Anti-ganglioside antibodies: experience from the Italian Association of Neuroimmunology

external quality assessment scheme

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Other centers and participants are listed in the Appendix
Abstract

**Background:** Anti-ganglioside antibodies are currently used in the differential diagnosis of suspected immune-mediated neuropathies. In-house and increasingly used commercial assays seem to perform suboptimally, and comparative information on their analytical performance are essentially lacking. Born within the frame of guidelines and standardization activities by the Italian Association of Neuroimmunology, this external quality assessment scheme (EQAS) is a real-life snapshot of the laboratory diagnostics in this field.

**Methods:** The EQAS consisted of 5 surplus, anonymized serum samples from patients with clinically-defined neuropathies, and 2 serum samples from healthy blood donors. Eight laboratories used commercial line-/dot-blots, 7 in-house/commercial ELISAs (in addition, 13 laboratories tested a recently released ELISA by Bühlmann). Only high levels of anti-ganglioside reactivities were considered, in accordance with consolidated recommendations.

**Results:** Large variations in anti-ganglioside antibody profiles were observed, even, although at a lesser extent, within homogeneous assays. Concordance between the profiles and clinical phenotypes was partial too.

**Conclusions:** Although conducted on a relatively small, but representative number of Italian laboratories, this EQAS shows a critical between-laboratory disagreement in test results of anti-ganglioside antibodies. Also considering the trend for using certified assays in generalist laboratories, strong efforts toward standardization and the identification of the best method(s) for their determinations are compellingly needed.

**Keywords:** autoimmunity; dot-blot; ELISA; immune-mediated neuropathies; line-blot; medical diagnostics
Introduction

After their discovery and for years, more than 20 antibodies to gangliosides, glycosphingolipid molecules densely expressed on the external side of neuronal membranes, have been representing possible pathogenetic agents, or biomarkers of disease at least, in inflammatory axonal neuropathies [1]. However, at present only a few anti-ganglioside antibodies associate with well-defined clinical phenotypes, and therefore good medical practice recommends to test these autoantibodies only when sufficiently typical inflammatory neuropathies are suspected [2-6]. On the other hand, there is a general trend for requesting anti-ganglioside antibodies in a wider spectrum of neuropathies by clinicians, and for testing extended autoantibody panels by laboratorists. Guidelines by the Italian Association of Neuroimmunology (AINI) have represented an attempt to rationalize such requests [6].

From the laboratory side, two methodological approaches are basically available: ELISA (either in-house, or commercial) and line-/dot-blot (commercial), in the absence of a feasible standard technique. The glycosphingolipid nature of the antigens makes it difficult to extract, purify, and use them to devise completely reliable methods, so that it is accepted that they perform suboptimally [7]. In 1999, the so-called INCAT-ELISA was proposed by an international group of experts in neuropathies [8], and is still reckoned as an eligible test [3, 6, 9], notwithstanding the not negligible inter-laboratory variability reported since its publication [8]. It is likely that the shortcomings only partially depend on the in-house nature of the test. However, also to overcome these issues, laboratory diagnostics is long moving toward the use of certified commercial tests in generalist laboratories. The analytical performances of the commercial tests have been never compared with those of the in-house ELISA so far. Anyway, the trend toward test centralization in large laboratories urgently needs careful quality controls and, ultimately, standardizations in this
neuroimmunology area, given the reported intra- and between-method differences in both in-house and commercial test results [8, 10].

We herein report our experience of external quality assessment (EQAS) on anti-ganglioside antibodies, which was promoted by AINI. The EQAS is a snapshot of the real-life diagnostics in this field, as serum samples from single patients with clinically-defined neuropathies were included, and as the participating laboratories used heterogeneous commercial or in-house assays.

**Material and methods**

**Control samples**

Controls consisted of 5 anonymized and blinded-to-the-participants sera from 5 patients with the following neurological diseases (defined with clinical, electrophysiological, and cerebrospinal fluid investigations): sensory-motor Guillain-Barré syndrome (GBS), control #1; GBS, control #2; IgMκ monoclonal gammopathy-associated polyneuropathy (anti-MAG antibody negative), control #3; CANOMAD syndrome (Chronic Ataxic Neuropathy, Ophthalmoplegia, Monoclonal IgM protein, cold Agglutinins and Disialosyl antibodies), control #4; multiple mononeuropathy, control #5. Following routine analysis, the leftover samples were aliquoted into plastic cryogenic tubes, and frozen at -20 °C at the Florence or Pavia laboratories, before in-house analysis, or distribution to the participating laboratories. Two additional control sera were from healthy blood donors (gift by Bühlmann).

