Journal of Neuroinfectious Diseases
ISSN: 2314-7326

Research Article Open Access

Serological Diagnosis Usefulness in Myasthenia Gravis: Study of a Group of Algerian Patients

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Abstract

Serological testing of presumptive myasthenia gravis (MG) patients for anti-acetylcholine receptor antibodies (anti-AChR Abs) is performed by radio-immunoprecipitation assay (anti-AChR RIPA) and to a lesser extent, by enzyme-linked immunosorbent assay (anti-AChR ELISA). Here we report results of autoantibodies detection by different assays in a group of 23 Algerian MG patients. All patients confirmed with generalized MG (16) were positive for anti-AChR by ELISA compared to 87.5% (14) by RIPA. 1 of the 7 (14.3%) ocular MG patients was positive by ELISA whereas 28.6% (2) were deemed positive by RIPA. These results suggest that some of the anti-AChR ELISA results might potentially be either false-negatives or false-positives. None of the 23 patients were seropositive for muscle-specific kinase antibodies (anti-MuSK abs) by RIPA or by a cell-based assay. Overall, the results suggest that in countries where RIPA is not available, anti-AChR ELISA may be useful in conjunction with clinical and electrophysiological findings for MG diagnosis.

Keywords: Myasthenia gravis; Radio immune precipitation assay; Enzyme-linked immunnosorbent assay; Cell-based assay; Acetylcholine receptor: Muscle specific kinase.

Introduction

Myasthenia gravis (MG) is an autoimmune disease caused by serum auto-antibodies (autoAbs) against components of the muscle membrane at the neuromuscular junction (NMJ). The disease is characterized by a failure of neuromuscular transmission and fluctuating, fatiguing muscle weakness [1]. Most MG patients present with ocular symptoms. About 75% patients will develop generalized weakness, typically within the first 2 to 3 years following presentation. When symptoms are solely ocular, MG is said to be ocular (OMG). Most cases of generalized MG (80% to 85%) and half the cases of ocular MG (50%) involve autoAbs directed against acetylcholine receptor (AChR), but a minority of patients with generalized MG (15% to 20%) instead reveal autoAbs directed against other components of the NMJ [2,3], most commonly Muscle Specific Kinase (MuSK) [4,5].

The initial diagnosis of myasthenia gravis is essentially based on clinical manifestation [6]. Typically this is followed by repetitive nerve stimulation electromyography (RNS-EMG). Then, serological testing is performed to consolidate the diagnosis. Radio-immunoprecipitation assays (RIPA) are the most commonly used assays for detecting AChR and MuSK autoAbs. Enzyme-linked immunosorbent assay (ELISA) is less frequently used but is technically simpler and less costly, and these are important considerations in many parts of the world. Cell-based assays (CBA) are labor-intensive but are occasionally used as a last resort for MG patients when no autoantibody is detected by RIPA [7]. A small fraction of clinically diagnosed patients remain sero-negative for both anti-AChR and anti-MuSK and might represent sensitivity limits of the assay or cases caused by antibodies against other synaptic antigens such as agrin and LRP4 [8,9]. Here we compare the anti-AChR and anti-MuSK status of 23 Algerian MG patients' sera as assessed by ELISA and RIPA assays [10].

Materials and Methods

Clinical material

Twenty three Algerian patients with a high suspicion for MG were included in the study. Patients were treated at Ait Idir Neurosurgery Hospital (Algiers, Algeria), Sidi Belloua Hospital (Tizi Ouzou, Algeria) or Mustapha Pacha Hospital (Algiers, Algeria). (Table 1) shows patients' demographical and clinical features. All patients were diagnosed with MG by their respective neurologists on the basis of both clinical and electromyography (EMG) criteria. All but three (3/23) revealed decrement in the compound muscle action potential during repetitive stimulation of the nerve. Five sera from Algerian patients with congenital myasthenic syndrome (CMS) were used as internal controls. Two sera from Australian healthy individuals were used as negative controls for anti-MuSK assays while two other sera from Australian patients with anti-MuSK MG were used as positive controls. All serum samples were stored at +4°C.

