Serologic diagnosis of NMO

A multicenter comparison of aquaporin-4-IgG assays

P.J. Waters, PhD*
A. McKeon, MB, MRCPI
M.I. Leite, MD, DPhil
S. Rajasekharan, PhD
V.A. Lennon, MD, PhD
A. Villalobos, MSc
J. Palace, DM, FRCP
J.N. Mandrekar, PhD
A. Vincent, MBBS, FRCPath
A. Bar-Or, MD, FRCP(C)
S.J. Pittock, MD*

Correspondence & reprint requests to Dr. Pittock: pittock.sean@mayo.edu or Dr. Waters: paddy.waters@imm.ox.ac.uk

ABSTRACT

Objectives: Neuromyelitis optica (NMO) immunoglobulin G (IgG) (aquaporin-4 [AQP4] IgG) is highly specific for NMO and related disorders, and autoantibody detection has become an essential investigation in patients with demyelinating disease. However, although different techniques are now used, no multicenter comparisons have been performed. This study compares the sensitivity and specificity of different assays, including an in-house flow cytometric assay and 2 commercial assays (ELISA and transfected cell-based assay [CBA]).

Methods: Six assay methods (in-house or commercial) were performed in 2 international centers using coded serum from patients with NMO (35 patients), NMO spectrum disorders (25 patients), relapsing-remitting multiple sclerosis (39 patients), miscellaneous autoimmune diseases (25 patients), and healthy subjects (22 subjects).

Results: The highest sensitivities were yielded by assays detecting IgG binding to cells expressing recombinant AQP4 with quantitative flow cytometry (77%; 46 of 60) or visual observation (CBA, 73%; 44 of 60). The fluorescence immunoprecipitation assay and tissue-based immunofluorescence assay were least sensitive (48%–53%). The CBA and ELISA commercial assays (100% specific) yielded sensitivities of 68% (41 of 60) and 60% (36 of 60), respectively, and sensitivity of 72% (43 of 60) when used in combination.

Conclusions: The greater sensitivity and excellent specificity of second-generation recombinant antigen-based assays for detection of NMO-IgG in a clinical setting should enable earlier diagnosis of NMO spectrum disorders and prompt initiation of disease-appropriate therapies. *Neurology*® **2012; 78:665–671**

GLOSSARY

AQP4 = aquaporin-4; **CBA** = cell-based assay; **E** = EUROIMMUN; **FACS** = fluorescence-activated cell sorting; **FIPA** = fluorescence immunoprecipitation assay; **IgG** = immunoglobulin G; **IIF** = indirect immunofluorescence; **M** = Mayo; **MS** = multiple sclerosis; **NMO** = neuromyelitis optica; **NMOSD** = neuromyelitis optica spectrum disorder; **O** = Oxford; **R** = RSR/Kronus; **ROC** = receiver operating characteristic curve.

Neuromyelitis optica (NMO) is a severe relapsing inflammatory CNS demyelinating disease that predominantly affects the optic nerves and spinal cord.[1] At presentation, the most common differential diagnosis is multiple sclerosis (MS). However, unlike MS, disability in NMO accrues with each attack. Hence, early diagnosis and treatment are critical.[2] The discovery of specific immunoglobulin G (IgG) antibodies binding to CNS astrocytic membranes identified the target as the water channel aquaporin-4 (AQP4), which has aided early recognition of the disease and broadened the clinical spectrum to include patients who have only optic neuritis or transverse myelitis (neuromyelitis optica spectrum disorders [NMOSDs]).[3,4] Studies in vitro and in vivo have demonstrated the pathogenic potential of these autoantibodies.[5–10] However,

Supplemental data at www.neurology.org

Supplemental Data

*These authors contributed equally to this work.

From the Neuroimmunology Group (P.J.W., M.I.L., J.P., A. Vincent), Nuffield Department of Clinical Neurosciences, Oxford, UK.; Departments of Neurology (A.M., V.A.L., S.J.P.), Laboratory Medicine and Pathology (A.M., V.A.L., S.J.P.), Immunology (V.A.L.), and Biostatistics (J.N.M.), Mayo Clinic, College of Medicine, Rochester, MN; Experimental Therapeutics Program (S.R., A. Villalobos, A.B.-O.), Montreal Neurological Institute, McGill University, Montreal, Canada; and Neuroimmunology Unit, Department of Neurology and Neurosurgery (A.B-O.), Montreal Neurological Institute and Hospital, McGill University, Montreal, Canada.

