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A novel triple mix radiobinding assay for the three ZnT8 (ZnT8-RWQ) autoantibody variants in children with newly diagnosed diabetes

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1. Introduction

The identification of islet autoantibodies has important implications in the diagnosis and prediction of type 1 diabetes mellitus (T1D). Autoantibodies directed against islet autoantigens such as insulin, glutamic acid decarboxylase 65 (GAD 65) and islet antigen-2 (IA-2) have been demonstrated to be markers of the islet autoimmunity that precede clinical onset of T1D (Kulmala et al., 1998; Krischer et al., 2003). These autoantibodies were able to predict T1D in most but not all of the patients. The recent description of zinc transporter 8 (ZnT8) as an islet autoantigen in T1D was therefore important since adding ZnT8 autoantibodies (ZnT8A) may improve the accuracy of prediction (Wenzlau et al., 2008a).

ZnT8 is one of ten members of efflux zinc transporters, belonging to the *SLC30A8* gene family (solute carrier family 30), (Chimienti et al., 2005). ZnT8 represents a membrane protein of islet beta-cell secreting granulae and consists of 369 amino acids with a molecular

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8. Conflict of interests

The authors declare no conflicts of interests.

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weight of 41 kDa (Seve et al., 2004). The single nucleotide polymorphism (SNP) rs13266634 at position 325 of ZnT8 causes an amino acid replacement from arginine (CGG) to tryptophan (TGG). Furthermore, another SNP (rs16889462) at the same position codes for glutamine (CAG) (Wenzlau et al., 2009).

ZnT8A were detected in 63% of newly diagnosed T1D patients, but also in 3% of T2D patients and 3% of healthy controls (Wenzlau et al., 2008b). These autoantibodies were also described to predict T1D in children born to T1D mothers and carrying high HLA genetic risk (Achenbach et al., 2009) as well as in first-degree relatives of T1D patients (De Grijse et al., 2010). These studies primarily analyzed ZnT8A against arginine (ZnT8-R) and tryptophan (ZnT8-W) variants while less information are available for the glutamine (ZnT8-Q) variant (Wenzlau et al., 2009). Relating the disease risk to the single amino acid polymorphism that produces three distinct variants (ZnT8-R, ZnT8-W or ZnT8-Q), which are detected by ZnT8A alone or in combinations is a challenge to precise and reproducible autoantibody assays. Most islet autoantibody assays for screening are radiobinding assays (RBA) and the radioactive tracer is produced by *in vitro* transcription-translation of autoantigen cDNA (Grubin et al. 1994). Several plasmids have been reported to accommodate ZnT8-R or ZnT8-W variants alone (Achenbach et al., 2009) or in tandem with more than one autoantigen in the cDNA, including a CW-CR construct (Kawasaki et al., 2008; Achenbach et al., 2009). To our knowledge we are the only laboratory that has all three variants (ZnT8-Q, ZnT8-R and ZnT8-W) mixed as separate constructs in a single assay. Other laboratories may have all three ZnT8-Q-R-W in one construct or a combination of two (dimers). These differences in combination assays might confer that sensitivity and specificity could not be directly compared between laboratories using various constructs. It is also not clear to what extent patients develop ZnT8A, which are mono-specific i.e. reactive only with one variant ZnT8-R, ZnT8-W or ZnT8-Q, or bi-specific (e.g. ZnT8-R-W and ZnT8-R-Q) or poly-reactive (ZnT8-R-W-Q).

The aims of the present study were to 1) re-clone the ZnT8-R cDNA into a high efficiency *in vitro* transcription-translation plasmid; 2) subject the ZnT8-R cDNA to site-directed mutagenesis to generate the ZnT8-W and ZnT8-Q variants, 3) test an autoantigen triple mix RBA (ZnT8-TripleA) to screen for autoantibodies against any of the three variants; 4) dissect the individual ZnT8-R, ZnT8-W and ZnT8-Q reactivity and 5) compare results for the ZnT8-TripleA assay with those for individual autoantibodies in controls and newly diagnosed diabetes among children and adolescents.

2. Material and Methods

2.1. Patients

A total of 2664 newly diagnosed patients with childhood diabetes (onset <18 years, 55% males, n=1470/2664) were recruited from the Better Diabetes Diagnosis (BDD) study (Delli et al. 2010), during the period May 2005 to August 2009. Serum samples were obtained at diagnosis for analysis of islet autoantibodies. The classification of diabetes was clinically confirmed six months after onset with T1D (n=2582; 97%), T2D (n=46; 1.7%), MODY (n=28; 1.0%) and secondary (due to treatment such as corticosteroids or other illnesses such as cystic fibrosis) diabetes (n=8; 0.3%) (Table 1).