Ethics statement

Informed patient consent was obtained from subjects in accordance of the Declaration of Helsinki. The study was approved by the University of Sciences and Technology Houari Boumediene (Algiers, Algeria) local ethics committee of the Algerian national agency of research development in health (ATRSS).

Enzyme-Linked Immune-Sorbent Assay (ELISA) for anti-AChR

AChR antibody titres were assessed by an enzyme-linked immune-sorbent assay, following the manufacturers' instructions (ELISA; ElisaRSR™ AChRAb kit, RSR, Cardiff, United Kingdom [11]. This assay depends on the ability of human anti-AChR Abs to compete for binding to the AChR with the two anti-AChR monoclonal antibodies provided in the kit, thereby inhibiting the ELISA signal. Only concentrations greater than 0.45 nmol/L were considered positive, as suggested by the manufacturer (RSR, Cardiff, United Kingdom).

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Received: 02-Jun-2022, Manuscirpt No. JNID-22-69292; **Editor assigned:** 04-Jun-2022, PreQC No. JNID-22-69292 (PQ); **Reviewed:** 20-Jun-2022, QC No. JNID-22-69292; **Revised:** 24-Jun-2022, Manuscirpt No. JNID-22-69292 (R); **Published:** 30-Jun-2022, DOI: 10.4172/2314-7326.1000396

Citation: Raache R (2022) Serological Diagnosis Usefulness in Myasthenia Gravis: Study of a Group of Algerian Patients. J Neuroinfect Dis 13: 396.

Table 1: Demographic and clinical characteristics of patients with myasthenia gravis (MG).

Patient	Sex	Age of	Onset	Fatigue distribution form	EMG	Treatment	Thymus
		onset (years)	form		decrement (%)		
1	M	44	LOMG	GMG	> 10	Pyr + Pred	Thymoma
2	M	18	EOMG	GMG	> 10	Pyr + Pred	N/A
3	M	49	LOMG	GMG	> 10	Pyr + Pred	N/A
4	M	54	LOMG	GMG	> 10	Pred	N/A
5	M	30	EOMG	GMG	> 10	Pyr + Pred	Thymoma
6	M	47	LO MG	GMG	> 10	Pyr	Thymoma
7	M	32	EOMG	GMG	> 10	Pyr + Pred	Thymoma
8	F	43	EOMG	GMG	> 10	Pyr	N/A
9	F	28	EOMG	GMG	> 10	Pyr	N/A
10	F	24	EOMG	GMG	> 10	Pyr	N/A
11	M	42	EOMG	GMG	> 10	Pyr	N/A
12	M	55	LOMG	GMG	> 10	Pyr	Normal
13	F	50	LOMG	GMG	> 10	Pyr + Pred	N/A
14	M	0.5	EOMG	GMG	> 10	Pyr	Normal
15	F	42	EOMG	GMG	> 10	Pyr	N/A
16	M	68	VLOMG	OMG	> 10	None	N/A
17	M	69	VLOMG	OMG	> 10	Pyr	N/A
18	F	14	EOMG	OMG	> 10	Pyr	N/A
19	F	53	LOMG	OMG	> 10	Pyr + Pred	N/A
20	M	23	EOMG	OMG	> 10	Pyr	N/A
21	F	84	VLOMG	GMG	< 10	None	Normal
22	F	25	EOMG	OMG	< 10	N/A	N/A
23	M	72	VLOMG	OMG	< 10	None	Normal

Abbreviations: EOMG = early onset myasthenia gravis; F = female; GMG = generalized myasthenia gravis; LOMG = late onset myasthenia gravis; M = male; N/A = not available/not applicable; OMG = ocular myasthenia gravis; Pred = Prednisone; Pyr = Pyridostigmine; VLOMG = very late onset myasthenia gravis.

