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Research article

The early detection of type 1 diabetes mellitus and latent autoimmune diabetes in adults (LADA) through rapid test reverse-flow immunochromatography for glutamic acid decarboxylase 65 kDa (GAD_{65})

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ABSTRACT

Background: Diabetes mellitus (DM) is a chronic and costly disease that has become a primary concern worldwide. Type 1 diabetes mellitus is categorized as an autoimmune disease, which results in islet cell apoptosis and insulin-dependent. GAD_{65} is known as a potential marker of impaired pancreatic β cell function that appears in the initial phase of type 1 DM and latent autoimmune diabetes in adults (LADA). This study aimed to develop a novel rapid test of anti-GAD_{65} autoantibodies in human serum samples.

Methods: We have developed a rapid test for anti-GAD65 autoantibodies in this assay based on the reverse-flow immunochromatography method. Human recombinant-protein antigen for GAD_{65} was attached as the control line over the nitrocellulose membrane. On the other side, the goat anti-mouse immunoglobulin G (IgG) was coated on the same membrane as a control line. The positive result for GAD_{65} was confirmed by a colloidal gold signal on the strip. Our novel assay analyzed 276 healthy subjects and 51 type 1 diabetes individuals serum samples from several ethnicities in Indonesia for this study.

Results: Among the 276 healthy samples, 225 samples were identified as positive for anti-GAD_{65} autoantibodies, while 51 samples were negative. Interestingly, the positive results for anti-GAD_{65} autoantibodies were linear to the decreasing of high-density lipoprotein (HDL) levels and inversely associated with triglyceride levels. A significant correlation with age was observed in all groups. The sensitivity and specificity test proved that this kit has higher accuracy (AUC value = 0.960).

Conclusion: The significant advantages of our rapid test for anti-GAD_{65} autoantibodies provide higher sensitivity, specificity, and stability compared to previous commercial kits. Therefore, it could be proposed as the future clinical diagnostic kit for patient management of type 1 DM.

1. Introduction

Type 1 diabetes mellitus (T1DM) has also been established as an incomplete clinical explanation for the etiology. Clinically, T1DM and T2DM cannot be distinguished easily, even though T1DM is more often observed in childhood than in adulthood. Indeed, the previous study showed that the incidence of T1DM in the younger age group (>15 years old) increased significantly, particularly among males [1]. T1DM pre-dominantly occurs in the younger population [2] related to the reduction of high-density lipoprotein (HDL) function [3] and the alteration of cardiovascular structure and function [4, 5], and strongly associated with mortality-linked cardiovascular disease [6, 7].

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In Indonesia, the prevalence of DM has increased gradually during the last two decades. Previous data reported an equal percentage of mortality caused by diabetes in males and females in Indonesia, consisting of 6% of the total population [8]. The significant changes of metabolic performance in the Indonesian population may correlate with this higher incidence [9, 10, 11]. Linear to the increase of diabetes prevalence, the community health program of the Indonesian Ministry of Health was launched to produce a robust biomarker for early detection of diabetes. Therefore, a reliable clinical prognostic biomarker, which is inexpensive, with higher specificity, sensitivity, and rapid detection, is required.

As a chronic autoimmune disease, T1DM is characterized by a lack of insulin production due to a decrease of the pancreatic β-cell population within the islet of Langerhans. Recently, the primary role of 65 kDa glutamic acid decarboxylase (GAD_{65}) has been thought to play a pivotal role in the pathogenesis of T1DM [12, 13, 14]. The clinical manifestation of higher levels of serum GAD_{65} in the asymptomatic subject to diabetes indicates the early onset of T1DM [15, 16]. GAD_{65} is known as a potential marker of impaired pancreatic β cell function that appears in the initial phase of T1DM [17]. GAD antibodies are also reported to have a robust capability in detecting the progressiveness of this disease in patients compared to other markers, including islet cell Antibody (ICA) and insulin autoantibody (IAA/I-A2) with a similar property to GAD_{65} [12,18]. Thus, GAD_{65} is the potential serological protein biomarker to detect pre-diabetic incident in patients, particularly in the younger population, and a promising clinical diagnostic factor development target [16].

