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An Update on the Laboratory Diagnosis of Neuromyelitis Optica Spectrum Disorders

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Neuromyelitis optica spectrum disorder (NMOSD) is an autoimmune disorder of the central nervous system that is specifically associated with demyelination of spinal cord and optic nerves. The discovery of specific autoantibody markers such as aquaporin-4 IgG and myelin oligodendrocyte glycoprotein IgG has led to several methodologies being developed and validated. There have been numerous investigations of the clinical and radiological presentations used in the clinical diagnosis of NMOSD. However, although various laboratory diagnostic techniques have been standardized and validated, a gold-standard test has yet to be finalized due to uncertain sensitivities and specificities of the methodologies. For this review, the literature was surveyed to compile the standardized laboratory techniques utilized for the differential diagnosis of NMOSD. Enzyme-linked immunosorbent assays enable screening of NMOSD, but they are considered less sensitive than cell-based assays (CBAs), which were found to be highly sensitive and specific. However, CBAs are laborious and prone to batch variations in their results, since the expression levels of protein need to be maintained and monitored meticulously. Standardizing point-of-care devices and peptide-based assays would make it possible to improve the turnaround time and accessibility of the test, especially in resource-poor settings.

Keywords neuromyelitis optica; laboratory diagnosis; aquaporin 4; myelin-oligodendrocyte glycoprotein; autoimmune disorder.

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INTRODUCTION

Neuromyelitis optica spectrum disorder (NMOSD), which is known as Devic's disease, is an autoimmune disorder characterized by chronic inflammation and demyelination of the central nervous system (CNS), and typically affects the spinal cord and optic nerves. NMOSD occurs worldwide, though epidemiological studies have estimated the prevalence of neuromyelitis optica (NMO) to be higher among the black population (10/100,000 population), followed by Asians (~3.5/100,000 population) and then White/Caucasian populations (~1/100,000 population). East Asian populations, namely Japanese (3.42/100,000) and Koreans (2.56/100,000), showed higher prevalence rates of NMOSD than do other Asian countries.[1-3] The etiology of NMOSD has remained elusive, but the understanding of the immunopathogenesis has improved following the discovery of related autoantibodies, namely aquaporin-4 (AQP-4) and myelin oligodendrocyte glycoprotein (MOG).

The present review focuses on the importance of these autoantibodies and their utility in the diagnosis of NMOSD using both traditional and newer technologies.

SERUM AUTOANTIBODIES IN NMOSD AS A BIOMARKER

NMO is often misdiagnosed as multiple sclerosis (MS), but in 2004 Lennon et al.[4] reported

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a NMOSD-specific biomarker AQP-4 IgG utilizing mouse brain and kidney as the substrate. Those authors applied dual immunofluorescence staining to brain tissues using serum samples of patients and found that AQP-4 IgG was detectable in patients with NMOSD but not in those with MS.[4] Detection of anti-AQP-4 IgG1 during the early disease phase will facilitate disease-specific therapies. However, a subgroup of NMOSD patients were recently reported to be negative for anti-AQP-4 antibodies but positive for MOG antibodies, delineating further a specific pathological entity referred to as "MOG-encephalomyelitis" (MOG-EM) or MOG antibody disease.

Aquaporins are a group of water-channel proteins expressed on the cell membrane that control the water flux of cells. Among 13 subfamilies of aquaporins, AQP-4 is highly expressed in the foot process of astrocytes. The AQP-4 tetramers organize on the cell membrane as orthogonal arrays of particles (OAPs), and each monomer has two isoforms: M1 and M23.[5] Detecting AQP-4 autoantibodies has changed the criteria for diagnosing NMOSD. Investigations of the pathogenic role of autoantibodies shows that upon binding to AQP-4, AQP-4 IgG activates the complement pathway resulting in lytic complex C5b-9 and leading to irreversible astrocyte damage. Furthermore, complement-mediated damage is increased by the activation of tumor necrosis factor α, interleukin (IL)-6, IL-1β, and interferon-γ in NMOSD.[5,6]

The integrity of the myelin sheath is maintained by cell-surface proteins such as myelin basic protein (MBP), proteolipid protein, and MOG synthesized by oligodendrocytes. MOG is a specific oligodendrocyte differentiation marker that mediates cytoskeleton formation and the stability of microtubules.[7] Numerous experimental studies performed during the late 1990s demonstrated that autoantibodies against MOG are associated with inflammatory demyelinating diseases of the CNS. Biopsies and postmortem studies of brain tissues of encephalomyelitis patients have demonstrated the pathogenic implications of anti-MOG antibodies, which are mediated by the activation of T- and B-cell responses.[8,9]

Several meta-analyses have revealed clinical and radiological aspects of the diagnosis of NMOSD, but there is still insufficient information about laboratory diagnoses of NMOSD, and a gold-standard test still needs to be identified.