Radio-immunopresipitation assay (RIPA) assay for anti-AChR and anti-MuSK

Commercially supplied ^{125}I-AChR and ^{125}I-MuSK, and RIPA kit including supplied standards were used, following the supplier's protocol (RSR Ltd., Cardiff, UK). Samples were considered negative for anti-AChR when values ranged from 0 to 0.25 nM, equivocal between 0.25 and 0.4 nM and positive above 0.4 nM. For anti-MuSK 0 to 0.05 nM was considered negative, 0.05 to 0.09 nM equivocal and >0.09 nM positive. In each case the criterion for considering a test sample positive was that it exceeded the mean + 3 SD of the non-myasthenic control values [12]. The kit positive controls for anti-AChR and anti-MuSK had values of 1.407 nM and 0.29 nM, respectively [13].

Cell-Based Assay (CBA) for anti-MuSK

HEK293 cells were grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1x antibiotic-antimycotic and 4 mM glutamine (Invitrogen) at 37°C, 5% CO_2. 1.7 x 10^6 HEK293 cells (50-80%) confluence were transfected with 5 µg of expression plasmid encoding MuSK-GFP [14]. complexed with Lipofectamine LTX® Reagent (Invitrogen). After 24hr of incubation, cells were replated onto coverslips in a 24-well tray (1 x 10^5 cells/well) for 24hr. Wells were incubated for 1 hr at room temperature with 300 µl of patient serum (1:20 dilution in 1% BSA/DMEM-20 mM HEPES), washed (x3) and fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS), rinsed with PBS, incubated 45min with anti-human IgG-Texas Red (1:750; Invitrogen), rinsed (x 4) with PBS, permeabilized (5min in 0.02% Triton X100- PBS) and washed (x 4). Nuclei were counterstained with 4',6-diamino-2-penylindole, dihydrochloride (DAPI; 2µg/ml in PBS). Coverslips were mounted on microscope slides using a fade-resistant mounting medium. A Zeiss Axio Imager fluorescence microscope and AxioCamHRm digital camera was used to collect photomicrographs of 3 to 12 microscopic fields containing cells with peripheral membrane GFP fluorescence for each sample. The CBA was performed twice (technical replicates). To avoid subjectivity, four raters were asked to blindly score all the randomized digital photomicrographs. Raters were instructed to score the presence of anti-human IgG immunofluorescence co-localized on the surface of MuSK-EGFP-positive cells in each image as either definitely positive (score of 1), possibly positive (scores of 0.25, 0.5 or 0.75) or definitely negative (score of 0) which were converted into percentages. The criterion for considering a test sample CBA positive was that it exceeded the mean + 3 SD of the non-null values obtained for the non-myasthenic controls.

Statistical analysis

Statistical analysis and histograms were performed using the software GraphPad Prism 6. Pearson's R was calculated to assess the linear correlation between the results from the RIPA versus ELISA assays.

Results

Anti-AChR ELISA

Table 2 shows the results of each assay for each patient. Table 3 shows serological results of each assay grouped by clinical classification. By ELISA, if the manufacturer's recommended threshold of 0.45 nM is applied, 17/23 (73.9%) of the samples were positive for anti-AChR. However, 16/16 (100%) of GMG patients were anti-AChR positive while only 1/7 (14.3%) of OMG patients were seropositive by ELISA.

Table 2: Antibodies titers by ELISA and RIPA and scores by CBA.

