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SHORT COMMUNICATION

Method Performance Verification of Anti-GAD65 and Anti-Insulin antibody Assays

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SUMMARY

***Background*: The aim of this study was to evaluate the performance of chemiluminescence immunoassays for anti-GAD65 and anti-insulin antibodies following user verification guidelines.**
***Methods*: The analytical performance of anti-GAD65 and anti-insulin antibodies using a MAGLUMI 2000 analyzer was verified following user verification guidelines by the Clinical and Laboratory Standards Institute.**
***Results*: Performance specifications including precision, linearity, carry-over, cutoffs for positive results, reference intervals, and comparability with pre-existing commercially available radioimmunoassays using patient specimens and certified reference material were verified (coefficients of variation for precision of anti-GAD65 and anti-insulin antibodies were 2.6% and 3.4%, respectively). Comparability assessed using clinical serum specimens showed overall agreement with radioimmunoassay of 87.2% (95% confidence interval 74.8% - 94.0%) for the anti-GAD65 antibody assay and 85.4% (95% confidence interval 71.6% - 93.1%) for the anti-insulin antibody assay.**
***Conclusions*: The results of this study verified the analytical performance of MAGLUMI anti-GAD65 and anti-insulin antibody assays for clinical use.**
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INTRODUCTION

Type 1 diabetes (T1DM) is an autoimmune disease characterized by destruction of the insulin-producing β-cells of the pancreas in genetically predisposed individuals [1]. The presence of anti-islet autoantibodies, including those against glutamic acid decarboxylase (GAD) 65, islet or insulinoma-associated antigen, insulin, zinc transporter 8, and islet cell autoantigen are used in clinical practice for diagnosis, prediction, and management of type 1 diabetes [2]. Meanwhile, among T2DM patients, there is a specific type with positive auto-antibodies characterized by adult onset, presence of islet auto-antibodies, insulin independence at the time of diagnosis, and rapid decline in β-cell function [3]. Measurement of anti-islet autoantibodies and anti-insulin an-

tibodies is important due to the need for insulin treatment in such patients [2,3]. In Korea, the overall incidence of T1DM has increased by 3% to 4% every year from 2007 to 2017 [3,4], suggesting increased importance of diagnostic modalities.

According to the Healthcare Bigdata Hub by the Health Insurance Review & Assessment Service, 21,186 anti-GAD antibody tests were performed in 2020, using radioimmunoassays, while only 1,210 tests were performed using other methods in Korea (https://opendata.hira.or.kr/home.do). The Islet Autoantibody Standardization Program, an international collaborative effort for improving the performance of assays measuring T1DM-associated autoantibodies, has reported that nonradioactive anti-GAD65 immunoassays are on par or superior to radioimmunoassays [5].

However, there have been few studies in Korea regarding the performance of chemiluminescence immunoassays for quantitative detection of anti-GAD65 and anti-insulin antibodies using MAGLUMI 2000 analyzers. Therefore, we followed user verification guidelines of the Clinical Laboratory Standards Institute (CLSI) to evaluate the analytical performance of anti-GAD65 and anti-insulin antibody kits in this study [6-10]. We aimed to evaluate the precision, linearity, carry-over, cutoffs for positive results, reference intervals, and comparability of anti-GAD65 and anti-insulin antibody assays on the MAGLUMI 2000 platform.

MATERIALS AND METHODS

Specimens

During March 2021, we obtained residual blood samples from Korean adults left over from clinical laboratory tests including anti-GAD65 and anti-insulin antibody tests requested to Green Cross Laboratories. In order to investigate the comparability between existing radioimmunoassays for anti-GAD65 and anti-insulin antibody test, aliquots from left over fresh serum specimens were analyzed the same day. Hemolyzed, turbid, clotted, and contaminated specimens on gross examination which can lead to false-positive or false-negative anti-GAD65 and/or anti-insulin antibody test results and were excluded from this analysis. This study was conducted according to the guidelines of the Declaration of Helsinki. The Institutional Review Board (IRB) of Green Cross Laboratories exempted this study (IRB No.: GCL-2021-1010-01).

