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Analytical evaluation of a GAD65 antibodies chemiluminescence immunoassay for CSF in neurological syndromes

https://doi.org/10.1515/cclm-2023-0072
Received January 18, 2023; accepted April 17, 2023;
published online May 1, 2023

Abstract

Objectives: Antibodies against glutamic acid decarboxylase isoform 65 (GAD-Ab) have been found in different severe neurological conditions associated with altered synthesis of γ-aminobutyric acid (GABA). Serum GAD-Ab can be found in up to 90 % of patients with type 1 diabetes mellitus (T1DM), mostly at relatively low concentrations, while high concentrations of GAD-ab are thought to be more frequently associate to a neurological condition, with levels 100-folds higher than those found in T1DM. Although CSF testing is recommended when suspecting a GAD-associated neurological syndrome, no commercial immunoassay is validated for this use and no cut-off is internationally recognized to support the diagnosis.

Methods: In this study we validated CSF testing of GAD-Ab on an automated chemiluminescence (CLIA) immunoassay that had previously shown good agreement with ELISA on serum.

Results: We tested 43 CSF from patients with typical GAD-associated neurological disorders and patients with other neurological conditions, identifying a clinical cut-off of 18 kIU/L that discriminated GAD-disease with an area under the curve (AUC) of 0.921. CLIA showed good analytical performances on repeatability and recovery tests in CSF and confirmed an excellent agreement with ELISA.

Conclusions: GAD-Ab associated neurological disorders are rare but CSF testing for GAD-Ab is a common request for neurologists when suspecting an insidious autoimmune central nervous system disease. CLIA platforms are expected to be increasingly adopted in clinical laboratories due to their flexibility and reliability, therefore studies on decisional levels should be implemented for improving the interpretation and utilization of laboratory data.

Keywords: analytical validation; autoantibodies; cerebrospinal fluid; chemiluminescence immunoassay; GAD antibodies; neurological syndromes.

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Introduction

Antibodies against glutamic acid decarboxylase isoform 65 (GAD-Ab) have been found in different severe neurological conditions associated to altered synthesis of γ-aminobutyric acid (GABA) from glutamate, where GAD is the rate-limiting enzyme [1]. GAD is an intracellular antigen found in the presynaptic neurons of central nervous system (CNS), β-cells of pancreatic islets, in the oviduct and the testes, and GAD-Ab can be found in up to 90 % of patients with type 1 diabetes mellitus (T1DM), mostly at relatively low concentrations [1, 2]. Neuronal disorders, now encompassed in a "GAD antibody-spectrum disorders" (GAD-SD) phenotype, particularly comprehend Stiff Person Syndrome (SPS) and its variants, including progressive encephalomyelitis with rigidity and myoclonus

(PERM), cerebellar ataxia (CA), epilepsy and limbic encephalitis (LE) [3]. Classical SPS, first described in 1956 [4], is a rare disorder with a prevalence of 1–2 cases per million, more frequent in women and mostly develops in adults of 30–50 years of age [1, 2]. It has an insidious onset of axial muscle stiffness progressively involving proximal and distal limbs, resulting in abnormal postures, and superimposed painful spasms accompanied by dysautonomic and psychiatric symptoms [2]. CA is the second most frequent GAD-associated neurological disorder [5], characterized by gait and limb ataxia, dysarthria and nystagmus and also affecting women in 80 % of cases, usually with a previously diagnosed organ-specific autoimmune disease [1]. GAD-associated epilepsy affects most often the temporal lobe and shows pharmacoresistance, and still is more common in women than in men [1], as it is LE, though the latter in younger age; GAD-LE can be either solely autoimmune and rarely is found as a paraneoplastic disease [2].

GAD-ab levels 100-folds higher than those found in T1DM are reported up to 80 % of SPS patients [6] and intrathecal synthesis in cerebrospinal fluid (CSF) is frequently described [7, 8] and support the correlation of autoantibodies with the neurological signs, though a direct pathogenic role is yet to be confirmed [1, 2].

Although high concentrations of GAD-ab are thought to be more frequently associated with a neurological condition [3], an international agreement on decisional level to support the diagnosis is still lacking [1].

Evidence for the presence of antibodies against GAD in both serum and CSF of an SPS patient was first described in 1988, thanks to a combined approach of light-microscopy immunocytochemistry and Western blot [9]. A radioimmunoassay was later validated reporting an SPS-specific cut-off for high level of GAD-abs in serum (>20 nmol/L) [10] and several enzyme-linked immunosorbent assays (ELISA) are now available [1]. Collaborative efforts for standardization and harmonization of nonradioactive tests have been recently depicted by The Islet Autoantibody Standardization Program (IASP) where ELISA assays showed the most homogeneous and accurate performances [11].