LABORATORY DIAGNOSTIC METHODOLOGIES STANDARDIZED TO DETECT AQP-4 AND MOG ANTIBODIES

All previous laboratory diagnoses of NMOSD and MOG-EM have relied on the serostatus of AQP-4 and MOG autoantibodies in the serum and cerebrospinal fluid (CSF). Diagnosing NMO using CSF is usually not recommended since sampling is invasive and serum samples are sufficient to provide the required information. Western blotting and the enzyme-linked immunosorbent assay (ELISA) were initially used to detect MOG IgG, and the results were controversial for MS and other demyelinating disorders. Taking into consideration these challenges, have researchers searched for alternative biomarker to differentially diagnose NMOSD, which revealed AQP-4 IgG. AQP-4 IgG has received more attention than MOG, but the quest for high-throughput laboratory techniques to detect AQP-4 and MOG IgG has continued (Fig. 1). Since the discovery of AQP-4 in 2004 using tissue-based indirect immunofluorescence (IIF) staining,[4] various diagnostic methods such as ELISA,[10-12] radioimmunoprecipitation assay (RIPA),[13] fluorescence immunoprecipitation assay (FIPA),[14] immunofluorescence cell-based assays (CBAs),[11,15,16] and fluorescence-activated cell sorting (FACS)[17-19] have been standardized, but their specificities and sensitivities have remained unclear (Table 1).[20,21] Various factors such as protein conformations, isoforms, and posttranslation modifications, as well as serum dilution are the common hurdles when designing a diagnostic procedure.

Indirect immunofluorescence assay

Tissue-based method

The first-generation diagnostic method used for the differential diagnosis of NMOSD was a tissue-based IIF method using frozen brain tissue sections of mice, rats, and nonhuman primates, and was initially considered as a gold standard, particularly for the monkey cerebellum. Lennon et al.[4] originally used mouse brain tissue as the substrate, and reported a sensitivity of 73% and a sensitivity of 91%, and suggested that the lower sensitivity could have resulted from the use of nonhuman substrates. However, studies have observed that using tissue section as a substrate enables efficient preliminary screening, since the antibodies bind to both the intracellular and extracellular motifs of AQP-4.

While studying the utility of AQP-4 IgG in diagnosing NMOSD, Apiwattanakul et al.[22] observed that a tissue-based immunofluorescence assay (IFA) detected only 40% of NMO seropositive cases, thus indicating its low sensitivity compared with ELISA and CBA.

Long et al.[23] deviated from the native protocol with the aim of improving the methodology by modifying the IIF assay using monkey brain sections as the substrate. However, the sensitivity was reported to be 62%, which was lower than that of the original test (70% sensitivity), and so they concluded that mouse brain sections are superior to monkey brain sections

Table 1. Sensitivities and specificities of assays reported in the literature standardized for the laboratory diagnosis of NMO, with AQP-4 IgG detection using a CBAIIF as the gold standard

Authors	Year	Number of samples tested	Tests performed	Sensitivity (%)	Specificity (%)
Lennon et al.[4]	2004	124	Brain tissue IIF for NMO IgG	61.3	90.9
Takahashi et al.[47]	2007	148	AQP-4 CBA with IIF	91	100
Paul et al.[13]	2007	291	RIPA	62.8	98.3
Waters et al.[61]	2008	114	Brain tissue IIF for NMO IgG	NA	100
			AQP-4 CBA with IIF FIPA	76–80	100
Hayakawa et al.[34]	2008	285	ELISA	71	87
			Brain tissue IIF for NMO IgG	62	85
McKeon et al.[62]	2009	6,335	Brain tissue IIF for NMO IgG	58	99.6
			FIPA	33	99.3
Fazio et al.[18]	2009	52	Mouse brain tissue IIF for NMO IgG	46.7	95.5
			Rat brain tissue IIF for NMO IgG	39.4	96.8
			AQP-4 CBA with IIF	39.4	100
			FACS	30.3	96.8
			RIPA	33.3	96.8
Jarius et al.[16]	2010	151	AQP-4 CBA with IIF	78	100
			Brain tissue IIF for NMO IgG	65.6	99.0
Kim et al.[10]	2012	300	M1/M23 AQP-4 ELISA	72	100
			AQP-4 CBA with IIF	78	100
Long et al.[23]	2012	168	Brain tissue IIF for NMO IgG	62	89.5
Apiwattanakul et al.[22]	2012	31	Brain tissue IIF for NMO IgG	40	NA
			AQP-4 CBA with IIF	60	NA
			ELISA	50	NA
Isobe et al.[11]	2012	170	ELISA	48.3	96.7
			AQP-4 CBA with IIF	41.4	97.1
			FACS	51.7	97.1
Kim et al.[12]	2012	300	Brain tissue IIF for NMO IgG AQP-4 CBA with IIF ELISA	44.4–55.6	87.0–92.2
Kang et al.[40]	2012	147	AQP-4 CBA with IIF	86	91
			FIPA	79	100
Waters et al.[37]	2012	146	Brain tissue IIF for NMO IgG	48	100
			FIPA	53	100
			AQP-4 CBA with IIF	68	100
			ELISA	60	100
			FACS	76.7	100
Fryer et al.[35]	2014	338	M1 AQP-4 ELISA	58	99
			M1 AQP-4 FACS	83	100
			M23 AQP-4 FACS	75	95
			M1 AQP-4 CBA	75	100
Ambika et al.[63]	2015	40	ELISA	68	100
			CBA	73	100
Yang et al.[64]	2016	225	M23 FACS	77.3	100
			AQP-4 CBA with IIF	69.7	100