Several previous studies have shown that developing an early biomarker of diabetes is crucial and urgently needed in the clinical setting. The early detection of T1DM using the rapid lateral flow immunoassay method for tyrosine phosphatase-like protein IA-2 (IA-2As) and GAD_{65} autoantibodies by electrochemiluminescence assay was found to be a fast, sensitive, and specific test for T1DM prevention [19, 20]. Further, the improvement of clinical diagnosis through a radio-binding assay using radiolabeled GAD_{65}(96–585) has been reported to be quite sensitive to detect individuals with high risk for T1DM [21]. Even though some findings proposed new methods for GAD_{65} autoantibody detection, a significant challenge of T1DM rises. The extremely high cost for the GAD_{65} test in Indonesia ($150 per analysis) results in the permanent problem of the patient regarding routine medical check-up in the clinic. Hence, the novel innovation of a sensitive GAD_{65} kit with a reasonable cost is needed. This study provides an alternative approach for rapid detection of anti-GAD_{65} autoantibodies and may decrease the expanding expense resulting from the higher clinical incidence of T1DM in the Indonesian population. Moreover, this study could offer a novel rapid test kit for autoimmune disease in particular T1DM.

2. Methods

2.1. Study population

The population study was an ongoing clinically-based case-control study. This work was conducted in collaboration with the Faculty of Veterinary Medicine of Brawijaya University, Biosains Institute of Brawijaya University, Biofarma Pharmaceutical Company of Bandung-Indonesia, Bromo Health Clinics of Malang-Indonesia, Dr. Syaiful Anwar Hospital of Malang-Indonesia, and the Laboratory of Clinical Pathology Faculty of Medicine of Brawijaya University. This study was conducted at the Biosains Institute of Brawijaya University from 2012 to 2017. The samples consist of 276 healthy subjects and 51 individuals with type 1 diabetes. The exclusion criteria for healthy samples was subject with type 2 diabetes mellitus (T2DM), overweight and obesity, active smoker, serious illnesses affecting the immune system, medications suppressing the immune system, and subjects with hypertension. The inclusion criteria for the same samples were individuals with normal fasting glucose, normal blood pressure, normal body mass index (BMI), and non-smoking 245 g. In addition, the exclusion criteria for 51 cases of a person with T2DM, overweight and obesity, active smoker, serious illnesses-related the immune system, patients with immunosuppressor medications, and subjects with hypertension. Moreover, the inclusion criteria were subjects with insulin-dependent, normal blood pressure, normal BMI, and non-smoking. This study was approved by the Research Ethics Committee (Institutional Review Board (IRB)) of Brawijaya University (No. 109/EC/KEPK/04/2012). Written informed consent was obtained from the local Indonesian patients/subjects.

2.2. Serum sample collection

A total of 20 healthy subjects participated in the first blood collection in the early stages of GAD_{65} kit manufacturing. Informed consent was obtained from these patients and followed by blood sample collection.

2.3. GAD_{65} gene isolation and amplification

DNA isolation was performed based on the manufacturer's protocol (DNA Nucleospin Tissue -Macherey-Nagel, cat. Number: 740952.50). Then, the amplification of GAD_{65} sequence was conducted using GAD_{65} primers. The polymerase chain reaction (PCR) was set up in the initial step (pre-denaturation) at 95 °C for 7 min and continued for 35 cycles of denaturation at 95 °C for 30 s with an annealing temperature of 60 °C for 20 s. In the final step of PCR, the elongation temperature was set to 72 °C for 30 s, and then it was maintained at 60 °C. The confirmation of specific sequence encoding GAD_{65} was performed by PstI enzyme and agarose gel electrophoresis.

2.4. Gene cloning of GAD_{65} by heat transformation

The GAD_{65} gene cloning was conducted using competent cell *Escherichia coli* strain *BL21*. In the initial step, 2 μL of pQE T-7® cloning reaction was added to the One Shot® solution and mixed gently. The sample was incubated on ice for 5–30 min. The second step was performed by combining 2 μL of PCR product with the previous sample solution (one Shot® solution and pQE T-7® cloning solution). The cells were conditioned to heat shock for 45 s at 42 °C without shuffling, then placed on ice for 2 min. This was followed by a normal temperature mixing process with 250 μL of Super Optimal broth with Catabolite repression (S.O.C.) medium and centrifuged at 200 rpm. A small volume of transformed cells (10–50 μL) was cultured on a selective solid agar medium and incubated overnight at 37 °C.