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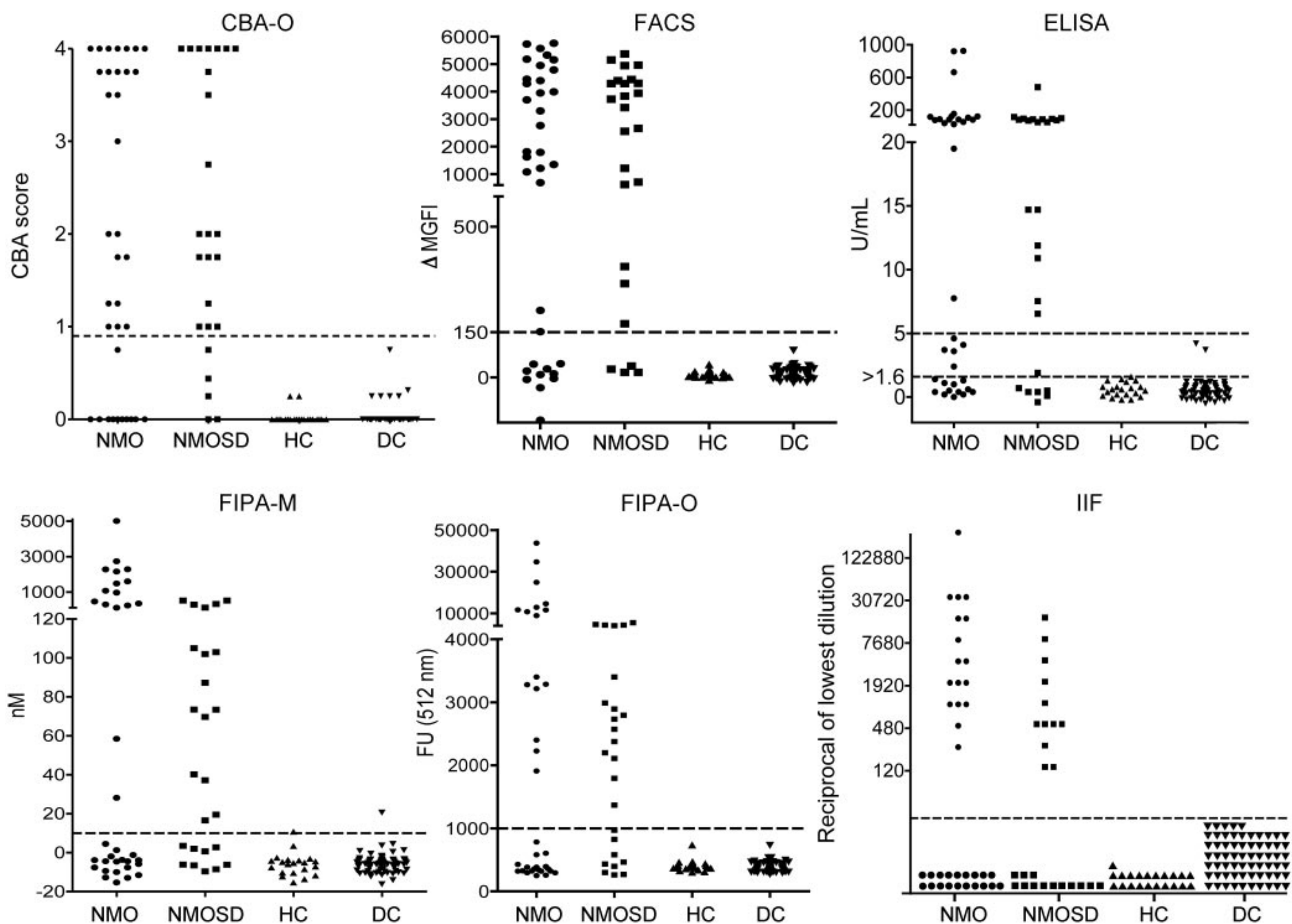
different assays for detecting AQP4-IgG in patients' sera differ in their sensitivities for NMO and other NMOSDs.[3,4,11–17] In this international multicenter study, 6 different AQP4-IgG assays were compared on coded samples.

METHODS **Standard protocol approvals, registrations, and patient consents.** This study was approved by all 3 institutional review boards.