Table 1. Sensitivities and specificities of assays reported in the literature standardized for the laboratory diagnosis of NMO, with AQP-4 IgG detection using a CBAIIF as the gold standard (continued)

Authors	Year	Number of samples tested	Tests performed	Sensitivity (%)	Specificity (%)
Waters et al.[24]	2016	209	Live-cell AQP-4 CBA with IIF	98–100	97–100
			Fixed-cell AQP-4 CBA with IIF	80.3–93.9	92.7–100
			FACS	69.7–100	90.6–100
			Brain tissue IIF for NMO IgG	51.5–98.5	81.3–94.3
			ELISA	83.3	92.2
Kim et al.[29]	2017	386	In-house AQP-4 CBA with IIF	80	100
Prain et al.[20]	2019	434	Brain tissue IIF for NMO IgG	78	100
			ELISA	60	97
			AQP-4 CBA with IIF	90–94	100
Pandit et al.[32]	2021	381	AQP-4 CBA with IIF	77.01	100
			In-house AQP-4 CBA with IIF	81.61	100

AQP-4, aquaporin-4; CBAIIF, cell-based assay indirect immunofluorescence; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FIPA, fluorescence immunoprecipitation assay; IIF, indirect immunofluorescence; NA, not available; NMO, neuromyelitis optica; RIPA, radioimmunoprecipitation assay.

as a substrate.[23]

To improve the sensitivity of the assay, Jarius et al.[14] designed an FIPA using mouse cerebellum cryosections as the substrate, and reported a sensitivity of 76%–80% and a specificity of 100%. Multicenter comparison studies have also suggested that a tissue-based IIF assay combined with ELISA and CBA can be used for screening, but not as a confirmatory assay.[24]

Cell-based assays

Rogers et al.[25] were the first to report on the use of high-sensitivity CBAs for detecting autoantibodies against glutamate receptor 3 in Rasmussen encephalitis. In 2005 Lennon et al.[26] found that NMO IgG specifically targets AQP-4 protein, which served as a background for the development of CBAs.

ELISAs for anti-MOG antibodies are not recommended for clinical practice since their results can be inaccurate. In general, ELISAs and other protein-based methods use unfolded or partial proteins as the antigenic substrate, which are not intact. The unmatched performance and reliability of CBAs in detecting AQP-4 antibody has prompted researchers to devise CBAs for detecting anti-MOG antibodies.[27] Furthermore, the utility of CBAs in neuroimmunology has led to the important conclusion that native protein is the key to improving the sensitivity of tests for detecting NMO IgG. CBAs have been considered the gold standard and are recommended in the diagnostic interpretation of NMOSD. In a CBA, transfected cells overexpress a specific target protein that serve as the substrate. Patient sera is then applied to the fixed cells, and after incubation the unbound antibodies are washed, and AQP-4 and NMO antibodies are detected using a fluorescent tagged secondary antibody.

Takahashi et al.[15] pioneered an indigenous antihuman AQP-4 antibody titration assay using AQP-4 transfected cells, which was reported to be sensitive since it was able to detect two AQP-4 positive samples that had tested negative for NMO IgG when using a standard tissue-based IFA. Those authors further expanded their CBA testing to a larger population (148 serum samples of clinically suspected NMOSD patients), and concluded that CBAs had a sensitivity of 91% and a specificity of 100% in detecting NMOSD.

While they are more sensitive than the native assays, CBAs have a few limitations such as the requirement of a fluorescence microscope, maintenance of cell lines, use of freshly transfected cells, variation in the quality of expressed epitopes and, most importantly, diagnostic laboratories possibly not being equipped to perform a recombinant technique. To overcome some of these limitations, in 2010 Jarius et al.[16] developed a highly sensitive recombinant IFA, wherein a large batch of transfected HEK293 cells was grown on cover slips and stored in liquid nitrogen, and the biochips were glued to microscopic slides when required. They reported a sensitivity of 78.1% and a specificity of 100%, and also showed that storing the slides did not impair the reproducibility of results.

