SHORT COMMUNICATION

Diagnostic relevance of ZnT8 autoantibodies in Type 1 Diabetes Mellitus: Insight from a Malaysian Cohort

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ABSTRACT

Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disorder marked by the destruction of insulin-producing pancreatic beta cells. The presence of diabetes-associated autoantibodies (DAAs) supports diagnosis; however, the utility of zinc transporter 8 (ZnT8) autoantibodies in Malaysian patients remains understudied. This study aimed to screen ZnT8 autoantibodies in Malaysian T1DM patients using an enzyme-linked immunosorbent assay (ELISA). ZnT8 autoantibodies were detected in 17.3% of all patients, with a higher prevalence in paediatric cases (36.7%) compared to adults (11.3%). Further analysis revealed variations based on gender, ethnicity, and the presence of other DAAs across age groups. These findings suggest differing autoimmune responses and disease mechanisms between paediatric and adult T1DM patients in Malaysia, highlighting the potential role of ZnT8 autoantibodies as a complementary diagnostic marker and the need for age-specific diagnostic and therapeutic strategies.
Malaysian Journal of Medicine and Health Sciences (2026) 22(3): 1-8.doi:10.47836/mjmhs.v22i3.1558

Keywords: Type 1 diabetes mellitus, Zinc transporter 8, Diabetes-associated autoantibodies, Malaysia, Human, Diagnosis, Biomarker

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INTRODUCTION

Type 1 diabetes mellitus (T1DM) is a chronic disease caused by an autoimmune attack on beta (β) cells of the pancreas, which produce insulin. Hence, the destruction of insulin-producing β cells causes hyperglycemia, diabetic ketoacidosis (DKA), and other complications in T1DM patients. The incidence of T1DM has been increasing globally (1), and Malaysia may also see an increase in T1DM incidence as observed worldwide. To our knowledge, no study has reported the burden of T1DM in Malaysia (2), therefore, it is possible that Malaysia may follow the global trend. This is particularly relevant given the recent review that suggested that new cases of T1DM may occur more because of environmental factors (3).

One of the methods to confirm the diagnosis of T1DM is detecting diabetes-associated autoantibodies (DAA), including insulin autoantibodies (IAA), glutamic acid decarboxylase autoantibodies (GADA), insulinoma-2-associated autoantibodies (IA-2A), islet cell autoantibodies (ICA) and zinc transporter 8 (ZnT8) autoantibodies as outlined by the Ministry of Health (MOH), Malaysia (4). Previous research reported that 68 of 213 T1DM patients (38%) were seronegative but presented with near or total β cell destruction (5). To our knowledge, no study has evaluated the utility of ZnT8 autoantibodies in confirming the diagnosis of T1DM in Malaysia. ZnT8 is a zinc transporter localized on the membrane of insulin-containing secretory granules of β

cells. It is crucial in insulin biosynthesis, storage, and release (6). ZnT8 is thought to be involved later in the development of T1DM in humans, suggesting that the antigen is recognized as part of the spreading rather than the initial autoimmune response (7). The ZnT8 autoantibody kit was approved by the United States Food and Drug Administration (FDA) in 2014 as the latest autoantibody for diagnosing and predicting T1DM (8). Therefore, there is a probability that some seronegative T1DM patients in the earlier study may be positive with ZnT8 since the study was performed before the FDA approved the kit.

ZnT8 could aid in confirming the diagnosis of T1DM in combination with other autoantibodies. Various frequencies have been reported of ZnT8 autoantibodies. Malaysia is a multi-ethnic country with 57.9% Malay, followed by Chinese (22.6%), Indian (6.6%) and others, which include Indigenous people in East Malaysia (12.2%), respectively (9). Considering Malaysia's diverse and multi-ethnic population, it is more suitable to examine literature that reports the frequency of ZnT8 autoantibodies in non-Caucasian individuals rather than focusing on Caucasian populations. ZnT8 autoantibodies were present in 73.3% of Indonesian T1DM patients (10), while 54.3% of Thai T1DM patients were positive for ZnT8 autoantibodies (11). On the other hand, ZnT8 autoantibodies were present among 24.1% and 31.8% of T1DM patients from China (12) and India (13), respectively. Besides, ZnT8 has been proposed to replace IA-2A in juvenile T1DM patients in India (13). Additionally, ZnT8 autoantibodies were detected in 13.5% and 16% as a single autoantibody among T1DM patients in China (12) and Thailand (11), increasing the sensitivity of the DAA test to confirm T1DM diagnosis. In conclusion, ZnT8 autoantibodies could be detected in 24 to 70% of non-Caucasian T1DM patients.

