ORIGINAL ARTICLE

ANTI -NMDA RECEPTOR ENCEPHALITIS: A POSSIBLE APPROACH TO DEVELOP A COST-EFFECTIVE TEST FOR ANTI NMDA RECEPTOR ANTIBODY DETECTION

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Background: Autoimmune encephalitis (AIE) has been ranked as the third most common cause of encephalitis after viral encephalitis and acute disseminated encephalomyelitis. The estimated incidence is 5–8 cases per 100,000 population. The objective of this study was to develop a cost-effective test for detection of anti NMDA R antibodies by using in house prepared rodent brain tissue sections which could facilitate timely diagnosis and management of anti NMDA R Encephalitis, which if left undiagnosed may prove fatal. **Methods:** A total of 500 samples sent for autoimmune encephalitis related antibody testing were included in this cross-sectional study from April 2019 to March 2021 at department of Immunology, Shifa International Hospital (SIH), Islamabad. Rodent brain was dissected to prepare tissue sections on which samples were tested by Indirect Immunofluorescence. Simultaneously samples were tested on cell-based assay (CBA) which is gold standard for testing anti NMDA R antibodies. Sensitivity, specificity, positive and negative predictive values were calculated. **Results:** Median age of patients who tested positive for anti NMDA encephalitis was 19 years (range: 1 to 57). Out these 76% were female and 24% males. 5% patients tested positive for anti NMDA antibodies out of those suspected to be suffering from autoimmune encephalitis. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of rodent brain IF for anti-NMDR antibodies taking CBA as gold standard was 92.6%, 98.5%, 78.1% and 99.6% respectively. The accuracy of the procedure was 98.2%. **Conclusion:** Indirect immunofluorescence (IF) on rodent brain tissue sections can be useful as a cost-effective alternate for resource constrained laboratories for timely detection of anti NMDA R antibodies facilitating timely diagnosis and management of anti-NMDA receptor encephalitis patients.

Keywords: Rodent brain; Indirect Immunofluorescence; Cell based assays; Cost effective

Citation: Sheikh S, Ahmed TA, Ahmad A. Anti -NMDA receptor encephalitis: a possible approach to develop a cost-effective test for anti NMDA receptor antibody detection. J Ayub Med Coll Abbottabad 2024;36(2):251–5.

DOI: 10.55519/JAMC-02-11655

INTRODUCTION

Autoimmune encephalitis (AIE) has been ranked as the third most common cause of encephalitis after viral encephalitis and acute disseminated encephalomyelitis. The estimated incidence is 5–8 cases per 100,000 population.[1] It is associated with autoantibodies directed against neuronal cell surface or synaptic proteins most commonly NMDA receptors and others being LGI, CASPR2, AMPA and $GABA_B$ receptors. Among these anti NMDA R encephalitis is the most common subtype and most responsive to treatment. NMDA receptor plays a significant role in synaptic transmission, neuroplasticity, memory, learning, and human behavior.[2] Anti NMDA R antibodies lead to receptor crosslinking and internalization, hence causing the clinical manifestations of memory deficits, anhedonia, depression-like behaviour, and a low seizure threshold.[3] It presents with psychiatric symptoms or cognitive dysfunction, seizure, speech dysfunction, movement disorder, decreased level of consciousness, and autonomic dysfunction or hypoventilation.[4] Prevalence of AIE is higher among women (65%), particularly young females and around 20% of anti-NMDAR encephalitis patients have an underlying neoplasm, i.e., ovarian teratoma.[5] Early in the course of the disease a range of psychiatric symptoms predominate therefore a lot of patients are either misdiagnosed as having psychiatric disorders or are diagnosed very late. A study by Wang *et al* showed that psychiatric symptoms were the initial symptoms in 57%, while the presenting symptom was neurological in 39% patients.[6]

Although NMDAR antibody can be detected in the serum and CSF, tests are most sensitive with CSF and provide a confirmatory diagnosis.[7] Antibody mediated neuronal destruction can be halted by early diagnosis and prompt treatment. First-line immunosuppressive therapy comprises of intravenous high-dose steroids (methylprednisolone), intravenous immunoglobulin (IVIG), and/or plasmapheresis. Upon unsatisfactory response to first line therapy,

second line therapy may be initiated which includes therapy with rituximab and cyclophosphamide.[8]