Commercial immunoassays commonly adopted in clinical laboratories were specifically developed for serum samples to support T1DM diagnosis [1], so that results above the upper limit of detection (e.g. 2,000 kIU/L for the most common ELISA kits) are usually not quantified.

Chemiluminescence immunoassays (CLIA) have been recently developed as immunoassays were the label is a luminescent molecule, having a wide dynamic range, high specificity, random access and being easily automated [12].

A CLIA assay for GAD-ab has recently proved good analytical performances compared to ELISA and has been validated for clinical practice in automated test systems [13], with linearity up to 280 kIU/L, for the intended endocrinological target.

Regardless of the peculiar technique used, the immunoassays have been mostly developed to quantify GAD-ab in serum, so that results in CSF so far have been interpreted with caution, although CSF testing is recommended when suspecting a neurological syndrome associated with either high serum level of GAD-ab [1] or low or seronegative results [3].

This study aims to validate CSF testing on a CLIA platform for neurological disorders and to determine a cut-off of GAD-ab in CSF for typical neurological GAD-related diseases.

Materials and methods

We collected 43 CSF samples from neurological patients previously classified at Neurology Unit O.S.A. of University-Hospital of Padova with GAD-related syndromes in preliminary work between 2009 and 2015 (Zoccarato M., unpublished results) and CSF leftovers samples from diagnostic routine workup between December 2019 and April 2022 at the Department of Laboratory Medicine from patients with other neurological conditions previously anonymized as control group; CSF was stored at −20 °C. Each patient had already undergone standard diagnostic procedures that also encompassed testing with commercial cell-based assay (CBA) and immunoblotting (Euroimmun, Germany) to exclude coexistence of other antibodies (LG1, CASPR2, GABABR, AMPAR, NMDAR and onconeural antibodies).

The occurrence of typical features of GAD-ab phenotypes [14–16] was reported. Patients were classified as “typical” when the clinical diagnosis was: (a) SPS (and variants) or (b) cerebellar ataxia or (c) encephalitis or (d) epilepsy, and if bearing clinical and paraclinical features recognized as common for immune-mediated diseases. This evaluation was blind to GAD-ab testing. Twelve patients had clinical features typically related to GAD-Ab (SPS, CA, temporal epilepsy, LE); 31 patients underwent lumbar puncture for the diagnostic workup of dementia, other phenotypes of epilepsy, infectious diseases, cerebrovascular disorders, headache, NMDAR encephalitis, oncologic diseases, and were classified as having “not-typical” GAD-Ab features (control group).

Samples were thawed at room temperature immediately before GAD-ab analysis. GAD-ab testing was performed on an automated CLIA system (MAGLUMI 2000 Plus by Snibe, China); this commercial sandwich immunoassay is validated for *in vitro* diagnostic use for serum only; manufacturer declares a measuring range of 1.0–280.0 kIU/L with a limit of blank of 1 kIU/L, it has a traceable calibrator for GAD65 (WHO 1st Reference Reagent 97/550).

CSF samples were also tested on an ELISA commercial kit (RSR Limited, UK or Euroimmun, Germany) with an automated system (DSX by Technogenetics, Italy or Triturus by Grifols, Spain) and results were compared to CLIA assay.

Precision evaluation

CSF samples with low GAD-ab concentration were pooled and divided into three aliquots, calibrator 1 (13.7 kIU/L) and calibrator 2 (169.3 kIU/L) were added to an aliquot each; precision was estimated with triple

measurements of each aliquot for three consecutive days, following a modified Clinical and Laboratory Standards Institute (CLSI) EP15-A3 protocol [17], and compared to the manufacturer declared results appropriately interpolated using a linear function. Recovery of spiked calibrators was calculated as %Rec = Measured concentration/Expected value * 100.

Linearity assessment

Three samples (patient 10 measured GAD-ab level of 212.5 kIU/L, patient 8,88.8 kIU/L, patient 11,74.9 kIU/L) were diluted with kit buffer (phosphate buffered saline containing bovine serum and NaN_3 <0.1 %, previously tested for GAD-ab); different linear dilutions were prepared with a fixed final volume and tested in duplicate, average values were compared to expected GAD-ab concentrations as explained in the CLSI EP06 A: 2003 guideline [18].

