and GADA, previously reported from our laboratory (Yu et al., 2012b, 2013; Miao et al., 2013, 2014) have been extensively validated in samples obtained from DAISY, TrialNet Pathway to Prevention, and very recently TEDDY study. ECL-IAA is superior to the current standard mIAA radioassay for its higher sensitivity and earlier identification of iAb seroconversion among young children (Yu et al., 2013). One of the reasons may be the ECL assays ability to detect autoantibodies in all immunoglobulin classes, including IgM. Our recently developed single ECL-TGA assay is also more sensitive than the standard TGA (IgA) radioassay and can identify TGA seroconversion earlier than the TGA radioassay among DAISY young children who converted to TGA positivity and were confirmed with clinical celiac disease by biopsy (unpublished data). Remarkably, both ECL-IAA and ECL-GADA assays are able to discriminate high-affinity, high-risk autoantibodies from those “low risk”, low-affinity signals in subjects who have not progressed to T1D (Yu et al., 2012b, 2013; Miao et al., 2013, 2014).

The assay conditions in the present multiplex ECL assay study were based on our established single ECL assay protocols with the exception that the serum samples were treated with acid as this improves IAA detection but does not affect the results of the other three autoantibody assays (GADA, IA-2A, and TGA). In general, the background and signals of one autoantibody did not interfere with autoantibody measurements of neighboring spots in a certain range. We did note three false positive results (out of 160 measurements), compared to single ECL measurements. Two false positive IAA results were likely caused by interference of very high signals (both above 25,000 counts) from neighboring spots. One false positive IA-2A level in the combined assay was also likely due to an extremely high signal (>40,000 counts) from its neighboring spot. We noticed that signals of less than 20,000 counts did not interfere with each other. Further work is required to optimize the multiplex assay conditions to avoid signal interference among antibodies in samples that have a very high level of one antibody. From our previous experiences in developing these ECL assays, optimization will likely require adjusting the ratio and amounts of two differently labeled antigen proteins. Alternatively, samples with a very high signal could be retested using single autoantibody assays if optimization is not ideal; however, we estimate that re-testing will be required in about 0.2% of samples positive for an iAb in light of general population screening, which would be tolerable.

As expected, the multiplex ECL assay did not lose iAb positivity among the new onset T1D patients compared to the radioassay results. Furthermore, the multiplex assay maintained perfect specificity in 50 healthy controls for all 4 autoantibodies.

An ELISA-based ElisaRSR™ 3 Screen ICA™ is now available from the RSR Limited (Cardiff, the U.K.). This combination assay detects GADA, IA-2A and ZnT8A, in separate wells, and is now available for research use only. It is acknowledgeable that the method with ELISA-based technology is more easily widespread to other laboratories in terms of equipment and expertise. The major advantages of our ECL multiplex assay compared with the 3 Screen ICA™ assay include: 1) ability to detect IAA — the primary iAb in children; 2)

ability to screen for two diseases — pre-T1D and celiac disease; and 3) much smaller required serum volume, for duplicate measurements — 12 µl vs. 150 µl. Both methods are radioactivity-free and high-throughput.

With the limitation of present multiplex assay, we did not include ZnT8A in the multiplex assay for two reasons. First, ZnT8A alone is present in only 1% of subjects followed to clinical diabetes (Yu et al., 2012c). In two large studies, TrialNet and TEDDY, ZnT8A is not used for initial screening and the ZnT8A assay is only performed if another iAb is positive. Second, we wanted to be able to screen for both islet autoimmunity and celiac disease risk. Celiac disease is present in up to 10% of T1D patients, making this a rationale pairing of autoimmune disorders. Although using anti-gliadin autoantibodies (AGA) in combination with TGA might slightly increase the sensitivity, but it will generate more false positivity (Benson et al., 2013) and only single TGA screening for celiac disease is recommended (AGA Institute, 2006). It remains to be established how many of the TGA positivities found in T1D patients by the multiplex-assay match with a biopsy proven clinically diagnosis of celiac disease. Interestingly, all TEDDY study participants are screened for TGA and have a higher incidence of TGA than iAb (Liu et al., 2014). In our recently completed pilot study of the general population screening for iAbs and TGA, based in general pediatric care offices in the Denver metro area, we also found TGA prevalence to be higher than that of iAbs (Bautista et al., 2015). In addition, participating parents and pediatric providers ranked the combined screening for iAbs and TGA as more valuable and attractive than screening for iAbs alone.

In conclusion, by using ECL detection on a platform from MesoScale Discovery we have developed a multiplex assay to accurately measure all four autoantibodies in a single well using a small blood volume, which will facilitate large-scale, general population screening simultaneously for T1D and celiac disease risk. Further validation of this multiplex ECL assay using already collected samples from prospective studies will help to optimize it for large-scale population screening.

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ECL 4-Ab Multiplex Assay on 4-Spot Plate

Fig. 1.

Illustration of the multiplex ECL assay. The autoantibodies in serum will link each Sulfo-tagged antigen to its corresponding linker labeled antigen, respectively, which will be captured on the solid phase of the 4-Spot plate. Detection of plate-captured Sulfo-tagged antigen is accomplished with electrochemiluminescence.

Fig. 2.

The levels of four autoantibodies between multiplex ECL assay and radioassay were compared among 40 newly diagnosed patients with T1D (A) IAA, (B) GADA (C) IA-2A, and (D) TGA. The two assays were correlated ($p < 0.0001$), but ECL-IAA and ECL-TGA assays detected more positives than radioassay in patients with 100% specificity in 50 healthy controls.

Fig. 3.

The levels of four autoantibodies between multiplex ECL assay and single ECL assay were compared among 40 newly diagnosed patients with T1D (A) IAA, (B) GADA (C) IA-2A, and (D) TGA. The two assays had an excellent correlation ($p < 0.0001$) while two low IAA and one low IA-2A in multiplex ECL assay negative in single ECL assay were likely false positives caused by signal interference of very high signal from another antibody.

Table 1

Individual radioassays and ECL multiplex assay.

	Individual radioassays for mIAA, GADA, IA-2, TGA	ECL multiplex assay for IAA, GADA, IA-2, TGA
Serum volume required (duplicate testing)	41 µl	12 µl
Radioactive reagents/waste	Yes	No
Cost (reagents and labor)	100	30 ^a
High-throughput	150 samples/instrument/day	500 samples/instrument/day
Immunoglobulins detected	IgG only for iAbs IgA only for TGA	IgM, IgG, IgA, IgD, IgE

^a30% of the cost per sample compared to individual radioassays for all 4 antibody tests.