The Regional Ethics Review Board in Stockholm approved the BDD study.

2.2. Controls

Sera from 613 healthy controls were analyzed for the three individual ZnT8A variants and with the ZnT8-TripleA assay. A total of 214 (50% males, n=107) were Swedish school children aged 11–13 years (mean age 12) described in detail elsewhere (Lindberg et al.,

1999a; Lindberg et al., 1999b; Agardh et al., 2001). The remaining controls were 399 healthy adult blood donors (65% males, n=259) aged 19–81 (mean age 44.3).

2.3. Preparation of the ZnT8 autoantigen variants

The C-Terminal ZnT8-R with Arg at position 325—The C-terminal cDNA construct (Arg325) from human islet ZnT8 (SLC30A8) cDNA, which was cloned into pcDNA3.1 directional TOPO vector (kit 45–0158; Invitrogen, Carlsbad, CA) as described (Wenzlau et al., 2007) was a kind gift from John C. Hutton (Barbara Davis Center for Childhood Diabetes, University of Colorado at Denver and Health Sciences Center, Aurora, CO).

Preparation of the pThZnT8-R vector—The pTnTTM vector (Promega, Madison, WI), which was designed with multiple cloning sites to support highly efficient expression of cloned genes, was used for *in vitro* coupled transcription translation system in the presence of ³⁵S-methionine (Vaziri-Sani et al., 2010). The C-terminal fragment with Arg at position 325 was cut from the pcDNA3.1 vector (2 µg) with KpnI (FastDigestTM, Fermentas GMBH, Helsingborg, Sweden) and XbaI (New England Biolabs, Inc. Herts, United Kingdom) using the restriction buffer, NEB2 10x (New England Biolabs, Inc.). Following 2.5 h digestion at 37°C, the pTnTTM vector was dephosphorylated with Calf Intestinal Alkaline Phosphatase (Fermentas) at a final concentration of 0.05 U/picomole of DNA termini according to instructions from the supplier. The linearized pTnTTM vector and ZnT8-R construct were separated and analyzed by gel electrophoresis in 1% agarose and the two bands extracted using a gel purification kit (QIAquick Gel Extraction Kit, QIAGEN AB, Solna, Sweden) according to the manufacturer's instructions. The ligation reaction with T4 ligase (New England Biolabs, Inc.), optimized to 1:1–1:5 vector: insert molar ratio was carried out at room temperature for 2 h. Subcloning efficiency DH5α competent cells were used for transformation according to manufacturer's instructions (Invitrogen AB, Stockholm, Sweden). The ligation reaction (5 µL) was added to the cells (100 µL), mixed gently, and after 30 min incubation on ice, the cells were heat shocked for 90 s in a 42°C water bath. The cells were immediately put on ice for 2 min, and then incubated for 1 h at 37°C in 500 µL LB medium with shaking (300 rpm). A total of 200 µL cell suspension were plated onto LB/ampicillin plates and incubated overnight at 37°C. White colonies were selected and transferred separately to a new set of LB/ampicillin plates for overnight culture before the cells were incubated for 16 h at 37°C in 5 mL LB/ampicillin medium. The bacterial cells were harvested by centrifugation for 20 min at 4°C at 3041xg. The pThZnT8-R plasmid DNA was extracted using the QiaPrep Spin MiniPrep Kit (QIAGEN AB). The insert was sequenced by RSKC (Region Skånes Kompetens Centrum, Malmö, Sweden) using a 3700/3730 BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Foster City, CA). The 5'-TTA CGC CAG CCC GGA TCC-3' and 5'-AAG GCT AGA GTA CTT AAT ACG A-3' were the reverse and forward primers, respectively (DNA Technology A/S, Risskov, Denmark).

Generation of pThZnT8-W and pThZnT8-Q by site-directed mutagenesis—A PhusionTM site-directed mutagenesis kit (Finnzymes Oy, Espoo, Finland) was used to generate the C-terminal constructs (Trp325, ZnT8-W and Gln325, ZnT8-Q) from the pThZnT8-R plasmid. Mutagenic oligonucleotides representing two phosphorylated primers were designed according to manufacturer's instructions (DNA Technology A/S) as forward primers, which includes the one base change to introduce the desired mutations for pThZnT8-W (5'-CT ACA GCA GCC AGC TGG GAC AGC CAA GTG-3') and pThZnT8-Q (5'-CT ACA GCA GCC AGC CAG GAC AGC CAA GTG-3'), respectively. The transformation was carried out in subcloning efficiency DH5α competent cells (Invitrogen) and the plasmid DNA was extracted with the QiaPrep Spin MiniPrep Kit (QIAGEN AB). The pThZnT8-W and pThZnT8-Q insert sequences were determined as described above.