Patient	AChR ELISA (nM)	AChR RIPA (nM)	MuSK RIPA (nM)	MuSK CBA (M ± SD%) 1st run	2nd run
1	15.68	18.4	0	0	04.1 ± 08.3
2	>20	13.18	0	0	0
3	8.9	10.86	0.01	0	08.3 ± 06.7
4	13.69	9.35	0	N/A	N/A
5	8.2	4.86	0	N/A	N/A
6	7.82	8.1	0.01	N/A	N/A
7	18.35	19.31	0	N/A	N/A
8	16.42	3.79	0	N/A	N/A
9	>20	2.16	0	N/A	N/A
10	13.34	3.88	0	N/A	N/A
11	11.94	3.96	0	N/A	N/A
12	5.2	1.24	0	06.2 ± 07.9	0
13	14.51	2.97	0.01	N/A	N/A
14	5.13	0.01	0	20.8 ± 24.0	04.1 ± 04.7
15	4.9	0.12	0.01	02.0 ± 04.1	0
16	0.37	0.98	0	12.4 ± 10.7	06.2 ± 04.1
17	0.34	0.76	0	06.2 ± 04.1	0
18	0.44	0.15	0	08.3 ± 11.7	16.6 ± 23.5
19	0.38	0.21	0	0	0
20	0.41	0.12	0.01	02.0 ± 04.1	0
21	15.57	16.42	0	N/A	N/A
22	0.25	0.05	0	24.9 ± 16.6	0
23	0.65	0.26	0.01	N/A	N/A
CMS 1	0.28	0	0.01	14.5 ± 14.2	0
CMS 2	0.42	0.14	0.01	12.5 ± 14.4	0
CMS 3	0.25	0.15	0	0	02.0 ± 04.1
CMS 4	0.25	0.14	0	0	0
CMS 5	0.3	0.12	0	0	04.1 ± 04.7
MuSKPC1	N/A	0.15	0.84	95.8± 8.3	100
MuSKPC2	N/A	0.13	0.83	100	100
MuSKNC1	N/A	0.39	0.01	0	02.0 ± 04.1
MuSKNC2	N/A	0.19	0.01	04.1 ± 04.7	0

Abbreviations: CMS = congenital myasthenic syndrome sample; M ± SD% = mean ± standard deviation in percentages; MuSKNC = anti-MuSK negative control; MuSKPC = anti-MuSK positive control; N/A = not available; nM = nanomole per liter.

15/20 (75%) of patients with an EMG decrement greater than 10% were anti-AChR positive by ELISA. On the age of onset basis, 6/7 (85.7%) of patients with LOMG, 9/12 (75%) of those with EOMG and 2/4 (50%) of those with VLOMG were anti-AChR positive by ELISA. None (0/5) of the CMS controls were positive by anti-AChR ELISA, neither were the anti-MuSK positive (0/2) and negative (0/2) controls.

Anti-AChR RIPA

By anti-AChR RIPA, and applying the recommended 0.40 nM threshold, 16/23 (69.6%) of all MG patients were positive (Table 3). Of all GMG patients, 14/16 (87.5%) were anti-AChR seropositive while of the OMG patients, 2/7 (28.6%) were anti-AChR positive by RIPA. 15/20 (75%) of patients with an EMG decrement greater than 10% were anti-AChR positive by RIPA. For patients with LOMG, 6/7 (85.7%) were anti-AChR positive by RIPA while for patients with EOMG and VLOMG, 7/12 (58.3%) and 3/4 (75%) were respectively seropositive for anti-AChR by RIPA. Similarly to anti-AChR ELISA, none of the CMS and the anti-MuSK controls was positive by anti-AChR RIPA.

Anti-AChR ELISA/RIPA correlation

Figure 1 compares anti-AChR concentrations for all 23 samples

Table 3: Serological results by clinical forms.

Clinical classification		Anti-AChR		Anti-MuSK	
and controls	N	ELISA positives	RIPA positives	RIPA positives	CBA positives
		n (%)	n (%)	n (%)	n (%)
Total MG	23	17 (73.9)	16 (69.6)	0	0
GMG	16	16 (100)	14 (87.5)	0	0
OMG	7	01 (14.3)	02 (28.6)	0	0
RNS-EMGd >10%	20	15 (75)	15 (75)	0	0
EOMG (<45 years)	12	09 (75)	07 (58.3)	0	0
LOMG (45 – 65 years)	7	06 (85.7)	06 (85.7)	0	0
VLOMG (>65 years)	4	02 (50)	03 (75)	0	0
CMS controls	5	0	0	0	0
MuSK negative controls	2	N/A	0	0	0
MuSK positive controls	2	N/A	0	02 (100)	02 (100)

Abbreviations: AChR = acetylcholine receptor; CBA = cell-based assay; CMS = congenital myasthenic syndrome; ELISA = enzyme-linked immunosorbent assay; EOMG = early-onset myasthenia gravis; GMG = generalized myasthenia gravis; LOMG = late-onset myasthenia gravis; MG = myasthenia gravis; MuSK = muscle-specific kinase; N/A = not available; OMG = ocular myasthenia gravis; RIPA = radio-immunoprecipitation assay; RNS-EMGd = repetitive nerve stimulation electromyography decrement; VLOMG = very late-onset myasthenia gravis.