2.5. Bacterial culture, protein extraction, and protein product analysis

In the first step, 400 μL of lysogeny broth (LB) medium was acclimatized at 37 °C for 1 h and mixed with 100 μL of cells from the selected colonies. Then, this sample was incubated at 37 °C with 250 rpm shift speed for 24 h. The next day, phosphate-buffered saline with Tween (PBST) containing 4 mM of phenylmethane sulfonyl fluoride (PMSF) as much as five times the volume. The tube was centrifuged at 6,000 rpm at 4 °C for 15 min to homogenize this sample. The supernatant was collected and added to cold ethanol with a ratio of 1:1, then incubated for 24 h in the refrigerator. After overnight incubation, the sample was centrifuged at 10,000 rpm for 10 min at 4 °C. The ethanol was removed, and the precipitate was dried and mixed with 50 μl of Tris-Cl buffer. The human-recombinant GAD_{65} protein was frozen at -20 °C. The pure GAD_{65} recombinant protein was obtained using Biologic DuoFlow (Biorad).

2.6. The preparation of goat anti-mouse (GAM) antibody

The goat anti-mouse antibody was produced from mouse serum and immunization in the Boer goat strain. Briefly, the antigen was isolated from mouse serum and mixed with complete and incomplete Freund's adjuvant before the immunization step. The mix solution was homogenized by vortex and emulsified for 60 min. Then, the antigen protein within Complete Freund's Adjuvant (CFA) was injected into Boer goats on

the dorsal subcutaneous area for the first immunization. The second and third immunization was done with the same method with incomplete Freund's Adjuvant. To obtain the antibody protein, goat serum was isolated from the jugular vein with a disposable syringe. The antibody purification was done by SAS (saturated ammonium sulfate) 50% precipitation. An immunoglobulin G (IgG) sample was collected and stored in the fridge before the next application.

2.7. The colloidal gold signal reagent preparation

The initial step for colloidal gold signal reagent preparation was performed by mixing 1.5 mL of 1% gold chloride [gold (III) chloride trihydrate, Sigma G4022] solution with 2.625 mL of Tri-Na citrate (citric acid trisodium salt, anhydrous, Sigma C3674) solution. The mixing solution was boiled and shaken gently. The sample was monitored until the solution changed to a purplish color. The sample was cooled, and the pH adjusted from 6.8 to 6.9. The colloidal gold was then coupled with Protein A from *Staphylococcus aureus* (Sigma P6031). For the final stage, the sample solution was added to 1% BSA from the total volume of sample solution (BSA protease-free, Sigma A3294).

2.8. The preparation of human-recombinant GAD_{65} to immunochromatography based test

The advanced development of the human recombinant GAD_{65} kit was performed as reverse-slow immunochromatography-based analysis shown in the schematic illustration in Figure 1.

This kit consists of two main sections. The first section is the tested area, which consists of the polyester membrane, nitrocellulose membrane for the sample placement test with control line area (coated by goat anti-mouse IgG), and a test line area (covered by the human-recombinant protein of GAD_{65}) and sample pad. The second part is the observation area with absorbent paper and signal reagent area. This section was prepared to allow the signal reagent solution to work on it, while the buffer solution was used to modulate the immunochromatography system. The general overview of rapid tests is generated based on the concept in Figure 1. The details of our manual protocol for this work were arranged by our previous work [22]. To obtain the best result, the kit was incubated at room temperature for a while to allow the sample and buffer to mix gently. At the final step, the test card was immediately closed and incubated for 20–30 min. The positive results were confirmed by two red lines in the control line and test line areas. In contrast, the negative results were observed by a single line in the control line area.

2.9. Data analysis

After data collection, all data were analyzed using statistical software (SPSS version 20.0, IBM). The data are presented as mean ± SD for continuous variables. Overall, to compare differences between groups, unpaired t-test analysis was used. The correlation between anti-GAD_{65} autoantibodies and other predictors was determined by Pearson's correlation (normally distributed data). For further statistical analysis, logistic regression was used to test the influence of clinical data and anti-GAD_{65} autoantibodies on healthy and diabetic patients. A P value less than 0.05 was considered statistically significant.

3. Results

3.1. Assay optimization of anti-GAD_{65} autoantibodies rapid test kit

A representative of negative and positive results with reverse flow rapid test for GAD_{65} autoantibodies is shown in Figure 2.