Statistical analyses

Median and interquartile ranges (25th–75th percentiles, IQR) were used as descriptive statistics, while the non-parametric Kruskall–Wallis test was used for comparing GAD-ab level between the two studied groups. Spearman rank correlation was used to test linear association between variables. Clinical threshold was calculated with receiver-operator characteristic (ROC) analysis and Youden index cutoff with confidence (CI) intervals 95 %. Proportional and constant bias between CLIA and ELISA were already previously estimated [13], therefore we evaluated agreement at manufacturers' declared cut-offs for serum (17 kIU/L for MAGLUMI, 10 kIU/L for RSR/Euroimmun) with Cohen's kappa. Analysis were performed using Stata v 16.1 (StataCorp, Lakeway Drive, TX, USA) and GraphPad Prism v 9.1.

Results

Comprehensive results of CSF CLIA and ELISA testing, demographic data and clinical diagnosis of the 43 patients are reported in Table 1. None of the patients with typical GAD-ab features had coexistent other neural antibody positivity; one of the patients in the control group had NMDAR-positive encephalitis.

GAD-ab concentrations were different between the group of patients with typical features and the control group with both assays. For CLIA median concentration was 150.7 kIU/L (36.8–280 IQR) vs. 5.6 kIU/L (5.3–6.1, χ^2=18.651, p=0.0001); for ELISA the median was 2,708 kIU/L (190–11,060) vs. 0 (χ^2=15.779, p=0.0001). Also, women had higher median concentrations both with CLIA (32.4 kIU/L vs. 5.7) and ELISA 190 kIU/L vs. 0. GAD-ab concentrations were not correlated to age (p= n.s. for both CLIA and ELISA).

Precision

Repeatability and intermediate precision of CLIA assay in a 3-days analysis are reported in Table 2, intra-assay and total CV% were both ≤11 %. Recovery of calibrator 1 ranged from 98 to 106 %, calibrator 2,124–128 %.

Linearity

Dilution linearity for three samples is shown in Figure 1.

Kit buffer, phosphate-buffered saline (PBS) and a pool of GAD-Ab negative CSF samples were previously tested as dilution means and Passing–Bablok regression and Bland–Altman analysis showed no constant bias between the three specimens (data not shown).

Clinical cut-off

CLIA ROC curve for outcome (GAD-typical features: SPS, CA, Epilepsy and LE, PNS vs. non-GAD pathology) had AUC=0.921 (95 % CI: 0.832–1.000) (Figure 2). Youden's cut-off identified was 18 kIU/L, sensitivity was 90.9 % (95 % CI: 58.7–99.8 %), specificity 93.5 % (95 % CI: 78.6–99.2 %); positive likelihood ratio 14.09 (95 % CI: 3.64–54.54), negative likelihood ratio 0.10 (95 % CI: 0.01–0.63).

ELISA ROC curve had AUC=0.909 (95 % CI 0.901 to 1.000) (Figure 2); at best cut-off of 93.5 kIU/L sensitivity was 90.9 % (95 % CI: 58.7 and 99.8 %) and specificity 93.3 % (77.9–99.2 %).

CLIA vs. ELISA

Agreement on positive or negative samples was excellent (100 %), with a Cohen's kappa of 1.00, SE=0.156, p<0.001.

Discussion

In this study we validated a commercial CLIA assay for GAD-ab testing in CSF as, at our knowledge, no commercial immunoassay has been previously validated for this use, being usually designed to detect low concentrations of GAD-ab in serum of T1DM patients [19]. CLIA showed good performances on CSF with intra-assay and total CV% comparable to what manufacturer declares for serum, with only a minor caveat on concentration level 5 of kIU/L, well below the reference upper level of 17 kIU/L, where imprecision results were 7.41 and 10.95 % compared to manufacturer's 5.33 and 8.26 %, respectively.

Additionally, the analytical recovery using the traditional spike addition test was acceptable (98–106 % at 13.7 kIU/L and 124–128 % at 169.3 kIU/L); based on these results, CSF seems a suitable matrix for this CLIA automated

Table 1: Antibody concentrations, demographic data and clinical features.