Anti-GAD65 and anti-insulin antibody assays

Specifications for analytical method characteristics are summarized in Table 1. Anti-GAD65 antibody was analyzed using GAD65 assay kits (Shenzhen New Industries Biomedical Engineering Co., Ltd. [SNIBE], Pingshan District, Shenzhen, China) traceable to the World Health Organization (WHO) 1st Reference Material by the National Institute for Biological Standards and Control (NIBSC) 97/550 and anti-insulin antibody was analyzed using IAA assay kits (SNIBE) traceable to unconjugated anti-insulin polyclonal IgG antibodies (bs-0056R, BIOSS Co., MA, USA) using MAGLUMI™ 2000 (SNIBE), according to the manufacturer's instructions.

Anti-GAD65 antibody assay using existing commercially available radioimmunoassay with the anti-GAD65 RIA kit (DIAsource, Louvain-la-Neuve, Belgium) on an r-counter (COBRA 5010 Quantum, Packard, Meriden, USA) in the laboratory for routine clinical use was a qualitative method with quantitative results expressed as U/mL (arbitrary unit). Anti-insulin antibody assays using an existing commercial radioimmunoassay with the AIA-100 kit (DIAsource) on an r-counter (COBRA 5010 Quantum) in the laboratory for routine clinical use was a semi-quantitative measurement for anti-insulin antibodies.

Analytical performance evaluation of chemiluminescence immunoassays

The precision was investigated according to guidelines from CLSI document EP12-A2 for qualitative results near the cutoff and EP15-A3 for quantitative results during a 5-day period [6]. The manufacturer's claims of precision for both anti-GAD65 and anti-insulin antibody assays were < 10% total CV. Cutoff verification for accuracy and precision performance of the anti-GAD65 antibody assay was performed using WHO reference material NIBSC 97/550. Linearity was evaluated according to CLSI EP06-ED2 [10], producing five equally spaced concentrations by equal-volume mixing of sample pools and using average measured values of two replicates with an allowable total error limit of 20% and 50% of the systemic error budget. Carry-over was tested using EP Evaluator software (build 12.2.0.7, Data Innovations LLC, Colchester, England). Reference intervals (cutoffs for positive results) of both assays were verified according to CLSI EP28-A3C [8]. Method comparability was performed using 47 clinical specimens for anti-GAD65 antibody assays and 41 clinical specimens for anti-insulin antibody assays with commercially available radioimmunoassays in accordance with the guidelines from CLSI EP09-A3c for quantitative results and CLSI EP12-A2 for qualitative results [6,9]. Although radioimmunoassays are use in current clinical practice by physicians in Korea, they are not reference measurement methods for anti-GAD or anti-insulin antibody detection [5]. Therefore, results obtained from the radioimmunoassay were considered only as results from a comparative measurement procedure instead of a reference diagnosis [9]. Positive percent agreement (PPA), negative percent agreement (NPA), overall percent agreement (OPA), and 95% confidence interval [CI] were calculated using 2 x 2 tables [6]. For use of qualitative results compared with those from the chemiluminescence immunoassay, anti-GAD65 antibody results were categorized as positive (≥ 2.0 U/mL) or not. p-values less than 0.05 were considered significant. Statistical analysis was executed using MedCalc software for

Table 1. Measurement method characteristics for anti-GAD65 and anti-insulin antibodies.

	anti-GAD65 antibody		anti-insulin antibody	
	Radioimmunoassay	Chemiluminescence immunoassay	Radioimmunoassay	Chemiluminescence immunoassay
Reagent kit (manufacturer)	anti-GAD65 (DIAsoure)	MAGLUMI GAD65 (SNIBE)	AIA-100 (DIAsoure)	MAGLUMI IAA (SNIBE)
Instrument (manufacturer)	r-counter (PACKARD)	MAGLUMI 2000 (SNIBE)	r-counter (PACKARD)	MAGLUMI 2000 (SNIBE)
Specimen storage stability	3 days (2 - 8°C)	5 days (2 - 8°C), 2 months (-20°C)	1 day (2 - 8°C)	7 days (2 - 8°C), 2 months (-20°C)
Min. required sample volume (mL)	300 µL	50 µL	300 µL	10 µL
Measurement unit	U/mL	IU/mL	%	IU/mL
Result interpretation	negative: < 1.0 grey zone: 1.0 - 1.9 positive: ≥ 2.0	negative: < 17.0 positive: ≥ 17.0	negative: 0 - 7 positive: ≥ 8	negative: < 20.0 positive: ≥ 20.0
Calibration point	5 - 6 points	2 points	5 - 6 points	2 points
Calibration frequency	every run	4 weeks	every run	4 weeks
Analytical measurement range	0.7 - 120.0	1.0 - 280.0	1.0 - 100.0	2.0 - 175.0
Reagent stability-sealed	6 - 7 weeks	12 month	6 - 7 weeks	12 month
Reagent stability-opened	2 weeks	4 weeks	8 days	4 weeks
Waste	radioactive waste	medical & chemical waste	radioactive waste	medical & chemical waste