Patients. Serum samples from 146 patients and control subjects were tested in duplicate, and 35 patients fulfilled the Wingerchuck diagnostic criteria for NMO (either 1999 or 2006 [excluding antibody status]).[2] Of the patients, 25 were classified by the investigators as having NMOSDs; this group included 14 patients with longitudinally extensive transverse myelitis (9 recurrent) and 8 patients with optic neuritis (5 recurrent; all patients with single attack cases of optic neuritis were seropositive). A total of 86 controls (22 healthy controls and 64 with miscellaneous diseases [fulfilling the McDonald criteria for relapsing-remitting MS, 39; Sjögren syndrome, 1; systemic lupus erythematosus, 4; rheumatoid arthritis, 1; spinal dural arteriovenous fistulae, 4; noninflammatory myelopathy, 3; short segment-longitudinal transverse myelitis, 2; sarcoid longitudinally extensive transverse myelitis, 1; polyclonal hypergammaglobulinemia, 1; and other, 8]) were also tested. The samples were provided by 3 institutions: Mayo Clinic, Rochester MN (93 patients), Neuroimmunology Group, Nuffield Department of Clinical Neurosciences, Oxford, UK (39 patients); and McGill University, Montreal, Canada (14 patients). For longitudinal analysis of antibody titers, 9 serum samples taken over 5 years from a single patient with NMO were analyzed.

All samples were submitted to McGill University, aliquoted, recoded, and returned frozen to the other 2 centers. Testing at Mayo Clinic included a tissue-based indirect immunofluorescence (IIF) assay for NMO-IgG,[3] ELISA-R (provided by RSR/Kronus, Ltd.; manufacturer's recommended level for seropositivity, 5 U/mL or greater), and GFP-AQP4 fluorescence immunoprecipitation assay (FIPA-M).[13,18] Testing at Oxford included a fluorescence immunoprecipitation assay (FIPA-O),[11] visual fluorescence-observation cell-based assay (CBA-O),[11,18] and a new quantitative flow cytometry (fluorescence-activated cell

Figure 1 **Distribution of neuromyelitis optica (NMO)/aquaporin-4 (AQP4)-immunoglobulin G (IgG) titers by 6 assays in serum samples of patients and controls**



Scatterplots of the 6 assays with cutoffs shown as dotted lines. The ELISA-R has 2 cutoffs, the recommended cutoff (5 U/mL) and a modified cutoff (1.6 U/mL). CBA = cell-based assay; DC = disease control; FACS = fluorescence-activated cell sorting; FIPA = fluorescence immunoprecipitation assay; FU = fluorescence units; HC = healthy controls; IIF = indirect immunofluorescence; M = Mayo Clinic; ΔMGFI = difference in median green fluorescence intensity; NMOSD = neuromyelitis optica spectrum disorder; O = Oxford.

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Figure 2 Concordance of assays in neuromyelitis optica (NMO) and NMO spectrum disorder (NMOSD) cohort