The wide variety of ZnT8 autoantibodies in non-Caucasian populations highlights the importance of investigating their frequency in Malaysian T1DM patients. This study aims to examine the frequency of ZnT8 autoantibodies among Malaysian T1DM, how it could improve the sensitivity of DAA to confirm T1DM diagnosis and explore its potential relationship with clinical parameters.

MATERIAL AND METHODS

This retrospective study used samples from the Allergy and Immunology Research Centre (AIRC), Institute for Medical Research, Malaysia, the reference laboratory for specialised autoimmune tests. The Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia (MOH), approved this clinical-serological cohort study with a waiver of consent for the clinical data obtained as part of serological test validation (NMMR ID-23-03921-V3W).

Sample size calculation

The sample size calculation was performed using G*Power version 3.1.9.7. The reference frequency used is 24.1% as it is the lowest frequency of ZnT8 reported among non-Caucasians (12). As shown in Figure 1, the sample needed for the experiment is 179 samples.

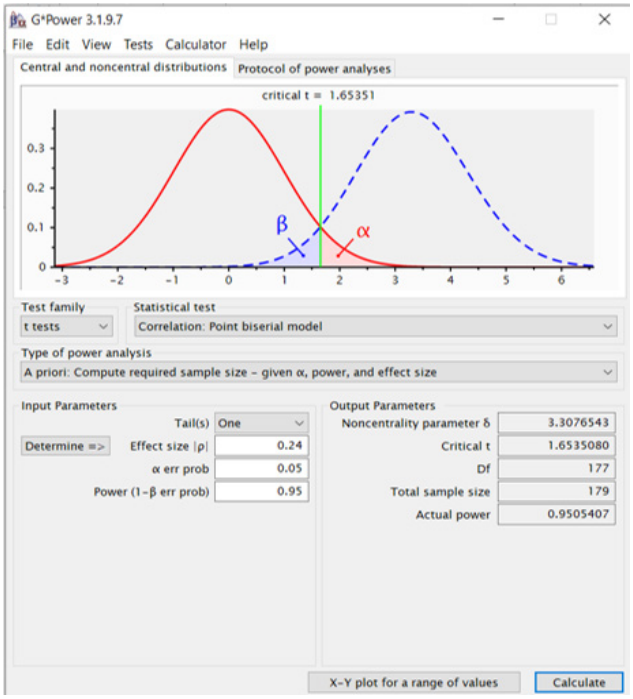

Figure 1: Sample size calculation using G*Power version 3.1.7.9. The sample size calculation was performed using the following software, with reference to 24% of T1DM patients being positive for ZnT8, as reported in a study (12). Therefore, the sample size needed for the experiment is 179.

Serum samples

Despite requiring only 179 samples, 509 archived serum samples were retrieved. The samples collected in 2019 were used in this investigation and stored at -20°C for three years. They had been previously screened for GADA, ICA, and IA-2A using ELISA kits (MEDIPAN Medizyme®, Germany, Cat. no. 3507 and 3506) for GADA and IA-2A. In contrast, ELISA kits from LS Bio were used for ICA (Cat no. LS-F55561-1). The detection ranges of the kits are as follows: GADA (5-250 IU/mL), IA-2A (10-400 IU/mL), and ICA (1-200 ng/mL). The following information was recorded in addition to DAA measurement: demographics details including age, sex, and ethnicity, as well as clinical parameters such as history of DKA and family history of diabetes (grandparents, parents, and siblings), were retrieved from the accompanying laboratory request forms. Incomplete data such as C-peptide, disease duration, and glycated hemoglobin (HbA1c) were excluded because not all samples contained this information.

Supplementary Table I provides detailed demographics and clinical characteristics. The diagnosis of T1DM was

based on the criteria outlined by MOH (4). The patients' age ranges from 1 to 76 years, with a median age of 26, as depicted in Table I. There were more adult and male patients compared to children and females. Most T1DM patients were Malay, followed by Chinese, Indian, and others. The most common antibody profile was the ICA and GADA combination. Most patients have no family history of diabetes, including siblings, parents, or grandparents. Notably, the majority of patients had a history of (DKA), a life-threatening complication of T1DM. The biochemical criteria for the diagnosis of DKA included hyperglycemia, where the blood glucose is more than 11 mmol/L or 200 mg/dL, venous pH less than 7.3 or bicarbonate less than 15 mmol/L, and ketonemia exceeding three mmol/L and/or ketonuria over two mmol/L (4).