To achieve improvements, researchers have experimented with a green fluorescent protein (GFP)-tagged AQP-4 recombinant system to ensure stable expression and colocalization, and reported that N-terminal or C-terminal tagged AQP-4 is cytotoxic and that they could modify the conformation of the antigen, which in turn may affect the assay sensitivity.[21,28] Kim et al.[29] subsequently standardized a novel CBA with M23-AQP-4-expressing HEK293 cells, which stably expressed the target M23 isoform as well as GFP using the internal ribosome entry site bicistronic vector. Their novel in-house assay reportedly had a sensitivity of 80% and a specificity of 100%. They also used a similar strategy to subsequently de-

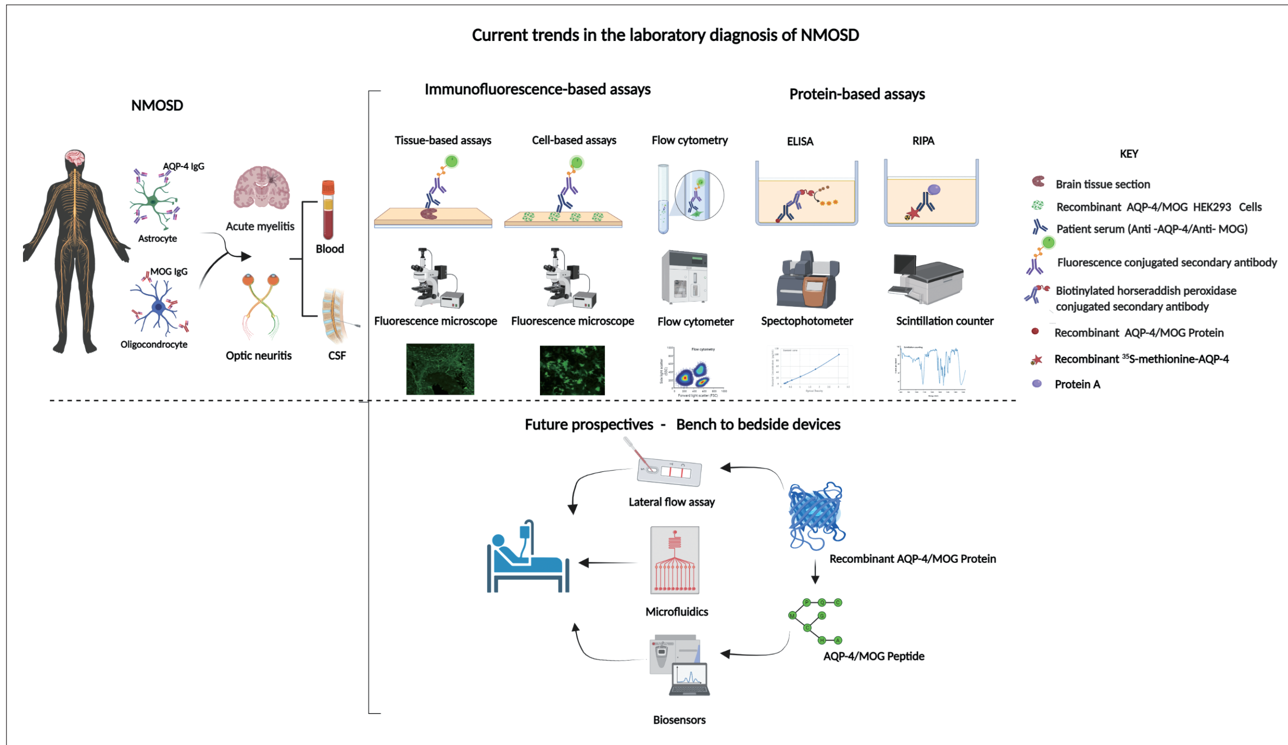

Fig. 1. Schematic overview of the standardized laboratory diagnostic assays and the future prospects in detecting NMOSD. AQP-4, aquaporin-4; CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; MOG, myelin oligodendrocyte glycoprotein; NMOSD, neuromyelitis optica spectrum disorder; RIPA, radioimmunoprecipitation assay.

velop a CBA for screening MOG IgG, which they used to investigate the serostatus of 355 CNS inflammatory diseases. The results showed that none of the patients exhibited seropositivity for MOG IgG and AQP-4 IgG.[30]

Waters et al.[31] investigated the conformational sensitivity of anti-MOG antibodies by performing a CBA with HEK293 cells expressing full-length MOG and C-terminus-truncated MOG as the substrate. Their results demonstrated that the use of truncated MOG interfered with the assay sensitivity whereas the native intact MOG improved the assay sensitivity. In 2021 Pandit et al.[32] generated a Chinese hamster ovary cell line expressing the M23 isomer of AQP-4 to devise an in-house CBA, and reported the assay to be more sensitive (81.61%) than a commercial CBA (77.01%) in detecting definite NMOSD.