A

Diagnosis	FACS	CBA-O	CBA-E	ELISA (<1.6)	ELISA	FIPA-0	FIPA-M	IF
NMO	+	+	+	+	+	+	+	+
NMO	+	+	+	+	+	+	+	+
NMO	+	+	+	+	+	+	+	+
NMOSD-TM	+	+	+	+	+	+	+	+
NMO	+	+	+	+	+	+	+	+
NMO	+	+	+	+	+	+	+	+
NMO	+	+	+	+	+	+	+	+
NMO	+	+	+	+	+	+	+	+
NMOSD	+	+	+	+	+	+	+	+
NMO	+	+	+	+	+	+	+	+
NMOSD-ON	+	+	+	+	+	+	+	+
NMOSD-ON	+	+	+	+	+	+	+	+
NMO	+	+	+	+	+	+	+	+
NMOSD	+	+	+	+	+	+	+	+
NMOSD-TM	+	+	+	+	+	+	+	+
NMOSD-ON	+	+	+	+	+	+	+	+
NMO	+	+	+	+	+	+	+	+
NMOSD-TM	+	+	+	+	+	+	+	+
NMO	+	+	+	+	+	+	+	+
NMO	+	+	+	+	+	+	+	+
NMOSD-TM	+	+	+	+	+	+	+	+
NMOSD-ON	+	+	+	+	+	+	+	+
NMO	+	+	+	+	+	+	+	+
NMO	+	+	+	+	+	+	+	+
NMOSD-ON	+	+	+	+	+	+	+	+
NMOSD-TM	+	+	+	+	+	+	+	+
NMO	+	+	+	+	+	+	+	+
NMO	+	+	+	+	-	+	+	+
NMOSD	+	+	+	+	+	+	+	-
NMOSD-TM	+	+	+	+	+	+	+	-
NMOSD-TM	+	+	+	+	+	+	+	-
NMO	+	+	+	+	+	-	-	-
NMOSD-TM	+	+	+	+	+	-	-	-
NMO	+	+	+	+	+	-	-	-
NMOSD-TM	+	+	+	+	+	-	-	-
NMO	+	+	+	+	-	-	-	-
NMO	+	+	+	+	-	-	-	+
NMO	+	+	+	+	-	-	-	-
NMOSD-TM	+	+	+	+	-	-	-	-
NMOSD-TM	+	+	+	-	-	-	-	-
NMO	+	+	-	+	+	-	-	-
NMOSD-TM	+	+	-	+	+	-	+	-
NMO	+	+	-	-	-	-	-	-
NMO	+	+	-	+	-	-	-	-
NMOSD-ON	+	-	+	-	-	-	-	-
NMO	+	-	-	-	-	-	-	-
NMOSD-TM	-	-	-	-	-	+	-	-
NMOSD-TM	-	-	-	-	-	-	-	-
NMOSD-ON	-	-	-	-	-	-	-	-
NMOSD-ON	-	-	-	-	-	-	-	-
NMO	-	-	-	-	-	-	-	-
NMO	-	-	-	-	-	-	-	-
NMO	-	-	-	-	-	-	-	-
NMO	-	-	-	-	-	-	-	-
NMO	-	-	-	-	-	-	-	-
NMO	-	-	-	-	-	-	-	-
NMO	-	-	-	-	-	-	-	-
NMO	-	-	-	-	-	-	-	-
NMO	-	-	-	-	-	-	-	-
NMO	-	-	-	-	-	-	-	-
Patients	46/60	44/60	41/60	42/60	36/60	32/60	32/60	29/60
Controls	0/86	0/86	0/86	2/86	0/86	0/86	2/86	0/86
AUC-ROC	0.883	0.867	0.842	0.838	0.800	0.767	0.755	0.742

(A) Positive samples are marked + with an orange background. Negative samples are marked − with a white background. Longitudinally extensive transverse myelitis (LETM, TM) samples are highlighted in blue; recurrent optic neuritis (rON, ON) in pink. (B) The results from the fluorescence immunoprecipitation assays (FIPAs), performed in both centers on 9 samples from a single patient plotted against time, demonstrate the consistency of this simple assay to monitor serial samples. AUC = area under the curve; CBA = cell-based assay; E = EUROIMMUN; FACS = fluorescence-activated cell sorting; FU = fluorescence units; IF = immunofluorescence; M = Mayo Clinic; O = Oxford; ROC = receiver operating characteristic curve.

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Table 1 **Sensitivity and specificity of 6 aquaporin-4-IgG assays[a]**

	NMO (n = 35)	NMOSD (n = 25)	Total (n = 60)	Controls (n = 86)	Sensitivity	Specificity	ROC-AUC
IIF	17	12	29	0	48.3	100.0	0.742
FACS	25	21	46	0	76.7	100.0	0.883
CBA-O	24	20	44	0	73.3	100.0	0.867
ELISA-R (5.0)	18	18	36	0	60.0	100.0	0.800
FIPA-O	16	16	32	0	53.3	100.0	0.767
FIPA-M	16	16	32	2	53.3	97.7	0.755

Abbreviations: AUC = area under the curve; CBA = cell-based assay; FACS = fluorescence-activated cell sorting; FIPA = fluorescence immunoprecipitation assay; IgG = immunoglobulin G; IIF = indirect immunofluorescence; M = Mayo; NMO = neuromyelitis optica; NMOSD = neuromyelitis optica spectrum disorder; O = Oxford; R = RSR/Kronus; ROC = receiver operating characteristic curve.
[a] Results for blinded study of 146 samples on 6 assays with calculated sensitivities and specificities. The final column is a measure of assay accuracy.

sorting [FACS]) assay. The results were sent to the McGill University coauthor (A.B-O.) for decoding. Subsequently, both laboratories performed, according to the manufacturer's instructions (EUROIMMUN), a visual fluorescence-observation cell-based assay (CBA-E) that incorporated fixed HEK293 cells transfected singly with either human AQP4-M1 or M23 isoform.[15] Details of published methods are found in appendix e-1 on the *Neurology*® Web site at www.neurology.org.