3.2. Baseline characteristics of the study participants

To validate our rapid test anti-GAD_{65} autoantibodies kit, we perform multiple tests in a more extensive population with different ethnicities in the Indonesian community and several ethnicities from different countries (data not shown). A total of 327 Indonesian subjects were proposed based on their age, ranging from 1 to 30 years old, and divided into three categories: healthy negative for anti-GAD_{65} autoantibodies, healthy positive for GAD_{65} autoantibodies, and diabetic individuals positive for anti-GAD_{65} autoantibodies. Approximately 70% of our samples were collected from female individuals, especially in the younger population

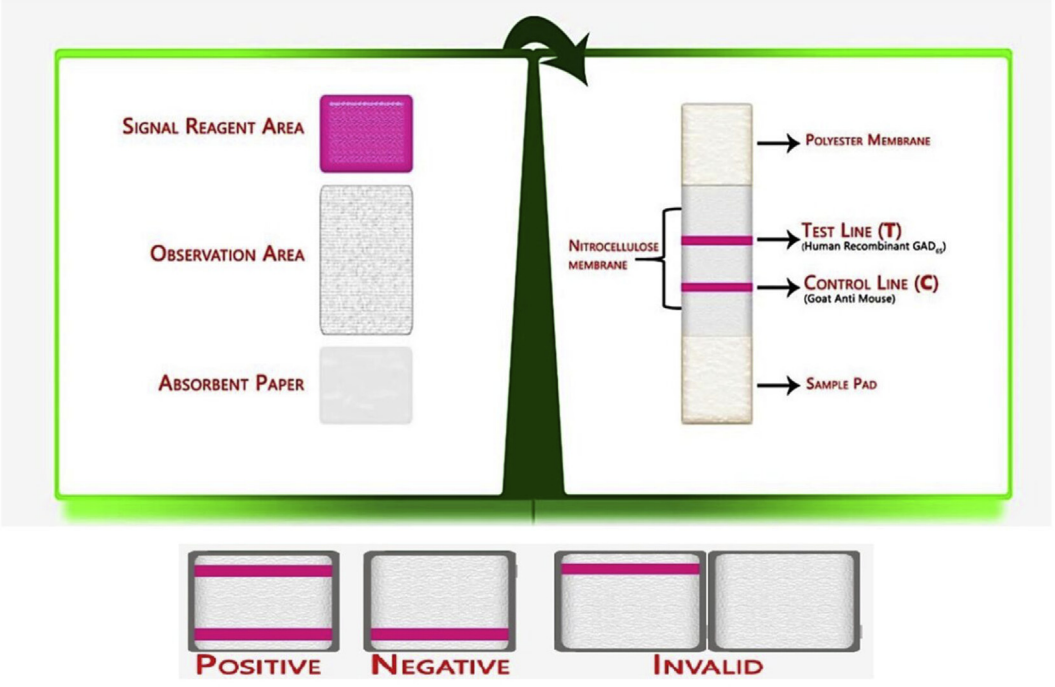

Figure 1. Reverse flow-immunochromatography-based test model for detecting anti-GAD_{65} autoantibodies antibody from human sera.

