Detection of NMO-IgG Antibodies in Thai NMO Patients Using a Recombinant *E. coli* AQP4-M23 ELISA

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Abstract

Neuromyelitis optica (NMO) is a severe inflammatory demyelinating disorder of the CNS. NMO was usually misdiagnosed as multiple sclerosis (MS) due to their overlapping clinical features. After the discovery of NMO-IgG antibodies and its target antigen (aquaporin 4, AQP4), antibody to AQP4 (also known as AQP4-Ab or NMO-IgG) has been used as a highly specific serum markers of NMO.

Objectives: To study a qualitative, indirect ELISA that uses recombinant protein of human AQP4 expressed in *E. coli* as an antigen for detection of anti-AQP4 antibodies in Thai NMO patients.

Methods: Serum samples were obtained from 56 patients who attended the neurology clinic at Siriraj Hospital with suspected CNS demyelinating diseases. AQP4 tagged with a green fluorescent protein (GFP) was expressed in *E. coli* BL21(DE3)pLysS. The NMO-IgG antibodies in the patients' sera were detected by ELISA. Crude *E. coli* lysates expressed AQP4-M23 isoform were used as antigens for the patients' sera in the ELISA.

Results: Detection of NMO-IgG antibodies by ELISA using synthesized recombinant AQP4-M23 antigen provides a low sensitivity (69.70%), low specificity (30.43%) test for the diagnosis of Thai NMOSDs patients. The strength of agreement was poor (kappa coefficient, k = 0.00137). The obtained ELISA data was interpreted in comparison to a CBA-Sendai.

Conclusion: These results suggested that AQP4-M23-GFP fusion protein is inappropriate for detection of NMO-IgG antibodies in Thai NMO patients.

Neuromyelitis optica (NMO) is a severe inflammatory demyelinating disease of the central nervous system (CNS). The episodes of the disease, inflammation and damages, almost exclusively affect in optic nerves (ON) and spinal cord leading to optic neuritis and longitudinally extensive transverse myelitis (LETM).[1-3] Patients who develop optic neuritis may have visual impairment from decreased visual acuity, loss of color vision up to complete loss of vision. Transverse myelitis leads to muscle weakness of arms and legs along with bladder and bowel dysfunction. More than half of the affected NMO patients become blind in one or both eyes or become disability within five years after disease onset.[2] The discovery of NMO-IgG as specific marker for NMO had been introduced to discriminating between NMO, MS and other demyelination disease. Early diagnosis and treatment could aid in controlling symptoms and prevent disability. Several assays were developed to achieve a high specificity and sensitivity for the detection of NMO-IgG antibodies.[4] These assays are cell-based assay (CBA),[5] radioimmunoprecipitation assay (RIPA), fluoroimmunoprecipitation assay (FIPA), and enzyme-linked immunosorbent assay (ELISA).[6] However, these assays are either high operating cost or require special skills to perform or interpret the data. This study demonstrates a qualitative, indirect ELISA that uses recombinant protein of human AQP4 expressed in *E. coli* as an antigen in the ELISA for detection of anti-AQP4 antibodies in the Thai NMO patient's and compares to other assays.

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Materials and methods

Sera samples
Fifty six sera samples were collected from study population in the MS clinic, Division of Neurology, Department of Medicine, Faculty of Medicine Siriraj Hospital. All participants gave a written informed consent and the experimental protocols were approved by the Siriraj Institutional Review Board of the Faculty of Medicine, Siriraj Hospital.

Construction of the pRSET-B-AQP4-M23-GFP plasmid
AQP4-M23 tagged with GFP was amplified by PCR

using Phusion DNA polymerase (Finnzymes OY, Finland) from a linearized pCMV-AC-GFP plasmid containing the human AQP4 variant a gene (RG204693) (Origene Inc., USA) using primer pair, AQP4-BglII (5'-GCGAGATCTATGAGTGACAGA CCCACAGCAA-3') and XL39 (5'-ATTAGGACA AGGCTGGTGGG-3'). PCR products were digested with restriction enzyme BglII and ligated to linearized pRSET-B plasmid using T4 DNA ligase (Finnzymes OY, Finland). Inserted sequence was confirmed by automated DNA sequencing.