Flow cytometry. Thirty-six hours after HEK293 cells were transfected with a plasmid encoding both human AQP4 and the fluorescent protein dsRed, the cells were trypsinized, resuspended in Dulbecco's modified Eagle's medium, 1% fetal calf serum, and 1 mM EDTA (FACS buffer) at 1.0×10^6 cells/mL, and rotated at 4°C for 2 hours. Patient serum (diluted 1:10 in FACS buffer) was mixed with 1.0×10^5 cells (100 μL). After holding at 4°C for 15 minutes, the cells were washed, and bound IgG was detected with Alexa 488–labeled antihuman IgG (diluted 1:500 in FACS buffer). The cells were resuspended 30 minutes later in 400 μL phosphate-buffered saline/5 mM EDTA and analyzed by FACSCalibur. The level of transfection was determined by measuring dsRed intensity (PE-Texas Red channel) in live cells (figure e-1, y-axis). Two gates were created: the higher gate captured cells expressing high levels of dsRed (labeled R5 in figure e-1); the lower gate captured untransfected or poorly transfected cells (labeled as R7 in figure e-1) and served as a negative control for each sample. Bound IgG was measured in the green channel (a shift to the right on the x-axis). A score for each serum was determined by subtracting the median green fluorescence in the lower gate from the median green fluorescence in the higher gate.

Statistical analyses. We used receiver operating characteristic curve (ROC) analysis to identify post hoc the optimal cutoff values for the ELISA-R assay to maximize disease sensitivity and specificity, and analyzed all data using SAS, version 9.1. The McNemar test for paired proportions was used to compare the sensitivities of the FACS, CBA-Oxford, and ELISA-R, with Bonferroni correction for multiple comparisons.

RESULTS Assays based on binding of IgG to HEK293 cells transfected with AQP4 proved to be the most sensitive (figures 1 and 2A): FACS (77%; 46 of 60), CBA-O (73%; 44 of 60), and CBA-E (68%; 41 of 60). The ELISA-R was the next most sensitive (60%: 36 of 60). FIPAs and the tissue-based IIF assay were least sensitive (48%–53%) (table 1, figure 2A). The 2 commercial assays combined (ELISA-R and CBA-E) identified 72% (43 of 60) of NMO/NMOSD samples as positive and no false positives (table 2). Interassay concordance overall was excellent (figure 2A); 28 of 60 samples were positive in all assays, and only 2 false-positive results were encountered (FIPA-M).

A post hoc ROC analysis of ELISA-R raw data revealed that by lowering the cutoff value from 5.0 U/mL to 1.6 U/mL, the sensitivity could be increased from 60% to 70%. An additional 6 of 60 patients with NMO or NMOSD were then scored as

Table 2 **Post hoc assay results: results with CBA-E and ELISA-R[a]**

	NMO (n = 35)	NMOSD (n = 25)	Total (n = 60)	Controls (n = 86)	Sensitivity	Specificity	ROC-AUC
CBA-E	21	20	41	0	68.3	100.0	0.842
ELISA-R (1.6)	23	19	42	2	70.0	97.7	0.838
CBA-E + ELISA	22	21	43	0	72.0	100.0	0.858

Abbreviations: AUC = area under the curve; E = EUROIMMUN; NMO = neuromyelitis optica; NMOSD = neuromyelitis optica spectrum disorder; R = R = RSR/Kronus; ROC = receiver operating characteristic curve.
[a] A reduction in cutoff from the recommended 5.0 U/mL to 1.6 U/mL improves the ELISA accuracy (demonstrated by an increase in the AUC-ROC score from 0.800 to 0.838). Combining the ELISA kit assay (using a cutoff of 5 U/mL) with CBA-E further improves the sensitivity (AUC-ROC 0.858).

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positive (figure 2A, table 2). All 6 were positive by FACS and CBA-O with 5 positive by CBA-E; however, there were also 2 false-positive results (1 healthy control and 1 patient with relapsing-remitting MS). Both of these latter false-positive samples tested negative on both cell-based assays.