**Materials**

The antigens considered, tested for IgG and IgM reactivities, were: GM1, GM2, GD1a, GD1b, GD3, GQ1b, GT1a, GT1b. Sulfatides were not included, as not present in some commercial tests, and as testing for anti-sulfatide antibodies substantially lacks of clinical utility [5].

**Methods**
The 15 participating laboratories, most of which being referral centers in Italy, are listed in the Appendix. The following tests were used (type and number of laboratories using each test in parentheses): Immunodot Dotzen-Zentec, Angleur Belgium (dot-blot, 3), Immunoline ganglio profile, Euroimmun, Lübeck, Germany (line-blot, 3), BÜHLMANN GanglioCombi\textsuperscript{TM} Light (ELISA, 1), Immunoline Generic Assays, Dahlewitz, Germany (line-blot, 2), in-house INCAT-ELISA [8]. Thirteen laboratories had the opportunity of testing the same control samples with the recently released BÜHLMANN GanglioCombi\textsuperscript{TM} MAG ELISA (rr-Bühlmann ELISA; lot, 0808; kits gently provided by Bühlmann). Commercial tests were performed in accordance with the manufacturer’s instructions. Following the local interpretative procedures, all the laboratories were expected to report ‘low’ and ‘high’ levels of positivity, but it was recommended to consider significant (positive test) only high reactivities (line-/dot-blot), or high levels of anti-ganglioside antibodies (ELISA), as low levels of positivity have non-specific meaning and should not be reported in routine diagnostics [2, 4, 6]. Each laboratory had a long-lasting expertise that allowed consistent interpretations of weak band/spot staining by visual inspection (line-/dot-blot), or appropriate cutoffs (ELISA), in order to recognize ‘low positive’ results (no consensus required for EQAS). As for rr-Bühlmann ELISA, samples with ODs corresponding to those within or higher than the ‘medium control’ range were considered as positive.

Results

Table 1 summarizes the results, taking into account only high levels of positivity; the 2 controls by healthy blood donors were not included, as they resulted negative on all the assays. Laboratory #14 was excluded from the analysis (EQAS not completed). On the basis of the main category of the tests used (solid-phase support), we analyzed the results between groups (line-/dot-blot vs ELISA), and within homogeneous groups. Sample #5 was interpreted as negative by all the
laboratories. A nucleus of agreement can be found for the IgM reactivity to GD1b in sample #3 (12/14 laboratories). There was no substantial agreement in the autoantibody profiles for samples #1-4, even within the small subgroups using line-/dot-blots from the same manufacturer.

As for the performance of rr-Bühlmann ELISA, two aspects were evaluated, namely, the inter-lab variability, and the comparison with the other ELISAs. At the given cutoff of positivity, all the laboratories were concordant in reporting the following samples as positive: #2 (anti-GM1 IgG), #3 (anti-GD1b IgM), and #4 (anti-GD1b and anti-GQ1b IgM), except for laboratory #14 (sample #2, anti-GD1b IgG, and sample #3, anti-GQ1b IgM, as additional reactivities). Sample #5 was interpreted as negative by all the laboratories. When considering ‘low positive’ results, only slight between-laboratories differences emerged for samples #2-4, except for sample #1, which was judged anti-GM1 IgG-positive by 8 laboratories, and negative by 5 (not shown). When comparing the results by rr-Bühlmann ELISA with those by in-house ELISA, only partial agreements were found (sample #1, negative in 4 cases; sample #2, positive for anti-GM1 IgG in 3 cases; sample #3 positive for anti-GD1b IgM in 5 cases; sample #4, positive for anti-GD1b and anti-GQ1b IgM in 2 cases; sample #5, negative in all cases).