Table I: The demographics and clinical characteristics of T1DM patients

Sample number (n)	509
Age (Median, range (year))	26±12.3 (1-76)
Paediatrics	120
Adults	389
Gender (n)	
Male	261
Female	248
Ethnicity (n)	
Malay	320
Chinese	72
Indian	60
Others	57
Autoantibody profile (n)	
ICA only	53
GADA only	8
IA-2A only	4
ICA & GADA	190
ICA & IA-2A	16
GADA & IA-2A	1
ICA, GADA & IA-2A	80
Autoantibody positivity, n (%)	
ICA	339 (66.6)
GADA	279 (54.8)
IA-2A	291 (57.2)
Autoantibody number (n)	
Zero	157
Single	65
Double	207
Triple	80
Family history of diabetes (n)	
Yes	92
No	417
History of DKA (n)	
Yes	314
No	195

ZnT8 autoantibodies ELISA

ELISA kits from Euroimun (Cat no. EA 1027-9601) were used to measure ZnT8 autoantibodies according to the manufacturer's instructions. The detection range of ZnT8 autoantibody is between 15-2000 Relative Unit (RU)/ml. Each serum sample was measured in duplicates.

Supplementary Table II shows intra- and inter-assay coefficient of variation (CV) results. The intra-assay CV was determined using calibrators 10, 25, 75, 500 and 2000 RU/ml; it was 20.3%. The inter-assay CV was determined using serum samples, which was 6.3%.

Table II: ZnT8 autoantibody in all T1DM patients

	ZnT8 positive	ZnT8 negative	p-value	Size effect	95% confidence interval
Sample number (n)	88	421			
Age (Median, range (year))	17.5 (4-48)	27 (1-76)	**<0.0001**	n.a	n.a
Age category (n)					
Paediatrics	44	76	0.4326	134.5	-1875 to 1542
Adults	44	345			
Gender (n)					
Male	46	215	**0.0096**	2.5	-198.3 to -134.7
Female	42	206			
Ethnicity (n)					
Malay	55	265	0.1438	42.31	-217.9 to 51.4
Chinese	16	56			
Indian	12	48			
Others	5	52			
Autoantibody profile (n)					
No DAA	2	155	0.2297	19.23	-72.78 to 21.35
ICA only	2	51			
GADA only	0	8			
IA-2A only	0	4			
ICA & GADA	29	161			
ICA & IA-2A	7	9			
GADA& IA-2A	0	1			
ICA, GADA & IA-2A	48	32			

CONTINUE

Table II: ZnT8 autoantibody in all T1DM patients (Cont.)

	ZnT8 positive	ZnT8 negative	p-value	Size effect	95% confidence interval
Autoantibody positivity (n, +/total)					
ICA	86 / 88	253 / 421	**0.0019**	0.5	-172.9 to -160.1
GADA	77 / 88	201 / 421	0.1596	42	-700.7 to 366.7
IA-2A	55 / 88	45 / 421	0.5167	176	-2403 to 2069
Autoantibody number (n)					
Zero	2	155	0.12	38.6	-201.6 to 39.62
Single	2	63			
Double	36	171			
Triple	48	32			
Family history of diabetes (n)					
Yes	20	72	0.3835	114.5	-1621 to 1288
No	68	349			
History of DKA (n)					
Yes	44	270	0.2185	59.5	-922.5 to 589.5
No	44	151			

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 10.3. The age and ZnT8 autoantibody concentrations between groups were compared using the Mann-Whitney test (two groups) and the Kruskal-Wallis test (more than two groups). In addition, two-way ANOVA was used to analyze two or more tables (column results). All tests were performed at a significant level of 0.05 (two-sided). In addition, the size effect of the difference and 95% confidence interval (CI) of the difference were added to each Table except Table I. The size effect of the difference is crucial because it provides a vital piece of information that complements the p-value. On the other hand, the 95% CI of the difference provides a tangible range of values that are likely to contain the actual difference between the two populations' means. A 95% CI of the difference without zero value means that the difference between groups is statistically significant at the 0.05 alpha levels.