As a diagnostic assay, the FIPAs were not very sensitive, but they were found to be suitable for serial determinations (figure 2B). Nine sera samples from a single patient taken over 5 years were also tested blinded. The FIPAs showed striking reproducibility between the 2 testing laboratories and a positive correlation between antibody level and treatment response. AQP4-IgG values fell after a single infusion of rituximab (Rituxan) and slowly increased in the following 20 months. A second infusion of rituximab (Rituxan) reduced the AQP4-IgG to an almost undetectable level.

Table 3 Improvement in ELISA sensitivity[a]

	FACS	CBA-O	CBA-E
ELISA-R (5.0)	0.0016	0.0047	0.0956
ELISA-R (1.6)	0.0455	0.1573	0.6547

Abbreviations: E = EUROIMMUN; FACS = fluorescence-activated cell sorting; O = Oxford; R = RSR/Kronus.
[a] The ELISA-R is significantly less sensitive than FACS or CBA-O (McNemar's test of paired proportions, corrected for multiple comparisons with a Bonferroni test) using the recommended cutoff of 5.0 U/mL, but not when the cutoff is reduced to ≥1.6 U/mL.

Comment: Sensitivity and clinical relevance of available anti-aquaporin-4 antibody assays

Anti-aquaporin-4 (AQP4) antibody occurs specifically in neuromyelitis optica (NMO), with various assays able to detect the autoantibody.[1] This international collaborative study compared the sensitivity and specificity of 6 different anti-AQP4 antibody assays, including 2 commercial kits, using coded sera from NMO, NMO spectrum disorders, multiple sclerosis, and control patients.[2] The study was properly done, although it is unclear how the authors selected samples with a wide range of antibody titers, which can substantially influence the sensitivity.

The result was that the specificities were excellent in all assays, but the sensitivities were different. Since anti-AQP4 antibody in NMO is undetectable in Western blot and stains the surface of cells transfected with AQP4, a transmembrane water channel, conformation of the extracellular AQP4 loops appears critically important for the antibody binding. Moreover, the amino acid sequences of extracellular AQP4 domains are somewhat different between humans and rodents. Thus, it was a logical result that human AQP4-transfected cell-based assays in which the antibody binds to the cells in solution were the most sensitive (73%∼77%).

The 2 commercial kits suitable to deal with a large number of samples were less sensitive (68% in fixed cell-based assay, and 60% in ELISA), probably because of some nonspecific antibody binding in the fixed cell-based assay and the use of nonmembrane-expressed human AQP4 in ELISA. Lowering the ELISA's cutoff improved the sensitivity, but also produced some false-positives. Manufacturing ELISA with membrane-expressed AQP4 would be desirable. Fluorescence immunoprecipitation assay (FIPA) and the mouse tissue-based immune fluorescence assay were the least sensitive (around 50%), but FIPA seemed useful in antibody titer follow-up.

This study is an important contribution to improve the clinical relevance of this highly specific biomarker for NMO. Although seronegativity does not exclude NMO, anti-AQP4 antibody serologic status has definite and important diagnostic and therapeutic implications,[1] so improving assay sensitivity without sacrificing specificity is crucial.

1. Jarius S, Wildemann B. AQP4 antibody in neuromyelitis optica and pathogenetic relevance. Nat Rev Neurol 2010;6:383–392.
2. Waters PJ, McKeon A, Leite MI, et al. Serologic diagnosis of NMO: a multicenter comparison of aquaporin-4-IgG assays. Neurology 2012;78:665–671.

Kazuo Fujihara, MD

Disclosure: Prof. Fujihara serves/has served on scientific advisory boards for Bayer Schering Pharma, Biogen Idec, and Merck Serono; has received funding for travel and speaker honoraria from Bayer Schering Pharma, Biogen Idec, Eisai Inc., Mitsubishi Tanabe Pharma Corporation, Astellas Pharma Inc., Takeda Pharmaceutical Company Limited, and Asahi Kasei Kuraray Medical Co., Ltd.; serves on the editorial board of *Clinical and Experimental Neuroimmunology*; receives publishing royalties from *Clinical Practice Guide of Orthopedic Surgery* (Bunkodo, 2007); and receives/has received research support from Bayer Schering Pharma, Eisai Inc., Kowa Pharmaceuticals America, Inc., Biogen Idec, Asahi Kasei Kuraray Medical Co., Ltd., The Chemo-Sero-Therapeutic Research Institute (KAKETSUKEN), Teva Pharmaceutical Industries Ltd., Mitsubishi Tanabe Pharma Corporation, Teijin Pharma, Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Technology of Japan, and Grants-in-Aid for Scientific Research from the Ministry of Health, Welfare and Labor of Japan.