Fluorescence-activated cell sorting

Improvements to CBAs and the development of more-specific techniques are still under consideration in the diagnosis of NMOSD. These approaches include FACS, which is considered as a high-throughput technique that enables quantitation of the autoantibody titer and an operator-independent platform. To perform FACS analysis, the AQP-4 transfected cells are trypsinized, washed with PBS, permeabilized at room temperature, and incubated with serum samples.

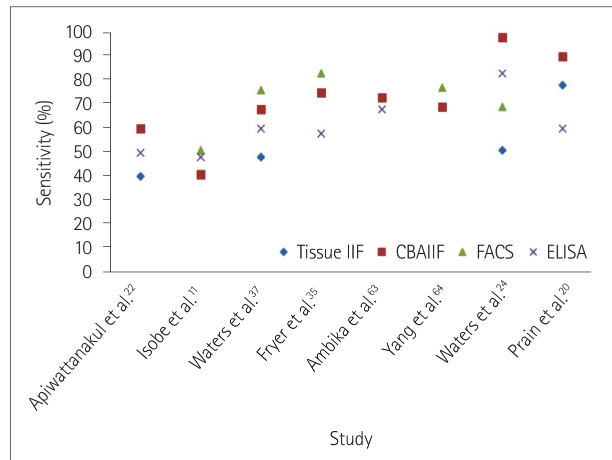

Fig. 2. Assay performance in diagnosing NMOSD, for comparing whether CBAs are more sensitive than tissue-based assays and ELISA. CBA, cell-based assay; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; IIF, indirect immunofluorescence; NMOSD, neuromyelitis optica spectrum disorder.

Unbound antibodies are washed and further incubated with phycoerythrin-conjugated secondary antibody, after which the cells are washed and analyzed in a flow cytometer.

In 2009 Fazio et al.[18] optimized an in-house flow cytometry assay using stable AQP-4-transfected HEK293 cells and screened 52 serum samples that were suspected to be NMOSD

positive, and found a sensitivity of 30% and a specificity of 96%. In 2010 Kalluri et al.[33] developed an FACS assay based on a native AQP-4 expression strategy. To mimic posttranslation modification during the expression of AQP-4, they used LN18 human glioma cells as the expression system. The novel substrate expressing native AQP-4 improved the sensitivity of the assay to 57.9% and its specificity to 100%.

In 2011 De Vidi et al.[17] standardized FACS by generating an AQP-4-transfected HEK293T cell line using an HIV-1-based vector to improve the expression stability and sensitivity. However, their results indicated that the CBA methodology using the same cell line was much more sensitive than FACS. Kim et al.[29] used a novel M23-AQP-4-transfected HEK293 cell line similar to that previous method CBA and also performed FACS analysis, which showed high sensitivity comparable to that of the CBA.

These findings indicate that while FACS is an advanced technology, its reported sensitivity has not been higher than those of CBA with IIF.

Protein-based assays

Enzyme-linked immunosorbent assays

ELISAs are classical immunoenzymatic assays used to detect antibodies, and they are user-friendly, have scope for automation, convenient, do not require expertise for result interpretation, and are cost-effective and ideal for simultaneous screening in larger numbers of patients.

The first ELISA for detecting anti-AQP-4 was designed by Hayakawa et al.[34] in 2008 using recombinant rat AQP-4 as the substrate, which had a sensitivity of 47% and a specificity of 85%. To quantitative and correlate the AQP-4 IgG subclass, Isobe et al.[11] subsequently developed an ELISA utilizing purified recombinant human AQP-4 as the substrate, which was later commercialized and manufactured by RSR™ (Cardiff, UK). Although the kit had a high specificity (96%), its sensitivities reported by various groups were variable (48−75%),[34-36] thus restricting its utility in diagnoses.

To increase the test sensitivity, ELISARSR™ AQP-4 Ab Version 2 was launched by RSR™ using the M23 isoform as the substrate, with a claimed sensitivity of 77%. In 2012 Kim et al.[12] developed an in-house M1 and M23 ELISA, and showed that the test sensitivity was independent of the isoform utilized.

While an ELISA can provide quantitative results, factors such as nonspecific antibody binding, irreproducible results, and 0.5%−1.2% false-positive results red flag its use as a diagnostic tool for NMOSD.

