Received: 1 August 2024 | Revised: 18 September 2024 | Accepted: 24 September 2024

DOI: 10.1111/dom.16002

RESEARCH LETTER

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# Development and validation of a novel method for evaluation of multiple islet autoantibodies in dried blood spot using dissociation-enhanced lanthanide fluorescent immunoassays technology, specific and suitable for paediatric screening programmes

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KEYWORDS: cohort study, islets, population study, presymptomatic diabetes, screening type 1 diabetes, type 1 diabetes

## 1 | BACKGROUND

Italy made history by becoming the first country to establish and fund a long-term screening programme for type 1 diabetes (T1D) and celiac disease (CD)[1]; indeed, the Istituto Superiore di Sanità (ISS) has been commissioned by the Ministry of Health to organize a pilot study that will be carried out in four Italian regions: Lombardia, Marche, Campania and Sardegna, called D1Ce (Diabete tipo 1 e Celiachia) Screen. This study is preparatory to the national screening programme and aims to assess the acceptability of a screening programme to parents and children and the problems associated with sampling procedures and dosage of autoantibodies.

T1D is a chronic autoimmune disease and has a known presymptomatic phase that can be detected by testing for the presence of circulating anti-islet autoantibodies (IA). These IAs include four markers: anti-glutamic acid decarboxylase (GADA), anti-zinc transporter-8 (ZnT8A), tyrosine phosphatase (IA2A) and anti-insulin (IAA).[2] Two or more autoantibodies and normal glucose levels mark stage 1 T1D, whereas stage 2 is marked by antibodies and dysglycemia without any symptoms of T1D. Stage 3 is marked by glucose

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levels consistent with the American Diabetes Association (ADA) definition of diabetes mellitus.[3] Studies have shown that the rate of progression to clinical Stage 3 diabetes is similar if islet autoantibody positivity at the early stage of T1D is identified through screening of the general population or a first-degree relative.[3] The screening programme for T1D has the goal of identifying children and adolescents at risk of developing T1D, thus preventing or reducing the associated complications, such as diabetic ketoacidosis, which affects over 40% of the T1D at onset in Italy.[4] Blood tests are generally used for screening purposes; however, conventional venous blood sampling is not always well accepted due to venipuncture, especially in children. In contrast, a simple, rapid and more tolerable capillary blood sampling by finger pricking could overcome these limitations and offer a valid alternative (WHO Guidelines on Drawing Blood: Best Practices in Phlebotomy 2010). Moreover, analysis of autoantibodies on capillary blood for T1D has the limitation of complex management of liquid samples in terms of biosafety, storage at controlled temperature, risk of haemolysis and insufficient blood volume collection.[5] An efficient and well-established micro sampling technique already used for neonatal screening of metabolic disorders is the dried blood spot (DBS).[6] This sample is easy to collect, requiring a simple prick of the finger, very stable and safe and has already been used for the measurement of antibodies.[7] Standard enzyme-linked immunosorbent assay (ELISA) tests, however, are usually not sensitive enough to analyse DBS samples,[8–10] and RIA-based assays have the problem of handling radioactive material.

The aim of this work therefore was the development of a multiplex, dissociation-enhanced lanthanide fluorescent immunoassays (DELFIA) based method for the detection of three of the autoantibodies (GADA, ZnT8A and IA2A) on DBS, a potentially simple matrix to collect and deliver samples to laboratories, suitable for a population screening programme in paediatric population. Moreover, the viability of an additional, reliable screening method would allow more laboratories to be included in the screening network. The use of the three antibodies included in the assay has been previously shown to be sufficient for identifying potentially affected individuals.[11] Positive samples will then be analysed for the presence of the three antibodies and the IAA separately to allow disease classification as described earlier.

## 2 | MATERIALS AND METHODS

See Supplementary Appendix; Data S1.