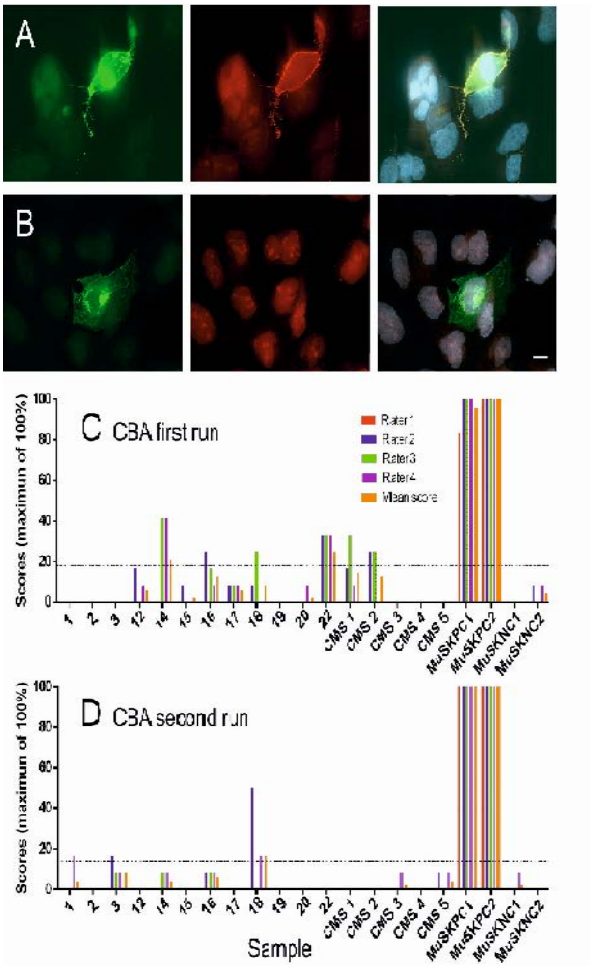

Figure 1: Anti-AChR ELISA/RIPA correlation.

as estimated by ELISA and RIPA assays. There was a clear correlation between anti-AChR titres estimated by RIPA and ELISA (Fig 1; slope = 0.61, Pearson R^2 = 0.47; p = 0.0003). The average value estimated by ELISA was 8.81 ± 1.49 nM, compared to 5.27 ± 1.31 nM by RIPA. However, despite the correlation between RIPA and ELISA results, the latter can differ substantially for some patient serum samples.

Anti-MuSK RIPA and CBA

None of the 23 Algerian MG patients and of the 5 Algerian CMS patients were positive for anti-MuSK by RIPA (Table 3). Both Australian anti-MuSK MG patients' sera were positive [2/2 (100%)] and both anti-MuSK negative controls sera were negative (0/2). We rescreened a subset of 12 of the Algerian MG cohort using a CBA (see Methods). Shows an example of cell surface immunofluorescence with anti-MuSK RIPA-positive serum from an Australian MG patient: human IgG immunofluorescence decorating the periphery of the MuSK-GFP-expressing cell). Negative control serum showed no such IgG immunolabeling. Four investigators blindly scored images for cell surface IgG labeling that co-localized with MuSK-GFP, after first being trained with positive- and negative- control micrographs. The CBA was run twice with the same samples, so as to provide technical replication. The first run cutoff was estimated at 18.2% and the second runs' was estimated at 14.3%. All four raters correctly scored images from anti-MuSK positive-controls sera (first run= 95.8 ± 08.3% and 100 ± 0% mean ± SD; replicate run= 100 ± 0% for both controls).