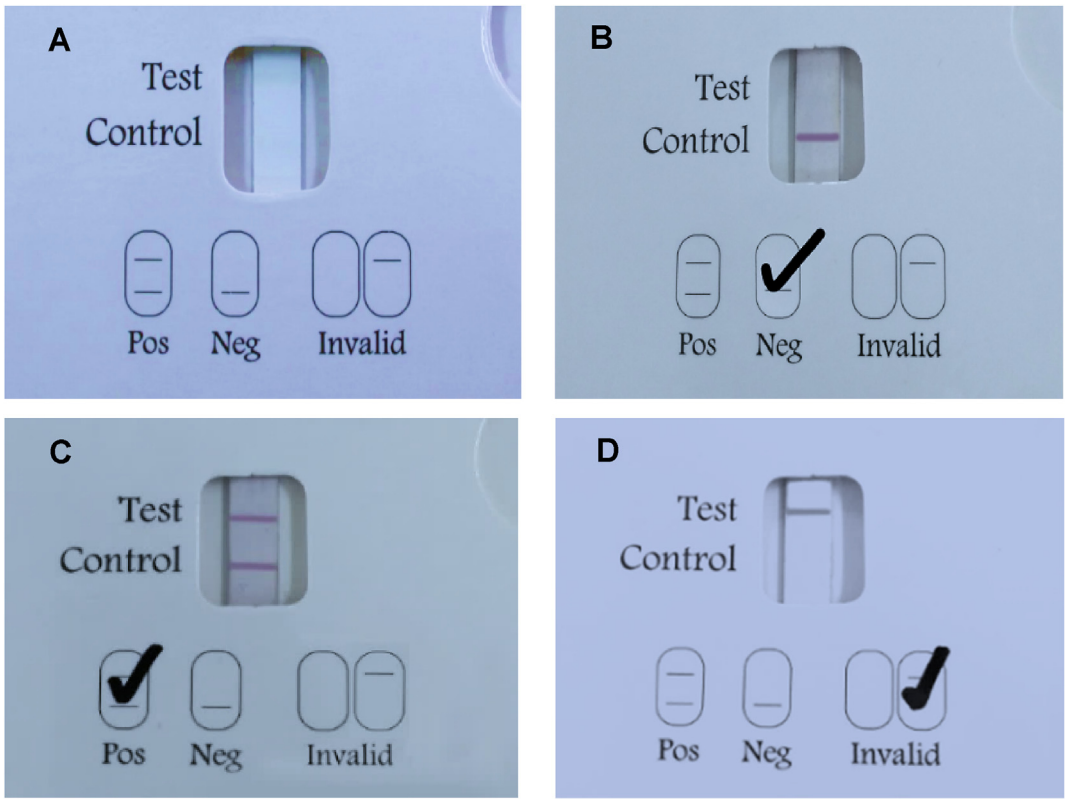

Figure 2. The representative results of anti-GAD_{65} autoantibodies detection from human sera by reverse flow-immunochromatography. (A). The kit without buffer/signal solution. (B). Negative/control result with a signal solution. (C). Positive signal is observed with two lines, and (D). invalid results are confirmed by one line on the test line area.

Table 1. Patients' baseline characteristics by group.

Characteristic	Healthy		Diabetic
	GAD_{65} (-)	GAD_{65} (+)	GAD_{65} (+)
Total Samples	51	225	51
Age[a]	23.51 ± 2.94	22.46 ± 4.14	11.67 ± 5.28
0–14 yrs	0 (0)	9 (4)	38 (74.5)
15–24 yrs	35 (68.6)	157 (69.8)	11 (21.6)
> 24 yrs	16 (31.4)	59 (26.2)	2 (3.9)
Gender[b]			
Male	14 (27.5)	58 (25.8)	22 (43.1)
Female	37 (72.5)	167 (74.2)	29 (56.9)
DM history[b]			
Yes	27 (52.9)	92 (45.1)	46 (90.2)
No	24 (47.1)	112 (54.9)	5 (9.8)
Ethnic[b]			
Java	44 (86.3)	190 (84.4)	48 (94.1)
Sumatera	4 (7.8)	17 (7.6)	0 (0)
Sulawesi	0 (0)	7 (3.1)	0 (0)
Kalimantan	1 (2)	4 (1.8)	0 (0)
Sumbawa	1 (2)	3 (1.3)	0 (0)
Other	1 (2)	4 (1.8)	3 (5.9)
GAD_{65} (+)[b]	0 (0)	225 (100)	51 (100)

[a] Data are presented as mean ± SD.
[b] Data are presented as the number (percentage) of study patients unless otherwise indicated.

Table 2. Univariate correlation with the anti-GAD_{65} autoantibodies level in healthy and diabetic subjects.

	Univariate Correlation		Multivariate Logistic Regression	
	r	*P-value*	OR	*P-value*
Age	-0.799	0.000*	0.513	0.000*
Gender	-0.164	0.099	0.092	0.027*
DM history	0.353	0.000*	25.339	0.101
Ethnic	-0.096	0.338	1.191	0.534

* Significance at level 0.05.

(Table 1) and were dominated by subjects with a positive family history of diabetes (40%–90%). Further, our samples originated from six different ethnicities, including Java (84–90%), Sumatera (7%), Sulawesi (3%), Kalimantan (2%), Sumbawa (2%), and others (2%–5%). In addition, the general serologic profile of healthy GAD_{65} (-), GAD_{65} (+), and type 1 DM GAD_{65} (+) subjects was analyzed to determining the early development of diabetes in our healthy patients compared to the diabetic individuals (Figure 3).