Coupled *in vitro* transcription-translation of the three plasmids (pThZnT8-R, pThZnT8-W and pThZnT8-Q)—The coupled *in vitro* transcription translation of the pCDNA3.1 and the pThZnT8-R, pThZnT8-W and pThZnT8-Q vectors were performed in a reaction mixture containing 2 µg of any of the four vectors, 50 µL TNT® rabbit reticulocyte lysate, 4 µL TNT® reaction buffer, 2 µL amino acid mixture without methionine, 2 µL RNasin® Ribonuclease inhibitor, 2 µL SP6 RNA Polymerase, (all from Promega), 4 µL ³⁵S-methionine (>1,000 Ci/mmol from Amersham Int., Amersham, Bucks., UK) and nuclease-free water to a final volume of 100 µL. The reaction mixture was incubated for 90 min at 30°C with shaking (300 rpm in an Eppendorf Thermomixer comfort, Eppendorf, AG Hamburg, Germany). The translation product was immediately subjected to gel filtration on Illustra NAP-5 Columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and radioactivity incorporated into protein was determined (1450 MicroBeta TriLux Microplate Scintillation-Luminescence Counter, PerkinElmer Life and Analytical Sciences, Shelton, CT).

Electrophoresis of ZnT8 vector products—The labeled antigen produced with the coupled *in vitro* transcription translation kit was analyzed by SDS-PAGE gel electrophoresis (ClearPage™ precast 8%, VWR, West Chester, PA, according to manufacturers' recommendations) to verify the specific translation products. The samples were incubated with sample buffer and reducing agent (ClearPage™ accessories, VWR) for 3 min at 100°C and were loaded on the gel with a [¹⁴C] labeled MW-marker (Promega, Madison, WI). The gel was dried for 72 h at room temperature using a Gel Drying Kit (Promega) according to the manufacturers' instructions. The gel was placed in contact with an X-ray sensitive film for 72 h at room temperature. The film was developed and fixed in DABdental developing and fixating solutions (DABdental, Malmö, Sweden) following the manufacturer's instructions.

2.4. Radiobinding Assay (RBA)

RBA for GAD65A, IA-2A and IAA—These autoantibodies were analyzed as described previously (Lynch et al., 2008).

RBA for ZnT8-R, ZnT8-W and ZnT8-Q autoantibodies—The RBA for each individual ZnT8 autoantibody was carried out over night at 4°C in duplicate samples of 5 µL of serum incubated with 60 µL labeled antigen at a final concentration of 425±25 cpm/µL after dilution in antigen buffer (150 mmol/L NaCl, 20 mmol/L Tris, pH 7.4, 0.15% (v/v) Tween 20, 0.1% (w/v) BSA). V-formed 96-well plates (catalog nr 442587, MicroWell™ plates, Nunc A/S, Roskilde, Denmark) were used. A total of 50 µL reaction mixture was then incubated for 1 h at 4°C with 50 µL Protein A Sepharose 4B conjugated (20%, washed five times at 4°C by sedimentation in antigen buffer) (catalog nr 10–1090, Invitrogen) in a 96-well MultiScreen-DV filtration plate (catalog nr MSDVN6B50, Millipore). The plate was then washed 8 times with wash buffer (150 mM NaCl, 20 mM Tris, pH 7.4, 0.15% Tween 20) using a Multiscreen vacuum manifold (MultiScreen^{HTS}, Vacuum Manifold, Millipore). Antibody-bound radioactivity was counted in a β-counter (1450 MicroBeta TriLux Microplate Scintillation-Luminescence Counter). Sepharose-bound radioactivity was converted into in-house units (U) using individual standard curves generated by six step doubling dilutions of high-titer T1D sera with high reactivity for each individual ZnT8 antigen.

RBA for Triple mix of ZnT8-R, ZnT8-W and ZnT8-Q (ZnT8-TripleA)—In the ZnT8-TripleA RBA 5 µL serum was incubated with 60 µL of a mixture of all three labeled ZnT8-R, ZnT8-W and ZnT8-Q, respectively, at equal proportions in antigen buffer and adjusted to a final concentration of 425±25 cpm/µL. The remaining procedure was carried out as

described above for the individual ZnT8 variants. Antibody-bound radioactivity was converted into in-house units (U) using a high-titer T1D serum reactive with all three variants subjected to a six step doubling dilution.