DISCUSSION Sensitive and specific detection of NMO-IgG (AQP4-IgG) has become an essential laboratory investigation in evaluating patients with inflammatory CNS demyelinating disorders, because seropositivity has diagnostic, prognostic, and therapeutic implications. Several different techniques have been used to detect these autoantibodies, and no previous international multicenter comparative study has been performed. In this fully blinded study, we assessed different assays, including a novel in-house FACS method and 2 recent commercially available kit assays (ELISA-R and CBA-E).

The in-house FACS and CBA-O assay proved to be the most sensitive. There was a significant difference in the sensitivity between these assays and the ELISA-R. However, reducing the recommended cutoff for the ELISA-R from 5.0 U/mL to 1.6 U/mL eliminated these significant differences (table 3) but slightly reduced the ELISA-R specificity. The tissue-based IIF assay and FIPA were not optimal for diagnostic assays because of lower sensitivities (48%–53%).

The demand for AQP4-IgG testing is increasing globally. In the past 12 months, the Mayo Clinic's Neuroimmunology Laboratory tested 20,334 patients' sera for AQP4-IgG on a service basis and the Oxford laboratory tested 3,500. The expertise and resources required to perform flow cytometry assays preclude its use in small-scale clinical diagnostic laboratories. This study affirms that commercially available kit assays (ELISA-R and CBA-E) are both sensitive and specific for AQP4-IgG detection. Their relative simplicity to perform, minimal reagent requirement, and amenability to currently available automation platforms allow nonspecialized laboratories to offer sensitive and specific AQP4-IgG testing. This study suggests that CBA-E could be used as a convenient routine assay. The ELISA-R, with the lower cutoff value of 1.6 U/mL, may be a sensitive screening tool, but sera yielding values between 1.6 and 4.9 U/mL would require confirmatory specificity testing by CBA-E.

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Assay refinement has reduced the frequency of NMO-IgG seronegativity. It is important to determine whether IgG of a different CNS antigen specificity might account for patients with NMOSD who lack detectable AQP4-IgG. An essential prerequisite is to maximize AQP4-IgG assay sensitivity to exclude false negatives. The FACS assay appears to be the most useful in this research context, whereas the FIPA may be more convenient for monitoring patients longitudinally. Functional assays are promising for yielding data to correlate with disease severity and possibly predict relapse.[19]

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AUTHOR CONTRIBUTIONS

Study design and conceptualization: P.J.W., A.B.-O., S.P. Drafting of manuscript: P.J.W., S.J.P. Acquisition, analysis and interpretation of data: P.J.W., A.M., M.I.L., S.R., V.A.L, A.V., J.P., J.N.M., A.Villalobos, A.B.-O., S.J.P. Statistical analysis: P.J.W., J.N.M., S.J.P. Critical revision of the manuscript: P.J.W., A.M., M.I.L., S.R., V.A.L., A.Villalobos, J.P., J.N.M., A.Vincent, A.B.-O., S.J.P.