RESULTS AND DISCUSSION

ZnT8 autoantibodies were detected among Malaysian T1DM patients.

Table II demonstrates that ZnT8 autoantibodies were detected in 88 of 509 (17.3%) Malaysian T1DM patients, indicating a lower frequency of ZnT8 autoantibodies than in other populations. The study aligned with ZnT8 frequency among Chinese (12) and Indian (13) populations, as Malaysia comprises Chinese and Indian descendants. However, despite being closer neighbors to Malaysia, ZnT8 frequencies in Indonesian (10) and Thai (11) populations may have been overestimated since the sample size was relatively small (30 and 81, respectively). An American study reported that ZnT8 frequencies in Asian Americans were lower than in Whites, Blacks, and Hispanics (14). However, other biological factors, including longer disease duration (incomplete data) (15), the absence of ZnT8-associated single nucleotide polymorphisms, SLC308A rs13266634, (16) and degraded antibodies because of extended storage (three years in -20° celsius) (17), could have contributed to the lower frequency of ZnT8 autoantibodies in Malaysian T1DM patients.

In addition to the lower frequency of ZnT8 autoantibodies, our data revealed that ZnT8-positive patients were younger than ZnT8-negative patients (Table II, median age: 17.5 versus 27 years, $p<0.0001$). The Indonesian data demonstrated a similar trend of younger patients being positive for ZnT8 compared to ZnT8-negative, although the difference is statistically insignificant (10). A Japanese study reported similar findings, in which ZnT8 autoantibodies were more frequently observed in younger patients (15). Additionally, more males were positive for ZnT8 than females (Table II, $p = 0.0096$), in contrast to a Japanese study, where more females were positive for ZnT8 than males (18). In conclusion, ZnT8-positive patients were more commonly younger males than older females. Furthermore, ZnT8 autoantibodies among Malaysian patients were co-expressed with ICA compared to ZnT8-negative patients, as shown in Table II (86 of 88 (97.7%), $p=0.0019$).

Different profiles between ZnT8-positive paediatric and adult T1DM patients

Given the difference between paediatric and adult T1DM patients [19], it is essential to focus on ZnT8 autoantibodies in both patient groups. Table III shows that ZnT8 autoantibodies were positive in 36.7% of pediatric patients, while Table IV demonstrates 11.3% of adult T1DM patients with ZnT8 autoantibodies.

As shown in Table III and IV, there is no significant difference between the ages of ZnT8-positive and ZnT8-negative populations in paediatric and adult patients, in line with previous studies (10,15).

Table III: ZnT8 autoantibody in peediatric T1DM patients

	ZnT8 positive	ZnT8 negative	p-value	Size effect	95% confidence interval
Sample number (n)	44	76			
Age (Median, range (year))	10 (4-17)	12 (1-17)	0.1574	n.a	n.a
Gender (n)					
Male	21	41	0.156	4	-66.82 to 34.82
Female	23	35			
Ethnicity (n)					
Malay	31	40			
Chinese	7	12	**0.0041**	1	-11.18 to -4.818
Indian	4	13			
Others	2	11			
Autoantibody profile (n)					
No DAA	1	15			
ICA only	1	8			
GADA only	0	1			
IA-2A only	0	1	0.3239	3.77	-12.92 to 4.915
ICA & GADA	8	31			
ICA & IA-2A	8	6			
GADA& IA-2A	0	0			
ICA, GADA & IA-2A	26	14			
Autoantibody positivity (n, +/ total)					
ICA	43/44	59/76	0.097	3	-57.12 to 19.12
GADA	14/44	46/76	0.5	16	-219.3 to 187.3
IA-2A	42/4	21/76	0.7042	37	-486.1 to 454.1
Autoantibody number (n)					
Zero	1	15			
Single	1	10	0.3422	7.106	-30.62 to 14.62
Double	16	37			
Triple	26	14			
Family history of diabetes (n)					
Yes	8	12	0.4097	12	-168.5 to 136.5
No	36	64			
History of DKA (n)					
Yes	24	47	0.2625	7	-104.9 to 72.94
No	20	29			

Table III focuses on the differences between Znt8-positive and ZnT8-negative groups in paediatric patients. The two-way ANOVA test revealed significant differences between ethnic groups, with a higher proportion of Malays being positive for ZnT8 (43.7%) compared to the other ethnic groups: Chinese (36.8%), Indian (23.5%), and Others (15.4%).