## 3 | RESULTS AND CONCLUSION

We collected sera and DBS samples from 68 paediatrics patients with T1D and 51 paediatric healthy controls (Power value was 0.99, as better described in Supplementary Appendix; Data S1). The clinical features of patients and controls are reported in Table 1. We initially

**TABLE 1** Demographic and clinical characteristics of children with T1D and healthy controls included in the study.

| Variable | Healthy controls ($n = 51$) | Children with T1D ($n = 68$) | $p$-Value |
|---|---|---|---|
| Gender (female), *n* (%) | 25 (49%) | 37 (54.4%) | n.s. |
| Age, mean ± SD | 14.05 ± 10.6 | 13.1 ± 4.7 | n.s. |
| Comorbid conditions, *n* (%) | | | |
| Celiac disease | - | 9 (13.2%) | n.s. |
| Atopic dermatitis | - | 1 (1.5%) | n.s. |
| Thyroiditis | - | 8 (11.8%) | n.s |
| Incontinent nephropathy | - | 1 (1.5%) | n.s. |
| Nocturnal enuresis | - | 1 (1.5%) | n.s. |
| Metabolic syndrome and obesity | 1 (1.9%) | - | n.s. |
| Sinus tachycardia | 1 (1.9%) | - | n.s. |
| HbA1c (%) | | | |
| Median value (min-max) | 5.3 (4.8–6.2) | 8.1 (5.6–16.4) | 0.001*** |
| BMI (Z-score) | | | |
| Median value (min-max) | 1.9 (0.01–3.9) | 0.6 (−1.9–2) | 0.001*** |
| Fasting Blood sugar (mg/dL) | | | |
| Median value (min-max) | 79.4 (53–109) | 156.1 (51–536) | 0.001*** |
| Insulin treatment, *n* (%) | - | 68 (100%) | n.a. |

*Note*: Results were obtained using the Mann–Whitney test for non-parametrical distribution and the chi-squared test for categorical parameters. Values are expressed as mean ± SD or median (min-max).
Abbreviations: BMI, body mass index; n.a., not available; n.s., not significant; SD, standard deviation.
***$p < 0.001$.

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**FIGURE 1** Comparison of enzyme-linked immunosorbent assay (ELISA) with dissociation-enhanced lanthanide fluorescent immunoassays (DELFIA) methods for detecting autoantibodies anti-GAD, anti-ZnT8 and anti-IA2 in paediatric diabetic patients. (A) Positive rate of single autoantibodies in paediatric patients analysed ($n = 68$). (B) Indirect ELISA performed on serum from diabetic paediatric patients ($n = 68$) and healthy paediatric controls ($n = 51$). (C) Indirect DELFIA performed on dried blood spot (DBS) extracts from the same diabetic paediatric patients ($n = 68$) and healthy paediatric controls ($n = 51$). Dotted line represents the positivity cut-off value calculated for DELFIA multiplex assay. Scatter plots for O.D. values (B) and Scatter plots for T.R.F. values (C) in DBS extracts show mean ± standard deviation. (D) Spearman's rank was calculated between T.R.F. values from DELFIA assay and O.D. values from ELISA assay. (E) ROC curve analysis of autoantibodies anti-GAD, anti-ZnT8 and anti-IA2 DELFIA test in the screening of diabetes. The ROC curve of the three autoantibodies in multiplex DELFIA assay provides an AUC of 0.98. Autoantibodies anti-GAD, anti-ZnT8 and anti-IA2 measured up to 30 days after the sample collection at room temperature (F), at +4°C (G), at −20°C (H). * means $p$-value 0.03.

evaluated the positivity of our samples for each autoantibody separately using a commercially available, clinically validated ELISA test (RSR Limited, Cardiff, United Kingdom) on serum samples. The frequency of autoantibodies detected by the three immunoassays is summarized in Figure 1A. We then compared the ability of a Ce-IVD commercially available ELISA multiplex assay for anti-GAD, anti-ZnT8 and anti-IA2 on serum samples (Figure 1B) and our DELFIA multiplex assay (Figure 1C) on DBS to detect diabetic patients. The cut-off for DELFIA multiplex was established for optimal sensitivity (95.59%), specificity (94.12%) and is shown in Figure 1C. The Spearman's rank