DISCLOSURE

Dr. Waters is a named inventor on a patent relating to assays for the detection of antibodies to Lgi1, Caspr2, and Contactin2 and may receive royalties for this technology. Dr. Waters receives research support from the Oxford NIHR Biomedical Research Centre. Dr. McKeon receives research support from the Guthy Jackson Charitable Foundation. Dr. Leite receives research support from the Oxford Biomedical Research Centre, the National Commissioning Group, and the Sir Halley Stewart Trust, UK. Dr. Rajasekharan reports no disclosures. Dr. Lennon is a named inventor on a patent relating to aquaporin-4 antibodies for diagnosis of neuromyelitis optica and receives royalties for this technology; is a named inventor on patents that relate to functional AQP4/NMO-IgG assays and NMO-IgG as a cancer marker; and receives research support from the Guthy Jackson Charitable Foundation. A. Villalobos reports no disclosures. Dr. Palace serves on scientific advisory boards for Merck Serono, Bayer Schering Pharma, Biogen Idec, Teva Pharmaceutical Industries Ltd., Novartis, and sanofi-aventis; has received funding for travel from Merck Serono; and receives research support from the Multiple Sclerosis Society and the Department of Health Risk Sharing Scheme (Clinical Coordinator). Dr. Mandrekar reports no disclosures. Dr. Vincent is a named inventor on a patent relating to assays for the detection of antibodies to Lgi1, Caspr2, and Contactin2 and may receive royalties for this technology. Dr. Vincent has served on scientific advisory boards for the Patrick Berthoud Trust and the Myasthenia Gravis Foundation of America; has received funding for travel and a speaker honorarium from Baxter International Inc.; serves as an Associate Editor for *Brain*; receives royalties from the publication of *Clinical Neuroimmunology* (Blackwell Publishing, 2005) and *Inflammatory and Autoimmune Disorders of the Nervous System in Children* (Mac Keith Press, 2010); receives research support from the European Union, the Oxford NIHR Biomedical Research Centre, and Sir Halley Stewart Trust; and has received Musk antibody royalties and consulting fees from Athena Diagnostics, Inc. and Musk antibody royalties from RSR Ltd., Cardiff, UK. The University of Oxford, where A.V. is based, receives royalties and payments for antibody assays in neurologic diseases. Dr. Bar-Or serves on scientific advisory boards for BioMS Medical, DioGenix, Inc., Ono Pharmaceutical Co. Ltd., GlaxoSmithKline, Roche, Guthy Jackson Greater Good Foundation, and NMO Research and Clinical Care Consortium; serves on the editorial boards of *Neurology*® and *Clinical and Experimental Neuroimmunology*; has received speaker honoraria from Biogen Idec, Bayhill Therapeutics, Bayer Schering Pharma (Berlex), Eli Lilly and Company, Genentech, Inc., GlaxoSmithKline, Merck Serono, Novartis, Wyeth, sanofi-aventis, and Teva Pharmaceutical Industries Ltd.; and receives/has received research support from BioMS Medical, Merck Serono, Bayhill Therapeutics, Biogen Idec, Genentech, Inc., and Teva Pharmaceutical Industries Ltd. Dr. Pittock is a named inventor on patents that relate to functional AQP4/NMO-IgG assays and NMO-IgG as a cancer marker; receives research support from Alexion Pharmaceuticals, Inc., the Guthy Jackson Charitable Foundation, and the NIH.

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Editor's Note to Authors and Readers: Levels of Evidence coming to *Neurology*®

Effective January 15, 2009, authors submitting Articles or Clinical/Scientific Notes to *Neurology*® that report on clinical therapeutic studies must state the study type, the primary research question(s), and the classification of level of evidence assigned to each question based on the classification scheme requirements shown below (left). While the authors will initially assign a level of evidence, the final level will be adjudicated by an independent team prior to publication. Ultimately, these levels can be translated into classes of recommendations for clinical care, as shown below (right). For more information, please access the articles and the editorial on the use of classification of levels of evidence published in *Neurology*.[1-3]

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Classification scheme requirements for therapeutic questions

Class I. A randomized, controlled clinical trial of the intervention of interest with masked or objective outcome assessment, in a representative population. Relevant baseline characteristics are presented and substantially equivalent among treatment groups or there is appropriate statistical adjustment for differences.

Class II. A randomized, controlled clinical trial of the intervention of interest in a representative population with masked or objective outcome assessment that lacks one criterion a-e in Class I or a prospective matched cohort study with masked or objective outcome assessment in a representative population that meets b-e in Class I. Relevant baseline characteristics are presented and substantially equivalent among treatment groups or there is appropriate statistical adjustment for differences.

Class III. All other controlled trials (including well-defined natural history controls or patients serving as their own controls) in a representative population, where outcome is independently assessed, or independently derived by objective outcome measurements.

Class IV. Studies not meeting Class I, II, or III criteria including consensus or expert opinion.

AAN classification of recommendations

A = Established as effective, ineffective, or harmful (or established as useful/predictive or not useful/predictive) for the given condition in the specified population. (Level A rating requires at least two consistent Class I studies.)

B = Probably effective, ineffective, or harmful (or probably useful/predictive or not useful/predictive) for the given condition in the specified population. (Level B rating requires at least one Class I study or two consistent Class II studies.)

C = Possibly effective, ineffective, or harmful (or possibly useful/predictive or not useful/predictive) for the given condition in the specified population. (Level C rating requires at least one Class II study or two consistent Class III studies.)

U = Data inadequate or conflicting; given current knowledge, treatment (test, predictor) is unproven.

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