Windows, version 19.1.5 (MedCalc Software, Ostend, Belgium) and EP Evaluator software.

RESULTS

A summary of the analytical performance specification study results in accordance with the design is presented in Table 2. The manufacturer's precision claims for the quantitative values and cutoffs were verified. The manufacturer's linearity claims for the quantitative values were verified to be 1.3 - 277.4 IU/mL for anti-GAD65 antibody and 2.3 - 172.8 IU/mL for anti-insulin antibody concentration ranges. Neither anti-GAD65 nor anti-insulin antibody assay had significant carry-over.

Because the anti-GAD65 antibody assay using a radioimmunoassay had arbitrary units (U/mL) for a quantitative value, comparison with anti-GAD65 antibodies using chemiluminescent immunoassays showed a significant difference between them. Deming regression analysis yielded a slope of 1.87 (95% CI, 1.41 - 2.33) and a y-intercept of 8.62 IU/mL (95% CI, 0.11 - 17.13) with standard error estimates of 15.11 IU/mL. The units of the two manufacturers are different and could not be converted. For anti-insulin antibodies, radioimmunoassay is a semi-quantitative method with units of %, which a comparison of quantitative values was not possible.

For qualitative results, PPA, NPA and OPA of anti-GAD65 antibodies between the chemiluminescence immunoassay and radioimmunoassay were 70.6% (95% CI 44.0 - 89.7), 96.7% (95% CI 87.8 - 99.9), and 87.2% (95% CI 74.8% - 94.0%), respectively. For qualitative results, PPA, NPA and OPA of anti-insulin antibodies between the chemiluminescence immunoassay and radioimmunoassay were 44.4% (95% CI 13.7 - 78.8), 96.9% (95% CI 83.8 - 99.9), and 85.4% (95% CI 71.6% - 93.1%), respectively.

DISCUSSION

In this study, we evaluated the analytical performance of anti-GAD65 antibody and anti-insulin antibody assay kits on a MAGLUMI 2000 analyzer according to user verification guidelines, in combination with a method comparison with preexisting commercially available radiommunoassays.

Table 2. Summary of the performance evaluation study results in accordance with design.