Patient	Age, years	Sex	GAD CLIA, kIU/L	GAD ELISA, kIU/L	Clinical diagnosis	Group
1	51	F	26.9	2,708	Encephalitis	GAD-Ab typical features
2	32	F	>280	55,698	Encephalitis	
3	65	F	>280	16,432	SPS	
4	27	F	38.0	2,963	Encephalitis	
5	32	F	>280	11,060	Encephalitis	
6	70	M	35.6	187	PERM	
7	44	M	5.7	0	PERM	
8	25	F	88.8	985	Epilepsy	
9	74	F	>280	3,710	Ataxia	
10	57	M	212.5	1,293	Epilepsy	
11	22	F	74.9	190	Ataxia	
12	52	F	259.7	n.a.	SPS	
13	51	F	> 280	10,933	Dysarthria	NOT GAD-Ab typical features
14	56	M	8.8	0	Corticobasal degeneration	
15	60	M	109	9,059	Epilepsy	
16	35	M	5.5	0	Encephalitis	
17	64	M	5.8	0	Encephalitis	
18	67	F	5.6	0	Encephalitis	
19	56	M	6.2	0	Epilepsy	
20	47	M	5.7	0	Epilepsy in obstructive sleep Apnea syndrome	
21	68	F	5.7	0	Status epilepticus	
22	36	M	6.1	0	NMDAR encephalitis	
23	31	M	5.8	0	Suspected vasculitis/Paraneoplastic encephalitis	
24	18	F	5.2	0	Headache	
25	70	M	3.5	n.a.	Behavioural changes	
26	74	M	5.3	0	Confusional, fever	
27	48	M	6.1	0	Cerebellar syndrome w colon cancer	
28	68	M	5.3	0	Alzheimer's disease	
29	71	F	5.5	0	Alzheimer's disease	
30	82	M	5.2	0	Cerebrovascular syndrome	
31	50	M	5.4	0	Epilepsy	
32	58	M	5.1	0	Epilepsy in glioma	
33	79	M	5.0	0	Epilepsy in cerebrovascular syndrome	
34	60	F	4.6	0	Dementia	
35	69	M	5.4	0	Epilepsy in cerebrovascular syndrome	
36	47	F	6.3	0	Viral cerebellitis	
37	50	M	5.8	0	Talamic stroke	
38	72	M	6.1	0	Viral encephalitis	
39	76	F	6.4	0	Infectious radiculopathy	
40	45	M	5.3	0	Cerebral venous thrombosis	
41	82	F	5.6	0	Alzheimer's disease	
42	67	M	5.4	0	Suspected neurodegenerative	
43	49	M	5.1	0	Epilepsy in glioma	

Positive GAD-Ab typical features, studied group; negative GAD-Ab typical features, control group. kIU, kilo International Units; SPS, Stiff Person Syndrome; PERM, progressive encephalomyelitis with rigidity and myoclonus; NMDAR, N-methyl-D-aspartate receptor.

assay and GAD-ab requests might be implemented in routine testing.

However, data from the serial dilution test did not provide a satisfactory linearity, except for sample patient 10; therefore, sample dilution in routine practice should be attempted cautiously.

A recent comprehensive review on GAD-ab neurological disorders enclose CSF GAD testing in the algorithm to determine the likelihood of an autoimmune cause when GAD-ab are found in serum, recommending that CSF should be tested when high levels are detected in serum, unless other clear alternative diagnosis can be defined. Additionally, for patients with classic SPS, the detection of GAD antibodies in the CSF should suffice to establish a definite autoimmune diagnosis, while for the other phenotypes the finding of GAD-ab in the CSF should be accompanied by a

Table 2: Precision evaluation obtained with pools of CSF samples and calibrators.

Design	Level, kIU/L	Measured repeatability CV%	Measured intermediate precision CV%	Manufacturer obtained repeatability[a] CV%	Manufacturer obtained intermediate[a] precision CV%
3 × 3 CLSI EP15-A3	5	7.41	10.95[b]	5.33	8.26
	9	3.38	3.38	5.18	7.86
	102	1.16	1.16	2.17	2.5

[a]Obtained from the MAGLUMI™ GAD65 (CLIA) insert, N 069 GAD65-en-EU, V12.0, 2020-02; precision results were interpolated by using a linear function. CLSI, Clinical and Laboratory Standards Institute. [b]Upper verification limit 8.57. kIU, kilo International Units; CV, coefficient of variation.