The serum level of triglycerides significantly increased ($p < 0.05$) in healthy patients with anti-GAD_{65} autoantibodies (+). By contrast, the concentration of HDL decreased gradually in the same group ($p < 0.05$). There is no significant difference for low-density lipoprotein (LDL), total cholesterol, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) between healthy individuals negative and positive for anti-GAD_{65} autoantibodies. In addition, a significant increase in LDL and ALT was observed in patients with T1DM.

3.3. Univariate correlation and multivariate regression analysis

In this study, we also aimed to explore whether anti-GAD_{65} autoantibodies have a significant correlation with the criteria of our baseline data. Interestingly, we found that anti-GAD_{65} autoantibodies level has a

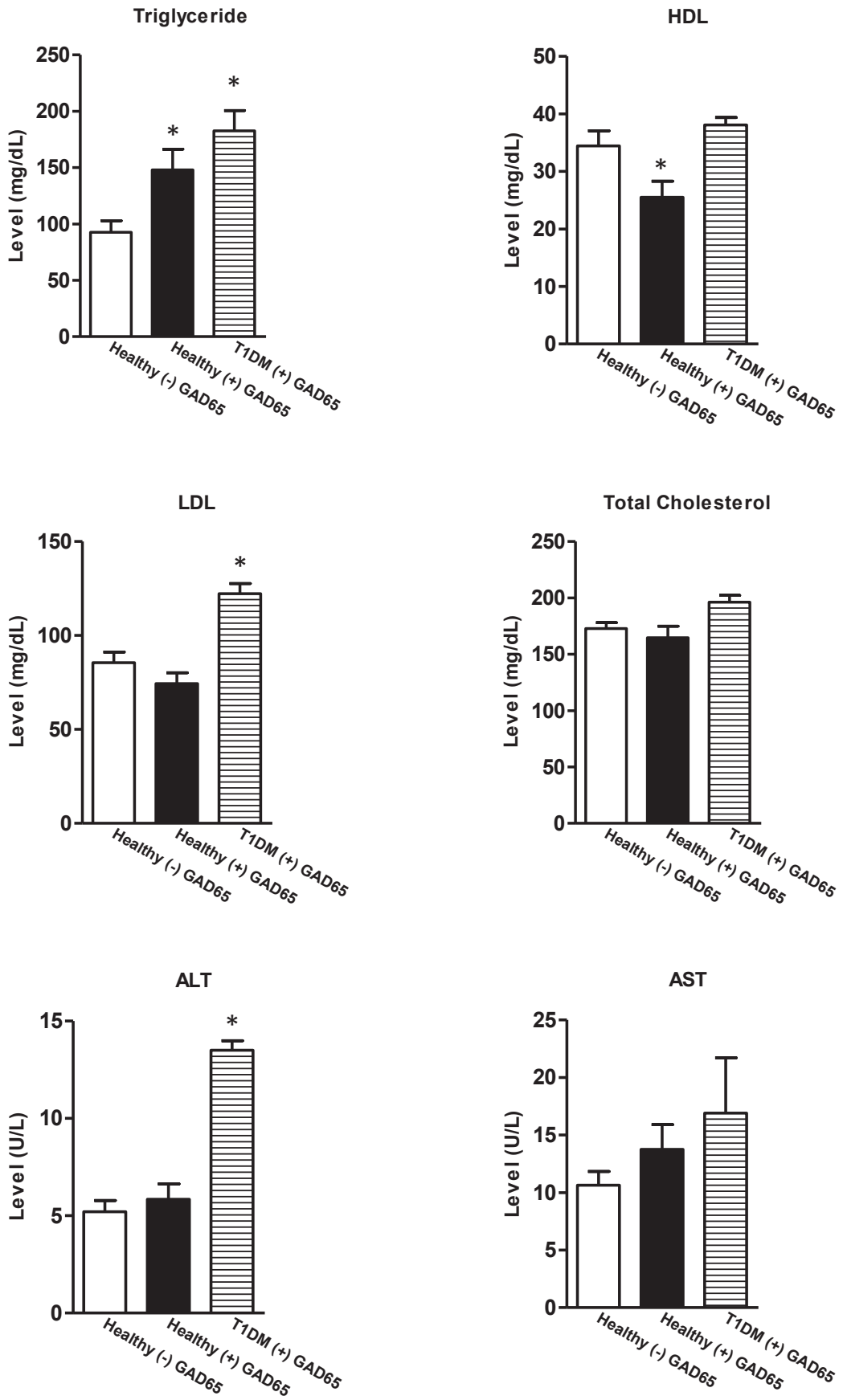

Figure 3. The metabolic profile of healthy subjects with anti-GAD_{65} autoantibodies (-) and anti-GAD_{65} autoantibodies (+). *P value is considered significant at 0.05.