Evaluation	Level of specimen	Experiment	Expected results	Test results
Anti-glutamic acid decarboxylase 65 antibody assay				
Precision (Qualitative)	**C50 (17.0 IU/mL) C50 - 20% (13.4 IU/mL) C50 + 20% (20.4 IU/mL)**	**20 replicates (each level)**	**% positive: 35 - 65% % negative: 90% % positive: 90%**	**% positive: 50% % negative: 100% % positive: 100%**
Precision (Quantitative)	**low (13.8 IU/mL) high (31.7 IU/mL)**	**25 replicates (5 runs x 5 days, each level)**	**CV < 10%**	**mean 14.4, SD 0.37 (CV 2.6%) mean 31.7, SD 0.63 (CV 2.0%)**
Linearity	**1.0 to 280.0 IU/mL (5 levels, equally distributed)**	**two replicates each level**	**TaE < 20% (50% systematic error budget)**	**linear from 1.3 to 277.4 IU/mL**
Carryover	**low (1.2 IU/mL) high (251.9 IU/mL)**	**specific sequence ***	**carryover < 3SD of low-low transition**	**0.08 < 0.42 (error limit)**
Reference interval verification	**negative (< 17.0 IU/mL)**	**24 Korean adults**	**< 10% of specimens fall outside**	**negative: 91.7% (fall outside 8.3%)**
Comparison with RIA	**not applicable**	**47 clinical specimens**	**OPA > 80%**	**OPA 87.2% (PPA 70.6%, NPA 96.7%)**
Anti-insulin antibody assay				
Precision (Qualitative)	**C50 (20.0 IU/mL) C50 - 20% (16.0 IU/mL) C50 + 20% (24.0 IU/mL)**	**20 replicates (each level)**	**% positive: 35 - 65% % negative: 90% % positive: 90%**	**% positive: 35% % negative: 100% % positive: 100%**
Precision (Quantitative)	**low (10.3 IU/mL) high (30.0 IU/mL)**	**25 replicates (5 runs x 5 days, each level)**	**CV < 10%**	**mean 10.7, SD 0.22 (CV 2.1%) mean 31.0, SD 1.05 (CV 3.4%)**
Linearity	**3.0 to 175.0 IU/mL (5 levels, equally distributed)**	**two replicates each level**	**TaE < 20% (50% systematic error budget)**	**linear from 2.3 to 172.8 IU/mL**
Carryover	**low (2.4 IU/mL) high (171.0 IU/mL)**	**specific sequence ***	**carryover < 3SD of low-low transition**	**0.18 < 0.66 (error limit)**
Reference interval verification	**negative (< 20.0 IU/mL)**	**24 Korean adults**	**< 10% of specimens fall outside**	**negative: 100.0% (full outside 0.0%)**
Comparison with RIA	**not applicable**	**41 clinical specimens**	**OPA > 80%**	**OPA 85.4% (PPA 44.4%, NPA 96.9%)**

Abbreviations: CV - coefficient of variation, C50 - the analyte concentration near the cutoff that yields 50% positive results and 50% negative results when many replicates of a single sample at that concentration are tested, NPA - negative percent agreement, OPA - overall percent agreement, PPA - positive percent agreement, SD - standard deviation.

According to recent guidelines by the American Diabetes Association, because T1DM and T2DM are heterogeneous diseases for which clinical presentation and disease progression vary considerably, traditional approaches based on age at onset of diabetes are no longer accurate, and appropriate classification using measurement of multiple autoantibodies is important for determining therapy [9,11-12]. Therefore, analytical verification of these antibodies in clinical laboratories is important for improving patient outcomes [5].

The precision, cutoff, linearity, and carryover performance of anti-GAD65 antibodies were comparable to those in previous studies performed in Italy and Denmark, the results of which were acceptable for clinical use [13,14]. Results of a comparison study using patient specimens with preexisting radioimmunoassays showed that an OPA of 87.2% (95% CI 74.8% - 94.0%) was comparable with the current global standardization efforts for anti-GAD65 antibodies using WHO reference standards [5]. For anti-insulin antibody assay, there was no international reference standard material allowing direct comparison of different assays for anti-insulin antibodies [10]. Because clinical information was limited for specimens used in the comparison study, clinical sensitivity and specificity could not be evaluated. Future studies to develop appropriate international reference material for islet cell autoantibodies with harmonization and implementation are needed [5,12].

The strength of this study is that it is the first to investigate the method performance of chemiluminescence im-

munoassays for anti-GAD65 and anti-insulin antibody detection verified in a Korean clinical laboratory using clinical specimens from Korean populations. The limitation of this study was a lack of detailed clinical information regarding diabetes and comorbidities. Limited numbers of specimens for comparison might be another limitation. However, a comparison study was performed in accordance with current CLSI guidelines. Future studies to investigate the clinical implications of anti-GAD65 and anti-insulin antibody assays with detailed clinical information are needed for more robust clinical performance of these assays.

In conclusion, we evaluated the analytical performance of chemiluminescence immunoassays for anti-GAD65 and anti-insulin antibody tests following user verification guidelines with comparison against radioimmunoassay results. Manufacturer's claims for performance specifications were verified. Overall agreement between the chemiluminescence immunoassay and radioimmunoassay was comparable with those reported in previous studies. Future studies regarding the clinical applicability of anti-GAD65 and anti-insulin antibodies are needed to clarify their clinical implications and the management of patient outcomes.

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Declaration of Interest:
All the authors declare that there is no conflict of interests

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