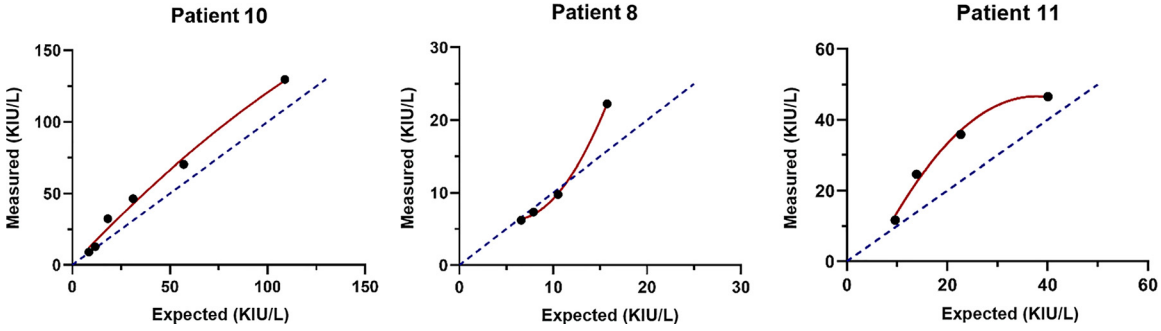

Figure 1: Linearity assessment: patients CSF samples were diluted with kit buffer at different linear dilutions and tested in duplicate, average measured values were compared to expected GAD-ab concentrations.

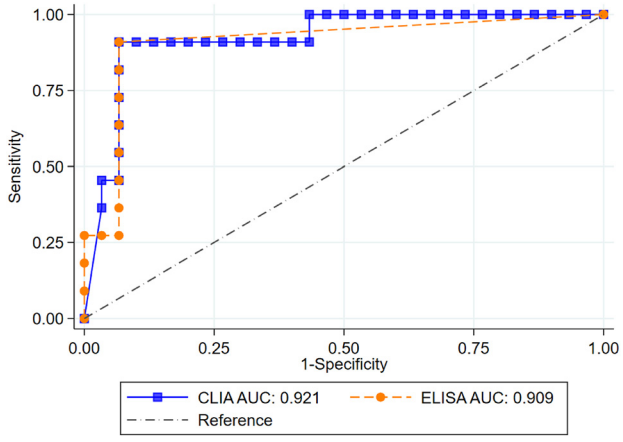

Figure 2: ROC curve for outcome (GAD-typical features vs. not-typical GAD features) for CLIA and ELISA. CLIA best cut-off=18 KIU/L (sensitivity 90.9 %, specificity 93.5 %); ELISA best cut-off=93.5 KIU/L (sensitivity 90.9 %, specificity 93.3 %).

demonstration intrathecal synthesis to establish a pathogenic link that is crucial for therapeutic management [1].

Some authors, however, suggest that CSF testing might not be necessary when GAD-abs concentration in serum is >10,000 kIU/L, while it could be useful at lower and/or negative concentrations [19]. The latter circumstances might benefit from the demonstration of intrathecal GAD-abs synthesis through a modified Link's formula that has already been proposed [7]. However, even if intrathecal antibody synthesis is not demonstrated, the mere presence of anti-GAD in CSF allows the definition of a probable autoimmune disease [1].

While previous studies excluded an association between serum GAD-abs concentration and disease severity or degree of clinical improvement after therapy [19, 20], Munoz-Lopetegi identified "high level antibody concentrations" with a cut-off of 100 kIU/L in CSF and 10,000 kIU/L in serum with an ELISA method and immunohistochemistry and cell-based assay as confirmatory qualitative methods for GAD-ab-associated neurologic syndromes that might favorably respond to immunotherapy [21]. We confirmed this report in our cohort as we identified a CSF best cut-off of 93.5 kIU/L for ELISA for typical GAD-related clinical features.

The corresponding CLIA best cut-off was 18 kIU/L, consistent with the established serum value of 17 kIU/L.

Finally, we demonstrated that CLIA had an excellent agreement with ELISA (Cohen's kappa=1) and ROC curve had similar AUC (0.921 vs 0.909), and, with the defined cut-offs, similar sensitivity and specificity, being the first report of harmonization and result comparability between two methods on CSF samples.

Although statistical power was reached, the relatively low number of subjects included is a limitation of this study; this is a common issue when addressing rare disorders that might be overcome in further multicentric evaluations.

A further limitation is that due to low sample volume CLIA results >280 kIU/L could not be diluted to fall within the measurement range.

Conclusions

Validation and standardization of GAD-ab testing for CSF are long-awaited goals for neurological clinical practice, due to the crucial impact of intrathecal synthesis on therapeutic management and long-term follow-up.

CLIA systems are expected to increase in clinical laboratories due to their performance advantages and their flexibility [22], agreement analysis and establishment of specific decisional levels should be pursued to facilitate clinical management particularly of complex patients, for whom a differential diagnosis could be challenging. The relative infrequency of these neurological syndromes might limit the statistical power of our results, so a more extensive application of a cut-off for CSF testing should prove its reliability.

Research funding: None declared.
Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.
Competing interests: Authors state no conflict of interest.
Informed consent: Not applicable.
Ethical approval: Not applicable.

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