2.5. Statistical analysis

The SPSS 18[®] statistical package (SPSS Inc. Chicago, IL) was used for statistical analysis. A *p* value <0.05 was considered as significant. Pearson Chi square test of independence (and Yates' correction for continuity value when applied) was used to assess differences in frequencies of autoantibody positivity. The ROC curve was used to calculate the sensitivity of assays for each ZnT8A variants as well as ZnT8-TripleA. The lowest autoantibody levels among patients were limited at 10 U/mL for ZnT8-RA and ZnT8-WA and 15 U/mL for ZnT8-QA while the maximum level was considered at 800 U/mL for all variants.

3. Results

3.1. Precision and reproducibility of the ZnT8-TripleA assay

The incorporated radioactivity (mean% $\pm$ SD) calculated for 10 separate experiments for each variant, was as follows: $35 \pm 12\%$ for pThZnT8-R, $33 \pm 9\%$ for pThZnT8-W and $39 \pm 8\%$ for pThZnT8-Q compared to $10 \pm 3\%$ for ZnT8-R in the pcDNA3.1 vector. The level of incorporated radioactivity (in one representative experiment) with ³⁵S-methionine in ZnT8-R with the pcDNA3.1 vector was 7% compared to 54% for pThZnT8-R, 50% for pThZnT8-W and 51% for pThZnT8-Q (Figure 1, Panel A).

The translation products demonstrated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography showed only the expected 11 kDa component corresponding to the C-terminal part of ZnT8 protein (aa268-aa369) for ZnT8-R, ZnT8-W and ZnT8-Q (Figure 1, panel B). No additional band was detected. The RBA, schematically presented in Figure 1, panel C for all four ZnT8 autoantibody assays showed comparable precision (intra-assay CV was 5.5% for ZnT8-RA, 5.3% for ZnT8-WA, 4.9% for ZnT8-QA and 4.4% for ZnT8-TripleA) and reproducibility (inter-assay CV was 13.8% for ZnT8-RA, 6.7% for ZnT8-WA, 11.0% for ZnT8-QA and 8.3 % for ZnT8-TripleA) which were measured in 15 experiments for all 2664 patients with childhood diabetes as well as known positive controls.

The cut-off levels for the positive tests of ZnT8-TripleA, ZnT8-RA, ZnT8-WA, and ZnT8-QA among 613 healthy controls were determined using quantile-quantile (QQ) plots at 98th percentile, in order to identify deviations from normality and was set at 75 U/mL for ZnT8-TripleA, ZnT8-RA, ZnT8-WA and at 100 U/mL for ZnT8-QA (Figure 2, Panel A1–4). We have calculated the area under the curve (AUC) for the ZnT8-TripleA, which was found to be 0.81 (95% CI = 0.79–0.82, SE=0.008) at specificity of 97% with corresponding sensitivity of 65% (Figure 2, Panel B1). The AUC for the ZnT8-RA variant was 0.77 (95% CI=0.75–0.78, SE=0.008), for the ZnT8-WA variant was 0.76 (95% CI=0.74–0.78, SE=0.008) and finally for the ZnT8-QA variant was 0.74 (95% CI=0.72–0.76, SE=0.009). The specificity was found to be 99% that corresponded to a sensitivity of 52%, 47% and 31% for the ZnT8-RA, ZnT8-WA and ZnT8-QA respectively (Figure 2, Panel B2–4). The cut-off levels were 75 U/mL for ZnT8-RA and ZnT8-WA and 100 U/mL for ZnT8-QA (Figure 2, Panel A2–4).

The performance of our ZnT8A assays (individual variant and triple mix assays) in the Diabetes Autoantibody Standardization Program (DASP) 2010 workshop on ZnT8A showed comparable results to our current study. The self-reported sensitivity and specificity of the ZnT8ATriple assay in our lab were 64% sensitivity at 94% specificity. The corresponding self-reported sensitivity and specificity for ZnT8-RA, ZnT8-WA and ZnT8-QA were 52%,

50% and 38% respectively at 100% specificity. The corresponding adjusted sensitivity at 95% specificity from DASP 2010 workshop were 64%, 60%, 56% and 42% for ZnT8-TripleA, ZnT8-RA, ZnT8-WA and ZnT8-QA respectively. The ZnT8-QA, however, did not have ROC AUCs significantly different from 0.5 and therefore could not significantly distinguish between patients from controls.