As for the coherence between clinical phenotypes and autoantibody profiles, the EQAS main results show that a) anti-GM1 IgG found in sample #2 by 3/7 laboratories of the line-/dot-blot group (BG), 3/7 of the in-house/commercial ELISA group (EG), and 13/13 of the rr-Bühlmann ELISA group (BEG) was compatible with GBS [4]; b) at least one reactivity for anti-GD1b and anti-GQ1b IgM found in sample #3 by 7/7 of BG, 5/7 of EG, and 13/13 of BEG was among the most frequently detected high-titer anti-ganglioside IgM reactivities in anti-MAG antibody negative, IgM monoclonal gammopathy-associated polyneuropathy [11]; c) as for sample #4, anti-GQ1b IgM plus a reactivity for at least one disialylated ganglioside, fulfilling the criteria for CANOMAD diagnosis [7], was found in 5/7 of BG, 4/7 of EA, and 13/13 of BEG.
Discussion

The main findings of the present EQAS on anti-ganglioside antibodies highlight a wide between-laboratory heterogeneity in both methods and results. The diagnostic shortcomings in this neuroimmunology area are well-known and difficult to solve, as long as they rely on both the nature of the antigens and other analytical- and manufacturer-related factors (reviewed elsewhere [6]).

This EQAS benefited from using single patient samples for comparison, thus allowing a real representation of the performance of each assay. Pooling samples can indeed alter the sample matrix and introduce bias. One fundamental point, previously established [2, 4, 6] and confirmed by our EQAS, is the recommendation of excluding ‘low positive’ results from the reports. If we had included ‘low positive’ results, further between-laboratory disagreement would have been emerged. On the other hand, a good test specificity, as sample #5, which was from a patient with a presumed non-autoimmune neuropathy, and the two samples from healthy controls were unanimously judged as negative.

The choice of well-defined cutoff values still remains a matter of standardization, whether for line/dot-blot assays, or ELISAs. Indeed, notwithstanding that only ‘high positive’ results were considered, considerable variations in anti-ganglioside antibody profiles were observed, even, although at a lesser degree, within homogeneous assays. Slight variations were found even in the results of rr-Bühlmann ELISA, whose kits had the same lot for all the laboratories. As a possible interpretation of these differences, a very interesting study on between-laboratory variability of an ELISA for neurofilament measurement demonstrated that, in addition to relatively more-difficult-to-control factors, such as analytical delays and reaction temperatures, the largely most
important cause of inaccuracy was preparation of standards [12]. The educational perspective of improving the human factor should be thus pursued.

In the specialized literature, there is still no clear-cut evidence for the best solid-phase support for anti-ganglioside antibody testing. Some authors, more involved in laboratory practice, favor hydrophobic membranes (line-/dot-blot), as they might allow better antigen-antibody interactions [10], others, more involved in clinical practice, keep choosing polystyrene (ELISA) [4]. A reappraisal of the original INCAT-ELISA, which involved 1232 patients and controls tested for GM1 IgG and IgM, showed that the ELISA was reliable and added diagnostic value in selected clinical situations only when high antibody titers were considered [4]. This study confirm that tests for anti-ganglioside antibodies should be requested only in selected populations of neuropathic patients.

The most common associations between specific autoantibody reactivities and well-defined clinical phenotypes include multifocal motor neuropathy and GBS variants (for more details, see [6]). In turn, this view follows the emerging idea that the total quality in laboratory should shift from pure internal indicators to clinical parameters spanning from rational requests to the test relevance for clinical effectiveness and patient outcomes (brain-to-brain loop) [12].

Diagnostics for anti-ganglioside antibodies, as well as for other neuroimmunological tests, is moving from small specialized laboratories, which preferentially use in-house tests, toward large generalist laboratories using certified commercial tests. The transition is per se critical, but the here-reported inter- and intra-method disagreements urge extra-efforts for the identification of the best standardized method(s). The role for laboratory, neuroimmunology scientific societies, and, possibly, specialized manufacturers in this virtuous process is fundamental.

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References


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Lab, laboratory number and type of laboratory test used: plain text, line-/dot blot (grouped by the same manufacturer); bold character, in-house/commercial ELISA. Results: plain text, IgG class; bold character, IgM class. Cntr, control sample; qr, qualitative result; Ag, antigen; nt, not tested