Expression of AQP4-M23-GFP recombinant protein

The pRSET-B-AQP4-M23-GFP plasmid was transformed into *E. coli* BL21(DE3)pLysS. Induction of AQP4-M23-GFP protein expression in the *E. coli* was done by 1 mM isopropyl β-D-thiogalactoside (IPTG) when optical density at 600 nm (OD600) of the bacterial culture reaches 0.5. Bacterial culture was grown for 5 hours and the *E. coli* cells were harvested by centrifugation at 12,000 g for 10 minutes at 4 °C using Eppendorf 5804R with rotor F-34-6-38. The amount of *E. coli* cells was adjusted according to OD600 in order to get an equal amount of *E. coli*. The *E. coli* pellets were washed twice in 10 ml washing buffer (1x equilibration/wash buffer; 50 mM NaH_2PO_4, 50 mM Na_2HPO_4 and 300 mM NaCl pH 7.0).

AQP4-M23-GFP crude lysate preparation

The *E. coli* cell pellets were resuspended in lysis buffer (1x equilibration/ wash buffer; 50 mM NaH2PO4, 50 mM Na2HPO4 and 300 mM NaCl pH 7.0, with 2% Triton-X100) by vortex to a final concentration of 40 OD/ml of *E. coli*. The resuspended *E. coli* cells were disrupted by the high-frequency oscillation (Bandelin sonoplus HD 200, Berlin, Germany). The cell debris and non-soluble proteins were removed by centrifugation at 12,000g for 20 minutes at 4 °C. Soluble proteins were stored at -20 °C until use. AQP4-M23-GFP protein expression levels were determined by measuring fluorescent emission levels at wavelength 509 nm of GFP protein (excitation at 395 nm). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to verify the expression of AQP4-M23-GFP soluble proteins.

ELISA for NMO-IgG

All samples were performed under blinded conditions. NMO patient sera were depleted of antibody against *E. coli* protein by treating the sera with an excess amount of acetone-precipitated *E. coli* BL21(DE3)pLysS containing pRSET-B plasmid lysate, at 4 °C overnight.[7]

ELISA was performed on 96-well plate (Nunc–immuno plate, Maxisorp, Nunc, Denmark). Each well was coated with 13 μl of AQP4-M23-GFP *E. coli* lysate (final concentration 0.54 OD/well) and 87 μl of coating buffer (0.285 mM Na_2CO_3 and 0.714 mM

$NaHCO_3$, pH 9.6) and incubated at 4 °C overnight. After washing off unbound antigens with PBS buffer (1.37 mM NaCl, 27 mM KCl, 10 mM Na_2HPO_4, and 2 mM NaH_2PO_4, pH 7.4), the plate was blocked with 0.5% skim milk in PBS for 1 hour at room temperature. Pre-adsorbed sera from the NMO patients were diluted at ratio 1:10 in 1x PBS buffer before adding into each wells and incubated overnight at 4 °C with shaking. After overnight incubation, the plate was washed with PBS buffer containing 0.01% Tween-20 and incubated with goat anti-human IgG-HRP conjugate, diluted to 1:5000 (Jackson ImmunoResearch, PA, USA) for 1 hour at room temperature. After incubation period, the plate was washed and treated with 100 μl BioFX TMB (3, 3′, 5, 5′ tetramethyl-benzidine) One Component HRP Microwell Substrate (Surmodics, MN, USA) for 15 min at room temperature. Finally, spectrophotometric reading was performed using a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT, USA). The optical density (OD) was determined at a wavelength of 620 nm. The assays were performed in triplicate for each serum specimen. A corrected OD of each sample was determined by subtracting the OD values obtained from the blocked wells without antigen (*E. coli* containing pRSET-B plasmid lysate). A patient's serum was considered to be AQP4-IgG positive if the corrected OD was greater than zero.