3.2 Type of diabetes and positivity in the ZnT8-TripleA assay

The results of the individual ZnT8A demonstrate that the frequency of positive autoantibodies was much higher for T1D patients than for T2D and MODY patients. All (n=8) secondary diabetes patients were negative (Table 1). Among the T1D (n=2582) patients, ZnT8-RA was the most common autoantibody detected in 1351 (52%) patients, ZnT8-WA was found in 1209 (47%) and ZnT8-QA in 790 (31%). Taken together 1661 (64%) of T1D patients had one or several ZnT8A (Table 1). These figures were compared to those in control subjects (n=613) who had almost 1% (n=6/613) ZnT8A positivity for one or more of the individual variants (Table 1).

ZnT8A may appear alone or in combination as is illustrated by the frequency (%) of positive results in Figure 3, panel A. In the 2582 T1D patients it was noted that 403 (15.6%) had ZnT8-RA alone, 267 (10.3%) had ZnT8-WA alone while ZnT8-QA alone was detected in only 3 (0.1%). In contrast as many as 702 (27%) of the T1D patients were positive for all three ZnT8 autoantibodies. Among the 46 T2D patients (Table 1), four patients were positive for ZnT8-TripleA, one patient had ZnT8-RA and ZnT8-WA and another had only ZnT8-WA and two patients had all three variants.

In the ZnT8-TripleA assay antibody positivity was detected in 1678 (65%) patients with T1D, in 4/46 (9%) with T2D, and in 3/28 (11%) with MODY but in none of the patients with secondary diabetes (Table 1). In the control subjects, 12/613 (2%) were positive in the ZnT8-TripleA assay (Table 1).

When data from the individual assays were compared with the triple test, the ZnT8-TripleA assay showed a false positive rate of 1.9% (49/2582) in T1D (Figure 3, panel B). The false positive rate for T2D (n=46) was 0% and for MODY was 3.6% (1/28) while it was 1.1% (7/613) for the control subjects. Only 1.2% (32/2582) of the T1D patients had false negative ZnT8-TripleA tests. The false negative rates for T2D and MODY were (0%) and 0.16% (1/613) in controls.

Since we were interested in discriminating between autoantibody positive from autoantibody negative patients, the end-titration of autoantibody levels was not considered in this analysis (maximum level was considered at 800 U/mL).

3.3 ZnT8A and occurrence of other islet autoantibodies

The analysis for other islet autoantibodies showed that among T1D patients (n=2582), a total of 1888 (73%) were positive for IA-2A, 1448 (56%) for GAD65A and 859 (33%) for IAA (Table 1).

We next analyzed the combination of islet autoantibodies after correcting for the false positive and false negative rates in the ZnT8-TripleA assay. The frequency of 6.2% (161/2582) of T1D patients who were negative for all autoantibodies remained unchanged after correction for false positive and false negative rates.

There were 9.8% (253/2582) T1D patients who were negative for the three conventional islet autoantibodies (IA-2A, GAD65A and IAA) only. Within this group, 83/253 (33%) were positive for ZnT8-TripleA including 6/253 (2.4%) who were false positive. This means that

adding the ZnT8-TripleA test to the three conventional islet autoantibodies would reduce the percentage of autoantibody-negative patients from 9.8% to 6.7% with only 0.2% false detection rate (6/2582).

4. Discussion

We describe a novel triple mix RBA for the measurement of serum ZnT8 autoantibodies, ZnT8-TripleA, and compare its performance with that of the individual tests for each of the three variants, ZnT8-RA, ZnT8-WA and ZnT8-QA. Our ZnT8-TripleA RBA was developed as recent studies have indicated that analysis of all three variants would increase the diagnostic sensitivity and specificity of T1D as well as increase the ability to predict the disease. The major finding of the ZnT8-TripleA RBA was a low false positive rate (1.9%, 49/2582) among T1D patients. Also only 1.2% (32/2582) T1D patients showed a false negative test in the ZnT8-TripleA RBA when compared to the individual tests. This means that the ZnT8-TripleA RBA would have overestimated the frequency of ZnT8A in T1D by some two percent while we would have missed only 0.2% (6/2582). The slight overestimation is easily resolved by analyzing the triple positive samples with each variant.