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correlation, calculated between time-resolved fluorescence (T.R.F.) values from DELFIA assay and optical density (O.D.) values from ELISA assay, is statistically significant ($r = 0.96$, $p < 0.0001$) (Figure 1D). The area under curve (AUC) using ROC analysis was 0.98 and is reported in Figure 1E. We evaluated the repeatability of DELFIA multiplex on DBS by performing intra- and inter-day assay tests. We evaluated the T.R.F. value variability by performing five measurements with a triple positive sample and reported the CV% with the standard deviation (SD) on Table S1 in Supplementary Appendix. Stability of autoantibodies anti-GAD, anti-ZnT8 and anti-IA2 in DBS samples was tested using a triple positive sample; stored at room temperature (RT), 4°C and −20°C up to 30 days from collection; and evaluated in triplicate (Figure 1F–H). Storage at RT induced a significant reduction in detectable autoantibody levels after 4 days to levels that remained stable for up to 15 days (Figure 1F) while levels were stable up to 30 days when samples were stored at 4°C, −20°C (Figure 1G,H). In conclusion, our results indicate that the DBS may be employed for T1D population screening; indeed the data obtained on DBS with our method are comparable to those obtained using classic ELISA tests on serum samples (Figure 1B,C) but overcome collection and shipment problems linked to the use of serum. This is of uttermost importance when sample collection has to be performed by general family paediatricians who also have to deal with sample delivery to centralized laboratories for analysis, overcoming the problems with the initial pilot project protocol, proposed by the ISS that requires the collection of both sera for evaluation of autoantibodies and DBS for the measurement of HLA genes for susceptibility to CD. The collection of two types of samples, however, reduces the patients and physicians' compliance and results in difficult management of the programme. In contrast the use of only one type of sample, that is easy to collect and ship, in particular, increases the chances of success.

It has to be mentioned, however, that there are some limitations in the use of DBS samples: the haematocrit (Hct) changes the blood volume per paper area and therefore the amount of blood analysed, as Hct increases the viscosity in relation to the higher percentage of red blood cells and finally the chromatographic effect can determine a heterogeneity of sample composition.[12] These analytical problems, however, are not relevant in screening programmes that do not require high accuracy, but only a semi-quantitative measurement that allows access to second tier analysis that confirms the diagnostic suspect.

In conclusion, our data show that the DELFIA multiplex assay performs well in terms of repeatability with an intra- and inter-assay CVs of ≤7.7% and ≤13.2%, respectively (Table S1, Supplementary Appendix). It shows a clear correspondence between classical serological tests and DBS tests with a Spearman test showing a $r = 0.96$ (Figure 1D), strongly supporting the use of DBS to determine autoantibodies in T1D screening programmes. Despite the low amount of blood in a 3.2 mm spot, the ROC curve indicates good sensitivity (95.59%) and specificity (94.12%) in subjects' reclassification (Figure 1E). Moreover, DBS samples are stable up to 30 days when stored at 4°C or at −20°C (Figure 1G,H), and can be safely delivered using a refrigerated bag, avoiding the problems related to sample treatment (centrifugation) and biosafety permits required for proper collection and shipment of serum samples (as required by Ministry of Health no. 3 May 2003, OMS Guidelines of 1997).

We have confidence that this method is simple and reliable, and it will make screening for T1D affordable and achievable for families and paediatricians. However, it is essential to broaden the analysis to include a larger sample, encompassing other prevalent types of diabetes in children and adolescents, like type 2 diabetes and monogenic diabetes, as well as a more extensive group of participants without diabetes.


## ACKNOWLEDGEMENTS

We kindly acknowledge Erika Pizzinato for their precious support of the sample management.

## FUNDING INFORMATION

Internal institutional funds were used to support this study.


## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/dom.16002.

## DATA AVAILABILITY STATEMENT

Data available in article supplementary material.


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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Dufrusine B, Natale L, Sallese M, et al. Development and validation of a novel method for evaluation of multiple islet autoantibodies in dried blood spot using dissociation-enhanced lanthanide fluorescent immunoassays technology, specific and suitable for paediatric screening programmes. *Diabetes Obes Metab*. 2024;1-5. doi:10.1111/dom.16002

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