Statistical Analyses

The percentages of sensitivity, specificity, positive predictive and negative predictive values of the ELISA assay were calculated by using a 2×2 table. The inter-rater agreement kappa test (Cohen's test) was applied to evaluate the agreement between 2 methods, ELISA and previously published data by cell based assay (CBA),[8] for positive and negative classifications (0.001–0.2 indicates a slight concurrence, 0.201–0.4 indicates fair agreement, 0.401–0.6 indicates moderate agreement, 0.601–0.8 indicates substantial concurrence, and 0.801–0.999 indicates excellent agreement).[9] All statistical analyses were done by using the MedCalc® version 12.3.0 (MedCalc Software, Mariakerke, Belgium).

Results

The expression of recombinant AQP4-M23-GFP in *E. coli* BL21(DE3)pLysS

SDS-PAGE was performed to verify the expression level of AQP4-M23-GFP protein in the *E. coli*. AQP4-M23-GFP protein was expressed in the *E. coli* at a molecular weight of about 114 kDa, as expected for a dimer of this fusion protein (Figure 1).

Expression level of green fluorescent protein (GFP)-tagged M23 was measured as a marker for gene expression using spectrofluorometer. Fluorescence signal of *E. coli* lysates with AQP M23-GFP had higher intensity than the control (Figure 2).
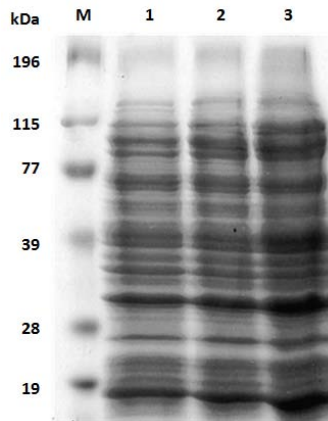
Figure 1 SDS-PAGE comparing the expression levels of the AQP4-M23-GFP recombinant protein. Lane M, protein standard markers; lane 1, soluble fraction of *E. coli* lysate harboring pRSET-B plasmid (control); lane 2 and 3, soluble fraction of *E. coli* lysate expressing AQP4-M23-GFP protein. The expected molecular weight of Aqp4-M23-GFP is 57 kDa, or 114 kDa for its dimeric form.
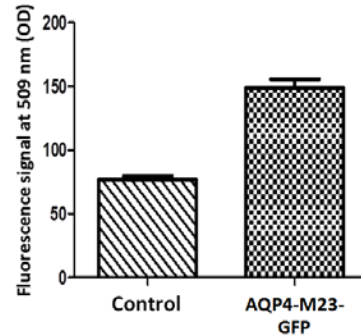
ELISA

ELISA was performed to detect the presence of NMO-IgG immunoreactivity against AQP4-M23 isoforms in serum of the patients. We evaluated a total of 56 random NMO serum specimens. Sera with positive results by AQP4-M23 ELISA were found in 39 patients and sera with negative results were found in 17 patients. Among 39 NMO-IgG positive patients, 23 were positive in CBA. Among 17 NMO-IgG negative patients only 7 were negative in CBA (Table 1). The corrected absorbances of all patients were shown in figure 3. These results correspond to a sensitivity and a specificity of 69.70% (95% CI 51.29 -84.41) and 30.43% (95% CI 13.21-52.92), respectively. The AQP4-M23 ELISA showed poor agreement with CBA-Sendai. The actual agreement was 54% (30/56), Cohen's kappa (K) = 0.00137.

Discussion

Neuromyelitis optica symptoms can be alleviated if the disease is treated promptly. Symptoms and signs in NMO and other demyelinating diseases such as MS may show overlapping conditions. After discovery of NMO-IgG, several methods have been developed to detect NMO-IgG in patients' serum. Although most of them showed high specificity, their technique required skillful technicians or costly materials.