The ZnT8-TripleA RBA, which was developed consist of a mixture of the three known variants of ZnT8 rather than using tandem plasmids as reported by others (Kawasaki et al., 2008; Achenbach et al., 2009) or even attempting to prepare a triple expression plasmid. More important to our approach was that each variant was produced at high and comparable specific ^{35}S radioactivity. To this end, we subcloned the original ZnT8-R cDNA into a high efficiency *in vitro* transcription translation plasmid (pTnT). This plasmid, pThZnT8-R, could then be subjected to site directed mutagenesis easily to create the pThZnT8-W and pThZnT8-Q plasmid, respectively. The incorporation rate of ^{35}S -methionine was consistently above 30% into one component only revealed by SDS gel electrophoresis also showing no difference between the three variants. This is important as an equal amount of each variant could then be present in the reaction mixture. We therefore do not expect that the ability to detect ZnT8A will differ between the three variants. Furthermore, in ZnT8-TripleA RBA the inter- and intra-assay coefficient of variations were 8.3% and 4.4%, respectively, which performed better than the precision and reproducibility of the individual tests (Section 3.1 in results).

The approach to define so-called cut-off levels or deviation from normal for ZnT8A varies markedly between laboratories and studies. Some investigators use control sera to compute mean and standard deviation to set a cut off at 3–5 standard deviations (Wenzlau et al., 2007). Others have demonstrated that islet autoantibody assays have binding levels that are not normally distributed and therefore use percentiles with cut-offs at 95–99 percentile (Wenzlau et al., 2007; Wenzlau et al., 2008b). We tested the three individual ZnT8A variants as well as the ZnT8-TripleA RBA among a large group of healthy controls (11–81 years). Using the 97th percentile as the cut-off in the ZnT8-TripleA RBA about 4% (24/613) were considered positive. When the patients' samples were analyzed for the individual variants we found a false-positive rate of 1.9% (49/2582) while only 1% (6/613) of controls was positive for one or more of the three ZnT8A variants. Additionally, 0% of controls (n=613) showed a false negative rate in the ZnT8-TripleA RBA (Table 1). These rates demonstrate that the ZnT8-TripleA RBA has a high diagnostic specificity in detecting ZnT8A among healthy subjects who are positive for at least one ZnT8A variant and would only miss less than 0.2%.

The individual assays for ZnT8A among T1D patients (2582) showed specific reactivity (detecting the specific variant alone) for each of the three ZnT8 variants. The specific reactivity for a single variant alone was 15.6%, 10.3% and 0.1% for ZnT8-RA, ZnT8-WA

and ZnT8-QA, respectively; while as many as 27% of the T1D patients were positive for all three ZnT8A. The ZnT8-QA assay may not be the best method to detect islet autoimmunity in healthy subjects or as a tool in the clinical classification of diabetes since only a few subjects are positive for ZnT8-QA alone. However, we believe that the triple assay should prove valuable when subjects are screened for islet autoimmunity or to improve classification at onset of clinical diabetes since many subjects are positive for ZnT8-QA in combination with ZnT8-RA or ZnT8-WA.

Among patients with T1D (n=2582), the ZnT8-TripleA RBA was positive among 65% with a false positive rate of 1.9%. However, among the T1D patients, frequency of ZnT8-RA was highest (52%) in comparison to ZnT8-WA (47%) and ZnT8-QA (31%). Altogether, 64% of these T1D patients were positive for one, two or all three ZnT8A variants. Considering the low false negative rate of 1.2% among T1D patients; the ZnT8-TripleA RBA would therefore be a reliable test in patients with T1D not only to verify diagnosis but also to exclude nearly one third of patients from being tested for individual ZnT8A variants since the ZnT8-TripleA tested positive in 65% of T1D patients.

Our data also showed that the ZnT8-TripleA RBA is able to detect ZnT8A among sera of patients who were clinically classified as T2D (6/46) and MODY (4/28) with false positive rates of 4% and 7%, respectively, and 0% false negative rates among both T2D and MODY patients. The ZnT8-TripleA RBA may therefore be useful, in addition to other islet autoantibodies, to validate the clinical diagnosis of childhood diabetes.

Of T1D patients 90% (n=2324) had at least one of the conventional islet autoantibodies (GAD65A, IA-2A and IAA) at diagnosis. When the same samples were analyzed with the ZnT8-TripleA RBA, the frequency of autoantibody-negative patients decreased from almost 10% (n=258) to 6.7% (n=173). Interestingly, this percentage remained unchanged after correction of false positive and false negative rates of the ZnT8-TripleA RBA indicating that the ZnT8-TripleA RBA is a reliable test for the identification of T1D patients negative for IAA, GADA and IA-2A.