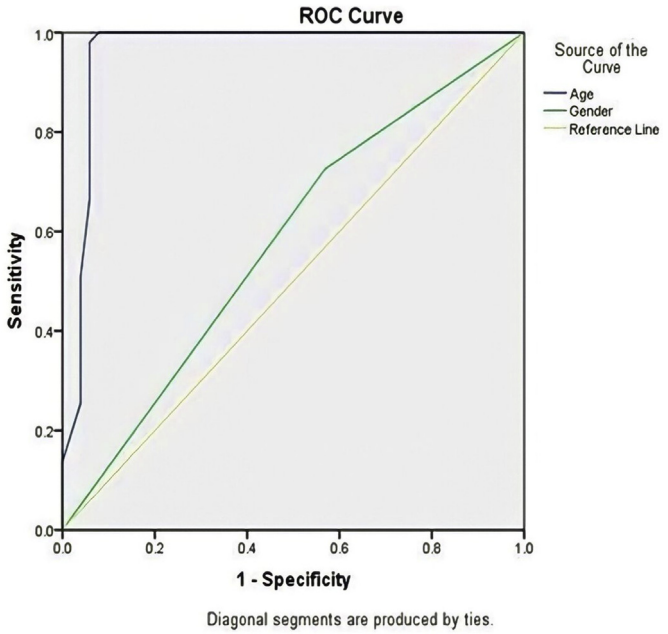

Figure 4. Receiver operating curve (ROC) analysis. The area under the curve (AUC) of reverse flow rapid test/kit for anti-GAD_{65} autoantibodies showed the sensitivity and specificity with age (AUC = $_{0.960}$) and less sensitivity and specificity for gender (AUC = $_{0.578}$).

significantly negative correlation with age (r = -0.799, p = 0.000) and is positively associated with the DM history of our patients (r = 0.353, p = 0.000) (Table 2). However, we did not find a significant association between anti-GAD_{65} autoantibodies and gender (r = -0.164, p = 0.099) or ethnicity (r = -0.096, p = 0.338). In addition, to verify the independent association between parameters, multivariate logistic regression analysis was performed. Importantly, our data showed that age strongly associated with anti-GAD_{65} autoantibodies in the circulation (OR = 0.513, p = 0.000) in line with gender (OR = 0.092, p = 0.027). The direct relationship between health conditions with anti-GAD_{65} autoantibodies level and its confounding variables was shown using the odds ratio (OR). The ORs are obtained by taking the exponential transformation on the estimates of regression coefficients, which are revealed in Table 2.

3.4. Rapid-test reverse flow immunochromatography for anti-GAD_{65} autoantibodies as a potential diagnostic kit for type 1 DM and LADA detection

Specificity and sensitivity are the essential requirements for a medical diagnostic and prognostic kit. Here, to confirm sensitivity and specificity, receiver operating curve (ROC) analysis was performed (Figure 4). Very importantly, age showed a significant correlation to anti-GAD_{65} autoantibodies (AUC = 0.960), while it was less sensitive and specific to gender (AUC = 0.578). Our data indicate the reverse flow rapid test kit for anti-GAD_{65} autoantibodies is potentially useful for the early detection of T1DM.

4. Discussion

The presence of higher titers of anti-GAD_{65} autoantibodies in the circulation could be positively associated with the progression of T1DM in the younger population and LADA in the older population. It is important to note that the activity of anti-GAD_{65} autoantibodies alone showed a pivotal role in T1DM pathogenesis. The higher level of anti-GAD_{65} autoantibodies was well-established to correlate with long-standing diabetes [23, 24]. We hypothesized that the prolonged exposure to higher anti-GAD_{65} autoantibodies titers in our patients significantly reduced the physiological homeostasis resulting from pancreatic beta-cell dysfunction and changes in serum lipid profiles. Our article reports for the first time an essential improvement in the early detection of T1DM, in particular for the Indonesian population using reverse flow immunochromatography for anti-GAD_{65} autoantibodies.