Radioimmunoprecipitation assay

Paul et al.[13] was the first to design a high-throughput RIPA using recombinant ^{35}S-methionine-AQP-4 as the substrate. The novel RIPA was reported to be an observer-independent method capable of providing reproducible results with a sensitivity of 62.8% and a specificity of 98.3%. However, the assay has major limitations, such as a laborious procedure, high cost, and the use of radioactive compounds.

ASSAY COMPARISON

Various single-center as well as multicenter studies have tested NMO serum samples using more than two methodologies in comparisons to determine the superior technique to be followed for the routine diagnosis of NMOSD (Fig. 2). In 2009 Fazio et al.[18] tested 33 serum samples using mouse and monkey CNS-tissue-based IFA, RIPA, CBA, and FACS, and concluded applying an IFA to monkey cerebellum was more sensitive but less specific than the other methods.

De Vidi et al.[17] constructed a novel CBA and FACS, and validated them against the standard primate cerebellum IIFA. They found that the concordance and likelihood of seropositivity was improved in the CBA compared with the IFA. Waters et al.[37] applied multicenter assessments of in-house and commercial tissue-based IIF NMO-IgG assay, ELISA (RSR™), GFP-AQP-4 fluorescence immunoprecipitation assay, CBA, and FACS to 146 serum samples. Their results showed that tests performed with AQP-4 transfected cells yielded the highest sensitivity (73%−77%), while the sensitivity was lowest for tissue-based IFA (48%−53%).

Due to the high prevalence of MOG-positive AQP-4-negative phenotypes of demyelinating disorders, Waters et al.[38] compared 3 CBAs (Euroimmun fixed-cell CBA, Oxford live-cell CBA, and FACS) in a large cohort of 394 patients for anti-MOG antibodies. It was particularly interesting that the positive predictive value was higher for assays performed on live cells than when using fixed cells. As mentioned above, MOG antibodies are conformation-sensitive, and fixing the cells could distort the conformation of the target epitopes of MOG.

Similarly, Tzartos et al.[39] compared the efficiency of an in-house live-cell anti-MOG CBA (for detecting IgG1) with an in-house live-cell CBA (detect IgG) and a fixed-cell commercially available CBA. Their results highlighted that the live-cell IgG1 CBA exhibited improved sensitivity and was also strongly correlated with the progression and relapse of the NMSOD. Kang et al.[40] compared the efficiency of CBAs and FIPAs by testing the AQP-4 serostatus of 36 patients, and their results supported the utility of the CBA since it was more sensitive (86%) than the latter approach.

Waters et al.[24] performed a detailed systematic comparison of 21 widely used AQP-4 assays under the Eugene Devic European Network project, which involved collaborating with 15 European centers. The 21 assays included live- or fixed-cell CBAs and FACS, tissue-based IFA, and ELISA. One major strength of their study was that instead of testing clinically defined NMO samples, to arrive at a sensitive assay they included random cases where the neurological defect was associated with autoimmunity. Three assays that employed live-cell CBAs with M32-AQP-4 and FACS showed a sensitivity of 100%, and all of the centers reported that overall the CBAs using either fixed or live cells were the most sensitive. However, wide variations in the sensitivities of assays were observed among the centers, which underscores the need for further standardization of the methodology.

Prain et al.[20] recently performed a blinded comparison of five commercially available assays: than tissue-based IFA, ELISA (RSR™), EI M1/M23, Euroimmun® M1/M23 biochip; Euroimmun® AQP-4 fixed-cell CBA; Oxford AQP-4 live-cell CBA; and MOG CBA (Euroimmun®, Germany). They observed that CBA was more sensitive (90%−94%) than tissue-based IFA (78%) and ELISA (60%), which they attributed to a high rate of false-positive results. Thus, the development of CBAs has enhanced the ability to identify MOG IgG and AQP-4 IgG in non-MS acquired demyelinating CNS syndromes.

CBAs are considered to exhibit superior specificity due to the expression of the native conformationally stable epitopes being identified by specific autoantibodies. However, studies have found varying sensitivities among the gold-standard CBAs, which could be due to the isoforms of AQP-4.

The M1 isoform was initially thought to bind to AQP-4 IgG, until studies performed by Pisani and his colleagues found that the OAPs formed by the M1-AQP-4/M23-AQP-4 heterotetramers on the cell membrane are specifically recognized by the AQP-4 IgG.[41,42] The M1 isoform (with an extra 22 amino acids) is capable of forming OAPs only when they congregate with M23, limiting its sensitivity and utilization in assays.[43] To explain this phenomenon, Crane et al.[44] used U87MG glioblastoma cells to study the binding specificity of NMOSD IgG to M1/M23 OAPs, and showed that the affinity was dependent on the OAP assembly, with the M23 isoform interaction with the autoantibody being stronger than that of M1. Furthermore, the large aggregates of M23 OAPs formed by tetramer–tetramer interactions provided a high affinity for AQP-4 IgG, which enhances the assay sensitivity and thus these OAPs are widely employed when designing CBAs and ELISAs.