To evaluate the workshop sensitivity and specificity of the ZnT8A assays, our laboratory participated in Diabetes Autoantibody Standardization Program (DASP) 2009 workshop on ZnT8A to determine workshop specificity and levels of autoantibodies against the two major allelic variants of ZnT8, ZnT8-R and ZnT8-W, in DASP patients and controls. It is noted that the workshop samples are selected to provide sufficient volumes of sera to be distributed to many laboratories. Young children are therefore not represented in the DASP effort. The final results from DASP 2009 workshop on ZnT8A showed a high level of workshop sensitivity of our assay when compared to the results of the other laboratories. The corresponding adjusted workshop sensitivity at 95% specificity were 66% for ZnT8-RA and 50% for ZnT8-WA respectively. The future clinical application and an extensive validation of both the individual (ZnT8-RA/ZnT8-WA/ZnT8-QA) and the ZnT8-TripleA RBA assay will be obtained through our future participation in DASP workshops. In DASP 2010, we used a detection limit of 58 U/mL for the ZnT8-TripleA assay, however, in this study; we used a detection limit of 75 U/mL at a diagnostic specificity of 97%, which corresponded to a diagnostic sensitivity of 65%. This limit conferred few false positive results from healthy controls. Because the threshold is a tradeoff between sensitivity and specificity, the ZnT8-TripleA assay also achieved higher diagnostic sensitivity compared with the three individual assays. If a subject is deemed positive by the screening method, the specimen is re-assayed for the three separate constructs.

Since our aims in this study were to examine the performance of the ZnT8-TripleA assay in terms of diagnostic sensitivity and specificity, end-titration of the ZnT8-TripleA assay as

well as individual variant assays were not determined and the maximum autoantibody-positive levels were limited at 800 U/mL. However, this limitation did not affect the performance or the results obtained for the ZnT8-TripleA assay.

The assessment of ZnT8A by utilizing a novel triple mix assay is simple to perform and we expect that this assay should represent a highly reproducible practical alternative to individual RBA to determine the frequency of ZnT8A in clinical routine to classify diabetes as well as in both prevention and intervention clinical trials in type 1 diabetes. This assay may also prove useful for the detection of ZnT8A in observational clinical trials such as TEDDY (2008), BABYDIAB (Schenker et al., 1999), DAISY (Rewers et al., 1996) and DiPiS (Larsson et al., 2004), ABIS (Ludvigsson et al., 2001) as well as in prevention and intervention clinical trials when islets autoantibody positivity is used to randomize participants (Larsson H E et al., 2010).

5. Conclusions

We conclude that our novel ZnT8-TripleA assay is useful for detection of circulating ZnT8A. This assay showed a low false positive rate and a negligible false negative rate with high precision and reproducibility. The triple mix assay would therefore be highly suitable not only to analyze patients with newly diagnosed diabetes but in particular for screening the general population, as it is likely not to miss individuals who have autoantibodies against any of the three ZnT8A variants.

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Abbreviations

BDD	Better Diabetes Diagnosis Study
GAD65A	Glutamic Acid Decarboxylase Autoantibodies
IA-2A	Islet Antigen-2 Autoantibodies
IAA	Insulin Autoantibodies
RBA	Radiobinding Assay
T1D	Type 1 Diabetes
ZnT8-TripleA	Zinc Transporter 8 Triple mix autoantibodies
ZnT8-RA	Zinc Transporter 8 Arginine autoantibodies
ZnT8-WA	Zinc Transporter 8 Tryptophan autoantibodies
ZnT8-QA	Zinc Transporter 8 Glutamine autoantibodies

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7. Appendix

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Panel A.

Panel B.

Panel C.

Figure 1.

Panel A. Illustration of the separation of incorporated radioactivity from free ^{35}S -methionine radioactivity in ZnT8-R with the pcDNA3.1 vector in relation to pThZnT8-R, pThZnT8-W and pThZnT8-Q. The level of incorporated radioactivity (in one representative experiment) with ^{35}S -methionine in ZnT8-R with the pcDNA3.1 vector (★) was 7% compared to 54% for pThZnT8-R (▲), 50% for pThZnT8-W (●) and 51% for pThZnT8-Q (⊙).

Panel B. The SDS-Page and autoradiography of single bands of the labeled ZnT8-R-W-Q antigens produced with the coupled *in vitro* transcription translation. The translation products showed only the expected 11 kDa component corresponding to the C-terminal part of ZnT8 protein (aa268-aa369) for ZnT8-R, ZnT8-W and ZnT8-Q. No additional band was detected.