Importantly, this study has proven that our rapid anti-GAD_{65} autoantibodies kit could detect the gradual alteration of anti-GAD_{65} autoantibodies levels in several ethnicities of the Indonesian community. This kit showed a rapid assay for the clinical diagnosis of T1DM with higher sensitivity and specificity to age in all subjects or participants by almost 96% accuracy. Linear to our hypothesis, the utilization of our anti-GAD_{65} autoantibodies rapid test kit can detect the first progression of T1DM in the younger population supported by the significant negative correlation between age and anti-GAD_{65} autoantibodies level. Increased anti-GAD_{65} autoantibodies levels in the circulation will be induced two times to fold probability to develop to T1DM in the younger population. Linear to the previous study, the increase of anti-GAD_{65} autoantibodies titers in our serum samples in healthy subjects with positive detection for anti-GAD_{65} autoantibodies were strongly and significantly associated with lipid profiles. Gradual changes of HDL function in the larger population appeared in the early symptoms of the younger age group with T1DM and became persistent in these patients [3]. In this study, we also found that the lower levels of serum HDL showed a negative association with the serum triglyceride levels, which were significantly higher in healthy subjects with positive anti-GAD_{65} autoantibodies. We speculate that the elevation of circulating triglyceride levels caused by the reduction of HDL function leads to its clearance and removal to the liver. However, further clinical study is required to clarify our theories regarding whether the higher titers of anti-GAD_{65} autoantibodies indirectly decreased the lipid clearance and induced lipotoxicity.

In addition, several studies have been performed in the last few decades to develop a rapid assay for clinical diagnosis based on a specific molecular biomarker for T1DM. Recent data has shown that the molecular test is available for the detection of LADA and DM development in the younger population. In general clinical diagnosis, the T1DM incident was predicted by radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), lateral flow immunoassay (LFIA), electrochemiluminescence assay (ECLA), micro-IAA radioassay, radioligand binding assay (RBA), proximity ligation assay, time-resolved fluorescence immunoassay (TRFIA), and other detection methods [13, 17, 19, 20, 25, 26]. However, all of the previous techniques were classified as time-consuming methods (45 min–24 h incubation and detection process), which required special equipment (LFIA reader, ELISA reader) and had a high cost. Therefore, developing a sensitive and specific rapid test for T1DM diagnosis is a primary goal for clinicians.

In contrast to some current findings for anti-GAD_{65} autoantibodies kit or detection method, the current study showed the definite progression for rapid detection of an autoimmune defect in T1DM. The advantages of our anti-GAD_{65} autoantibodies rapid test can reduce the testing time (20–30 min incubation at room temperature), does not require specialized equipment for the serological measurement and is cheaper than all previous assay/methods. In this study, the anti-GAD_{65} autoantibodies kit is a non-radioactive assay with a single-step procedure, which eliminates the washing process, and can be directly visually observed after the last step of the assay. Taken together, our rapid test kit for anti-GAD_{65} autoantibodies can be useful in the regular clinical screening of subjects with a high risk of developing T1DM. The package could facilitate serum screening or collection directly in the medical field. In future investigations, we plan to expand our clinical study by involving several ethnicities in another region, such as the Association of Southeast Asian Nations (ASEAN), Hispanic, or American populations. The measurement of anti-GAD_{65} autoantibodies in these ethnicities may underlie our kit's universal sensitivity and specificity as a future clinical diagnostic tool with higher accuracy and precision.

5. Conclusion

In summary, we have developed and invented a novel rapid test kit based on the chromatography model, which detects significant changes of anti-GAD_{65} autoantibodies in human circulation, especially in the younger age group. The essential key point is its fast and straightforward readout (20–30 min) assay and lower cost than other methods and commercial kits. Thus, we recommend this kit for routine screening of at-risk patients and diagnostic assessment of T1DM directly as the prevention of a severe condition in patients due to autoimmune disease.

Declarations

Author contribution statement

Aulanni'am Aulanni'am, Dyah Kinasih Wuragil, Hendra Susanto: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Anita Herawati, Yulianto Muji Nugroho, Wahyu Nur Laili Fajri, Perdana Finawati Putri, Susiati Susiati: Contributed reagents, materials, analysis tools or data.

Jerry Dwi Trijoyo Purnomo, Ahmad Taufiq: Analyzed and interpreted the data; Wrote the paper.

Djoko Wahono Soeatmadji: Conceived and designed the experiments; Analyzed and interpreted the data.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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