However, laboratory studies have produced conflicting results when using the M1 and M23 isoforms for detecting NMOSD. Jarius et al.[36] reported that the performance of commercial M1 ELISA was significantly better than immunohistochemistry for murine brain tissue sections, and attributed this to the higher recombinant antigen concentration in an ELISA.

In order to quantify AQP-4 IgG in NMOSD, Kim et al.[12] evaluated an in-house ELISA using the M1 and M23 isoforms in a large cohort of patients, and observed that the NMOSD detection rate was similar when using either isoform, although the signal-to-noise ratio was higher when using M23. A significantly higher sensitivity of 97% was exhibited by the M23 CBA in detecting definitive NMOSD, compared with only 70% sensitivity for the M1 isoform. They also reported a delineating staining pattern, wherein the antibodies binding to M23 OAPs demonstrated a laminar pattern and the antibodies binding to non-OAP were observed as a pointed pattern. It was particularly interesting that their investigations of the immune responses to the M1 and M23 isoforms revealed that they were associated with the disease status of the patients: elevated M1 AQP-4 IgG positivity was noted in patients with longer disease duration and relapse, while M23 AQP-4 IgG positivity was recorded in early NMOSD, endorsing these two isoforms as a significant biomarker.

CLINICAL PERSPECTIVES OF LABORATORY DIAGNOSIS OF NMOSD

According to various multicenter and single-center studies, CBAs are considered the gold standard since they are highly sensitive (mean sensitivity of 76.7%) and specific (100%) in the diagnosis of definite NMOSD.[24,38] The International Panel for NMO Diagnosis (IPND) performed an extensive study in 2015 that included an AQP-4 CBA as a diagnostic criteria for NMSOD.[44] The IPND has also consented the use of ELISAs and IIF assays (mean sensitivity of 64%) when CBAs are not available, but with an alert on low titer values, in which case the assay should be repeated, the result validated by a different assay, or the samples sent to a referral laboratory.[20,45,46]

As discussed in the previous section, the sensitivities of commercial as well as in-house CBAs have varied among studies. The live-cell CBA was found to be superior to the fixed-cell CBA, yet unpredictable parameters such as the transfection efficiency, stable transfection, expression of native epitopes, isoforms, IgG1-specific secondary antibodies, and interpreting personnel are all challenges to be addressed when reporting results.

Important preanalytical factors include the type of sample, since serum has a higher diagnostic value than CSF (recommended only when seronegative for both AQP-4 IgG and MOG IgG), and the disease course, regarding whether it is monophasic/relapsing/remitting disease and the administration of immunosuppressive therapy should be considered

while interpreting NMOSD. However, the IPND categorization insisted on considering the combinatorial results of the clinical presentation, neuroradiology testing, and serology when making a definitive decision about NMOSD.[45]

The titer levels of NMOSD IgG have always been a concern to clinicians when they are performing diagnoses and deciding about treatments. Since the advent of AQP-4 as a biomarker, few studies have correlated the relationship between the disease activity and AQP-4 IgG titer. Low titers have been observed as a consequence of effective immunotherapy, and have fluctuated to high titers during relapse. Additionally, patients with low titers did not experience relapse, and so previous studies concluded that continuous follow-up of the AQP-4 IgG titer will aid effective management by preventing relapse.[47,48] However, Jitprapaikulsan et al.[49] recently contradicted this hypothesis when testing the AQP-4 IgG titer in a large cohort of 336 consecutive samples collected from 82 NMOSD patients grouped according to their clinical status into preattack, attack, and remission. They observed no significant difference in titers among 81 pairs of samples collected during attack and remission, and among 13 pairs of preattack and attack samples. A similar negative correlation was observed in titers of samples collected from treated and untreated NMOSD patients, thereby questioning the clinical utility of the AQP-4 IgG titer in diagnosing the disease status.[49] Thus, monitoring the disease severity by longitudinal sampling might not provide clinicians with any valuable information.[49,50]