Panel C. Graphic illustration showing the procedures of both individual and the ZnT8-TripleA radiobinding assay for ZnT8 autoantibodies. In the ZnT8-TripleA RBA, all three ZnT8 antigens were mixed at equal proportions in antigen buffer. The antibody-bound radioactivity of the ^{35}S -Labeled ZnT8 protein was measured with a β-counter.

Panel A.

A-1

A-2

A-3

A-4

Panel B.**B-1** **B-2**

B-3

B-4

Figure 2.**Panel A 1–4.**

Identification of the deviation from normality and determination of the cut-off levels using the quantile-quantile (QQ) plots at 98th percentile on samples from 613 healthy controls aged 11–81 years.

Panel B. Determination of the diagnostic sensitivities and cut-off levels for ZnT8-TripleA and ZnT8A variants using ROC curves.

B-1

ROC curve showing the area under the curve (AUC) for the ZnT8-TripleA corresponding to 0.81 (95% CI = 0.79–0.82, SE=0.008). The specificity was found to be 97% with corresponding sensitivity of 65% sensitivity with respective cut-off levels of 75 U/mL.

B-2

ROC curve showing the area under the curve (AUC) for the ZnT8-RA corresponding to 0.77 (95% CI=0.75–0.78, SE=0.008). The specificity was found to be 99% that corresponds to 52% sensitivity with respective cut-off levels of 75 U/mL.

B-3

ROC curve showing the area under the curve (AUC) for the ZnT8-WA corresponding to 0.76 (95% CI=0.74–0.78, SE=0.008). The specificity was found to be 99% that corresponds to 47% sensitivity with respective cut-off levels of 75 U/mL.

B-4

ROC curve showing the area under the curve (AUC) for the ZnT8-QA corresponding to 0.74 (95% CI=0.72–0.76, SE=0.009). The specificity was found to be 99% that corresponds to 31% sensitivity with respective cut-off levels of 100 U/mL.

Panel A.

Panel B.

Figure 3.

Panel A. Venn diagram analysis showing the individual assays for ZnT8-RA, ZnT8-WA and ZnT8-QA (% positive) among T1D patients only (n=2582). There were 15.6% (n=403) patients who had ZnT8-RA alone, 10.3% (n=267) ZnT8-WA alone while ZnT8-QA alone was detected in only 0.1% (n=3).

Panel B. Ellipses Venn diagram analysis of the triple mix radiobinding assay for ZnT8 autoantibodies (ZnT8-TripleA) compared to the individual assays for ZnT8-RA, ZnT8-WA and ZnT8-QA among T1D patients only (n=2582). Among T1D patients, the ZnT8-TripleA showed 1.9% (n=49) false positive and 1.2% (n=32) false negative tests.

Table 1

Demographic and autoantibody data analysis of the diabetes patient (n=2664) and controls (613) used to validate the triple mix radiobinding assay for ZnT8 autoantibodies.

	T1D n (%)	T2D n (%)	MODY n (%)	Secondary n (%)	Controls n (%)
Subjects n=2664 (100%)	2582 (97)	46 (1.7)	28 (1)	8 (0.3)	613
Age					
Mean	9.9	15.1	11.9	13.3	Adults=44.3, 19–81
Range (y)	0.15–18	6.3–17.8	1.5–17.3	5.7–10.7	Children=12 11–13
Males	1436 (56)	19 (41)	11 (39)	4 (50)	366 (59)
ZnT8-RA	1351 (52)	3 (6.5)	1 (3.6)	0	3 (0.5)
ZnT8-WA	1209 (47)	4 (8.7)	1 (3.6)	0	3 (0.5)
ZnT8-QA	790 (31)	2 (4.3)	1 (3.6)	0	3 (0.5)
One or more ZnT8-A	1661 (64)	4 (8.7)	2 (7.1)	0	6 (1.0)
ZnT8-TripleA	1678 (65)	4 (8.7)	3 (10.7)	0	12 (2.0)
GAD65A	1448 (56)	3 (6.5)	2 (7.1)	0	19 (3.1)
IA-2A	1888 (73)	2 (4.3)	2 (7.1)	0	8 (1.3)
IAA	859 (33)	1 (2.2)	2 (7.1)	0	4 (0.7)
Negative for all Aab	174 (6.7)	42 (91)	24 (86)	8 (100)	577 (94)

T1D = type 1 diabetes, T2D = type 2 diabetes, MODY = Maturity Onset Diabetes of the Young, Secondary = diabetes secondary to other chronic disease, Aab = autoantibodies