Negative-controls images were also correctly identified in most cases (first run= 0% and 04.1 ± 04.7%; replicate run= 02.0 ± 04.1%). Thus all 4 blinded scorers were able to clearly distinguish positive and negative control samples. When assessing the CBA images for the 12 Algerian samples, 3 were scored were scored slightly above the cutoff in the one run and scored below in the other. The 4 blinded scorers revealed greater inter-rater and inter-run variability. In summary, neither RIPA nor CBA revealed anti-MuSK positive MG patients among the Algerian cohort studied.

Discussion

Some differences were observed. On one hand, less GMG patients sera were anti-AChR positive by RIPA compared to ELISA (100% vs. 87.5% respectively). The 2 anti-AChR seronegative patients' sera by RIPA had relatively high titers by ELISA (5.13 and 4.9 nM by ELISA vs. 0.01 and 0.12 nM by RIPA) which might suggest an unspecific measurement by the latter assay. These results are in line with those found by Oger et al. (6) where ELISA yielded 5% false positives. On the other hand, 2 OMG patients sera' were anti-AChR positive by RIPA while negative by ELISA. In these cases, RIPA values In this study, serum samples from 23 Algerian myasthenia gravis patients were tested for anti-AChR by ELISA and RIPA. Both assays yielded comparable results for most patient samples. Nevertheless, when grouped by clinical classification where relatively low (0.98 and 0.76 nM) which might suggest that ELISA may be less likely to detect low titre/low affinity anti-AChR when compared to testing by RIPA [15]. Taken separately, our AChR-RIPA results were comparable to those reported in various reviews [1,16]. (GMG: 87% pos*i*tive; OMG: 28.6%) and confirm that Algerian MG patients' seropositivity ratio is not obviously different from the reported ratio from other populations. Overall, both assays found comparable anti-AChR seropositivity ratios when patients were classified by EMG decrement (>10% or <10%) and by age of onset form (EOMG, LOMG and VLOMG). Nevertheless, an opposite trend is observed in regards to EOMG and VLOMG seropositivity rates: RIPA showed greater anti-AChR detection rate among patients with VLOMG compared to ELISA while the latter showed a higher seropositivity rate among patients with EOMG.

The percentage of MG patients reported to express autoAbs targeting MuSK rather than AChR varies widely (range: 10% to 70% in anti-AChR seronegative patients) [5,17]. In the present study, the anti-MuSK RIPA assay revealed no anti-MuSK-positive serum samples among our 23 Algerian MG patient cohort. Similarly none of the 12 patients serum samples re-tested were positive by CBA despite the strong labeling observed with serum from two Australian anti-MuSK seropositive control patients.

Our results should be considered with caution due to a number of limitations. First, given the small size of the Algerian patient cohort tested, no definitive conclusions could be drawn and the lack of any anti-MuSK patients is not surprising. Second, sets of clinically positive and negative controls should be run in order to separately assess sensitivity and specificity of each assay then an inter-assay sensitivity and specificity analysis should be performed using a receiver operating characteristic curve. Finally, it is worth noting that 2 patients sera (2 and 9) had above the linearity limit titers by ELISA (>20 nM) thus ELISA/RIPA correlation might be slightly biased and a serial dilution for these sera should yield a better quantification, therefore, a better correlation.

Conclusion

The present results show that Algerian MG patients have a typical serological MG profile. We also show that in a cost-constrained health care system, the anti-AChR ELISA kit used in this study can be applied for diagnostic purposes with a reasonable level of accuracy. While RIPA is considered the referential assay for anti-AChR(6), many diagnostic laboratories around the world lack facilities for handling and assaying radioisotopes.

We suggest that, in cost-constrained health systems, ELISA can be used as the primary screening test. Retesting or sending away samples for secondary analysis by RIPA and/or CBA might be limited to cases of diagnostic doubt when positive by ELISA, or for cases of acquired MG that are well characterized by clinical and electrophysiological criteria yet seronegative by ELISA. The availability of commercial anti-AChR ELISA kits and the relative simplicity of its realization make the anti-AChR ELISA assay useful, when used in combination with clinical observations and EMG, for diagnosis of MG.

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