FUTURE PROSPECTS

The laboratory diagnosis of autoantibodies along with the clinical presentation play an integral role in the early diagnosis of NMOSD, which will aid appropriate management and improve the prognosis of the disorder. Although various sensitive diagnostic tests are commercially available for detecting NMO IgG, several clinically unmet needs remain. Unfortunately, the usage of these commercial CBA kits is restricted by their exorbitant costs, which makes them unaffordable for low-income populations and restricts their availability since they need to be imported by most laboratories. Moreover, the results of CBAs are interpreted based on the observed fluorescence, which requires both expensive instrumentation and experienced staff, hence making them unsuitable for point-of-care applications. Apart from commercial CBA kits, referral laboratories may have standardized in-house CBAs, which are cost-effective, but again specialized tissue culture setups are required to establish and maintain transfected cell lines. On the other hand, low titers of NMO IgG and double seronegativity (for AQP-4 IgG and MOG IgG) also represents challenges for clinicians attempting to conclude about definitive NMOSD. Screening for specific novel biomarkers other than AQP-4 IgG and MOG IgG, such as glial fibrillary acidic protein and neurofilament light chain, may be of diagnostic value in the near future.[51,52]

ADVANCED METHODOLOGIES

As an initiative to address the above-mentioned issues, researchers have been focusing on ultrasensitive methodologies such as biosensing and peptide-based assays for the early diagnosis of NMOSD. Biosensors are self-reliant analytical biomedical devices that are commonly used for the sensing and quantitation of specific target biomolecules. A biosensor consists of a bioreceptor (nucleic acid or protein), a signal transducer, and an electrical circuit with a display. Several biosensors have been developed for diagnosing MS and other demyelinating diseases, especially for quantitate analytes such as MBP, anti-MBP autoantibody, miR422, miR-223, miR-126, miR-23a, and IL-12, each using either aptamers or peptides as bioreceptors.[53,54]

Short-chain amino acids are designated as peptides, which can be manufactured synthetically on a large scale. In recent years synthetic peptides have been widely used when designing immunodiagnostic tests, since they have several advantages over complex native proteins.[55] Multiple epitopes in the whole protein can result in cross reactivity, but this can be minimized by screening and utilizing specific epitopes. Although the process of designing, scanning, and chemically synthesizing a peptide remains expensive, once standardized, their large-scale production is more cost-effective than synthesizing recombinant protein.

Few studies have attempted to predict the peptides specifically targeted by AQP-4 IgG. Crystallographic studies predicted that AQP-4 was a transmembrane protein with three extra cellular loops (designated as loops A, C, and E), which were considered potential antigenic determinants.[56] Mutagenesis studies of these three extracellular loops revealed the importance of key amino acids in each loop, among which loop A (amino acids 61−64) and the superficial part of loop C (amino acids 146−150) formed the interacting epitope.[57] Iorio et al.[58] screened peptides of the loops and observed that the peptides of loop C were more disease-specific than those of loops A and C, but they also concluded that an assay with a high sensitivity for NMOSD IgG can only be achieved by using native proteins.

Synthetic peptides are short, rigid, and do not exhibit posttranslational modification, and researchers have attempted to develop peptide-based assays for the rapid detection of AQP-4 autoantibodies in the sera of NMOSD patients. In 2016

Son et al.[59] standardized a peptide-based carbon nanotube biosensor by fabricating nanotubes with AQP-4 extracellular loop peptides, and reported that a sensor coated with the E-loop peptide exhibited high sensitivity in detecting AQP-4 IgG at up to 1 ng/L.

In 2019 de Souza Moraes et al.[60] generated a novel peptide-based atomic force microscopy nanoimmunosensor that detected the force of the interaction between the serum sample and the bioreactor with an AQP-4 peptide. Initially they tested four extracellular loop peptides for sensitivity and specificity (peptide positions AQP-4 61−70, 131−140, 141−150, and 201−210), among which AQP-4 61−70 was highly specific.

Further studies are needed to screen more peptides to identify potential conformational epitopes targeted by NMOSD IgG. The low assay sensitivity reported by Iorio et al.[58] when using peptide-based ELISA could be due to the masking of the epitopes. Conjugating short peptides with bovine serum albumin or keyhole limpet hemocyanin or ovalbumin, or thyroglobulin or synthetic carriers such as multiple antigenic peptides and then immobilizing it to the substrate will aid the successful display of the epitopes for NMOSD IgG binding.

Furthermore, utilizing specific peptides rather than proteins can be cost-effective in resource-poor settings and also improve the assay sensitivity, since this does not require the production of recombinant AQP-4 protein or monitoring of the conformational changes of expressed proteins.

CONCLUSION

Biomarkers for delineating different phenotypes of NMOSD are still being investigated, and yet it is undeniable that the detection of autoantibodies remains the gold standard in laboratory diagnoses. Current methods demand skilled personnel, accessory equipment, dedicated laboratory setups, and a prolonged reporting time. Developing a sensitive and specific point-of-care device that can generate results more rapidly is needed for the prompt initiation of therapy, especially in low-resource settings. Developing and implementing such a point-of-care device for the differential diagnosis of NMOSD will aid in successfully providing predictive and customized curative therapies.

Availability of Data and Material

All data generated or analyzed during the study are included in this published article.

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Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

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