On the other hand, adult T1DM patients showed a different ZnT8 autoantibody profile compared to paediatric patients. A higher proportion of males among adult T1DM patients were positive for ZnT8 autoantibodies compared to females, as shown in Table IV (p=0.0106), in contrast to a previous study (15). The difference could be explained by the fact that Malaysia consists of a more heterogeneous ethnic group compared to Japan, which has a more homogenous ethnic group. Furthermore, ZnT8 autoantibody is strongly associated with ICA positivity, as shown in Table IV (p=0.0063). Although ICA was the first autoantibody discovered in confirming T1DM diagnosis, it lacks the specificity compared to ZnT8, which is in the insulin secretory granule membrane (20).

Table IV: ZnT8 autoantibody in adult T1DM patients

	ZnT8 positive	ZnT8 negative	p-value	Size effect	95% confidence interval
Sample number (n)	44	345			
Age (Median, range (year))	27 (18-48)	29 (18-76)	0.1521	n.a	n.a
Gender (n)					
Male	25	173	**0.0106**	2.5	-182.3 to -118.7
Female	19	172			
Ethnicity (n)					
Malay	24	225			
Chinese	9	43	0.1709	41.97	-208.8 to 58.31
Indian	8	36			
Others	3	41			
Autoantibody profile (n)					
No DAA	1	139			
ICA only	2	43			
GADA only	0	7			
IA-2A only	0	5			
ICA & GADA	20	129	0.0954	19.53	-83.81 to 8.556
ICA & IA-2A	1	3			
GADA& IA-2A	0	0			
ICA, GADA & IA-2A	20	19			

CONTINUE

Table IV: ZnT8 autoantibody in adult T1DM patients (Cont.)

	ZnT8 positive	ZnT8 negative	p-value	Size effect	95% confidence interval
Autoantibody positivity (n, +/total)					
ICA	42/44	194/345	**0.0063**	1.5	-169.6 to -131.4
GADA	40/44	155/345	0.1475	35.5	-601.6 to 300.6
IA-2A	21/44	27/345	0.4781	144.5	-1987 to 1686
Autoantibody number (n)					
Zero	1	139	0.0935	30.99	-173.9 to 23.37
Single	2	55			
Double	21	132			
Triple	20	19			
Family history of diabetes (n)					
Yes	12	64	0.3689	98.5	-1402 to 1101
No	32	281			
History of DKA (n)					
Yes	20	224	0.2174	53.5	-830.3 to 529.3
No	24	121			

Study limitations

Our study has several limitations. Firstly, as the samples included in this study were archived, some clinical parameters were incomplete. Some relevant clinical data, such as HbA1c, fasting blood glucose (FBG), C-peptide, and disease duration, could not be included; therefore, some of the associations between ZnT8 and clinical parameters could not be analyzed. Secondly, the IAA measurement was not carried out as part of the DAAs testing at the Allergy and Immunology Research Centre (AIRC), Institute for Medical Research (IMR); therefore, this study may have missed other T1DM patients. The reason why IAA measurement was not performed is a technical reason since no IAA kit was approved by the Medical Device Agency (MDA) during the sample collection. Finally, since the disease duration data is missing, some adult patients may be classified as latent autoimmune diabetes in adults (LADA) patients instead of T1DM patients.

CONCLUSION

ZnT8 autoantibodies were detected in 17.3% of Malaysian T1DM patients, with a significantly higher prevalence in paediatric (36.7%) compared to adult (11.3%) patients. Among paediatric patients, positivity was more common in Malays, while in adults, ZnT8 positivity was associated with female gender. The findings suggest that ZnT8 autoantibodies may serve as a potential alternative to ICA testing in adult patients but not in children. Given the overall low prevalence, routine inclusion of ZnT8 in DAA screening panels for all Malaysian T1DM patients is not recommended. However, ZnT8 testing may be beneficial in selected seronegative cases, particularly when considering patient age, gender, and ethnicity. These results highlight the importance of personalized diagnostic approaches in T1DM management.

ACKNOWLEDGEMENT

The authors thanked the Director-General of Health Malaysia for permission to publish this article. Furthermore, the authors thanked the Ministry of Higher Education for financial support under the Fundamental Research Grant No. FRGS/1/2022/STG03/UMP/02/1 (UMPSA reference RDU220106).

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