We have successfully expressed AQP4-M23-GFP fusion protein on *E. coli,* as a soluble protein which can be detected on SDS-PAGE and fluorescent signal measurement. The distinct band of protein around 114 kDa in *E. coli* harboring pRSET-B-AQP4-M23-GFP plasmid suggested that AQP4- M23-GFP in the *E. coli* might form dimeric complexes.

The AQP4-M23 ELISA results showed low sensitivity and specificity (69.70% and 30.43%,

Figure 2 Fluorescence signals of the control *E. coli* lysate *vs* AQP4-M23-GFP *E. coli* lysates.
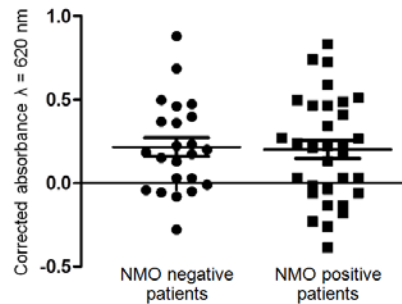
Figure 3 Serum NMO-IgG detection by AQP4-M23 ELISA. Data plotted from the corrected absorbance of all patients (N = 56). NMO negative patients and NMO positive patients were determined by CBA-Sendai method. Corrected absorbances were calculated by $OD_{AQP4-M23}$ $-OD_{pRSET-B}$.

Table 1 Contingency table analysis of agreement between the CBA and the AQP4-M23 ELISA in detecting serum NMO-IgG antibodies against AQP4-M23 isoform in NMO patients (N = 56)

	NMO patients AQP4-M23	
	CBA-positive	CBA-negative
AQP4-M23-ELISA-positive	23	16
AQP4-M23-ELISA-negative	10	7
Total	33	23

respectively) when compared to CBA-Sendai. The agreement between AQP4-M23 ELISA and CBA-Sendai assays is poor (54%) with a Cohen Kappa of 0.0 (95% CI -0.254 to 0.256).

Ten patients were found to be negative by AQP4-M23 ELISA but positive by CBA-Sendai. It might be that AQP4-M23 expressed in the *E. coli* did not show epitopes recognized by NMO-IgG from these patients' serum, since the binding affinity of NMO-IgG to the AQP4-M23 isoform is greater than that of the AQP4-M1 isoform.[10] Epitopes that can be recognized by NMO-IgG include AQP4-M23 assembled into orthogonal array of particles (OAPs), extracellular loops and N terminus.[11] The critical epitopes to be recognized by NMO-IgG antibodies are epitopes during formation of OAPs.[12] Due to the large C-terminus tag by GFP and expression in *E. coli* system, it is possible that AQP4-M23-GFP could not form OAPs. In addition, N-terminal epitopes that could be recognized in AQP-M1-GFP were missing

in AQP-M23-GFP. This might also lead to a decrease in sensitivity of this test.

Moreover, another report demonstrated that quaternary structure epitope of OAP formation was recognized on the surface of transfected cells expressing only AQP4-M23 isoform.[13] These results suggested that OAP formation by AQP4-M23 isoform generally presented in eukaryotic cells may not be recognized by NMO-IgG antibodies when expressed in prokaryotic cells.

Sixteen patients were found to be positive by AQP4-M23 ELISA but negative by CBA-Sendai. This study attempted to eliminate non-specific antibodies from NMO patients' sera by pre-adsorption of the patients' sera with acetone-precipitated *E. coli* lysate. Since this procedure was performed on the same serum samples, but with AQP4-M1-GFP as the antigen in ELISA, and could yield 96.7% sensitivity and 92.3% specificity (unpublished), therefore residual non-specificity should be virtually negligible. It is possible that high false positive rates occurred from poor antigenicity of AQP4-M23-GFP protein that can cross react with other IgG in patients' serum.

Another possibility of these poor results might be that AQP4-M23 protein expression levels and amount of protein used for coating the microplate were too low to detect the antibodies in the serum. Increasing the amount of protein for coating might increase non-specific antigen in the plate, thus protein purification might improve sensitivity and specificity of this test.

Acknowledgements

Conflict of Interest
None.

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