Currently there are four methods for detection of anti NMDAR antibodies which include indirect immunofluorescence (IIF) on rodent brain tissue sections (either in house or commercially prepared), immunohistochemistry (IHC) on rodent brain tissue sections, culture of dissociated hippocampal neurons from rats and IIF on cell-based assays (CBA) that use commercially prepared cells transfected with genes of NMDA Receptors (Euroimmun, Lubeck-Germany).[9] We aimed to prepare in house rodent brain tissue sections which may be used as an antigen substrate for anti NMDA receptor antibody detection by IIF. In this study we aimed to find out the sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy of IIF on in house rodent brain tissue sections for detection of anti NMDA receptor antibodies keeping indirect immunofluorescence on CBA as the gold standard. CBA are expensive and are available in very few centres in Pakistan due to which a lot of anti NMDA R Encephalitis patients are either misdiagnosed or the diagnosis is delayed resulting in fatal outcomes.

In this way timely detection of anti NMDA R encephalitis may help in prompt initiation of immunotherapy which may help prevent the deadly complications of untreated NMDA R encephalitis which include permanent cognitive deficit and death.[10]

MATERIAL AND METHODS

After taking approval from Institutional review board Shifa International Hospital 500 patients were enrolled in this cross-sectional study by non-probability consecutive sampling technique at department of Immunology, Shifa International Hospital, Islamabad (SIH) from April 2019 to March 2021. All samples sent for anti NMDA R antibody testing were included in the study (serum and CSF) and samples from patients who did not provide consent were excluded from the study. Sample size was calculated by using WHO sample size calculator. Informed consent was taken from all the patients and all data was kept strictly anonymous. Study proforma was used to record all relevant information.

For rodent brain IIF, adult female Wistar rats were anesthetized and decapitated. Brain was removed and washed with 0.1 M Phosphate buffer saline. Brains were bisected sagitally at midline and placed in cold 4% formaldehyde in PBS for 1 hour (Rats were procured from NIH-Islamabad and dissected at the Armed Forces Institute of Pathology, Rawalpindi-Pakistan). Rest of the procedure was carried out at the study site. Brains were transferred to 40% sucrose in 0.1M PBS for 48 hours. Cerebellum and hippocampus were identified and separated from rest of the brain. (Figure-1) Block was made using OCT (Compound Embedding for Cryostat, Biotech, Milano-Italy). Frozen sections were cut at 7 microns mounted on glass slides, air dried and stored at -20 until used. Slides with sections of rat brain were incubated with the patient's serum (dilution of 1:10) and/or CSF at room temperature for 30 minutes. Slides were washed in phosphate buffered saline (PBS) for 5 minutes and fluorescein isothiocyanate (FITC) conjugated anti human immunoglobulin (IgG) was applied to tissue sections and incubated in dark for 30 minutes. Slides were washed again in PBS for 5 minutes and mounted with cover slip using PBS-Glycerol as the mounting medium. For CBA testing, Euroimmun IIFT kit for NMDAR (FA112d-1005-51 or FA112d-1010-51, Euroimmun Lubeck-Germany) was utilized according to the manufacturer's instructions.

Anti NMDA R antibodies bind to their respective receptor/antigen resulting in a specific staining pattern (on the rat cerebellum and rat hippocampus) which is observed under IIF microscope. Results were recorded as positive or negative. This enabled the detection of anti NMDA R antibodies associated with autoimmune encephalitis based on specific staining patterns produced, using indirect immunofluorescence technique. (Figure 2) Positive results showed characteristic fluoresence pattern in granular layer of rat cerebellum (Figure 2A) and molecular layer of rat hippocampus. (Figure 2B)

Data analyses was performed using SPSS version 20. Quantitative variables like age were measured as mean ±SD. Qualitative variables like Gender, True positive, True negative, False negative and False positive were measured as frequency and percentages.

RESULTS

A total of 500 patients were enrolled. All patients were tested for anti-NMDAR antibody with CSF and/ or serum. Overall, 323 (64.60%) patients were females, and 177 (35.40%) were males. Mean age of patients included in this study was 40.70±14.44 years. Minimum age was 10 years and maximum age was 70 years. Twenty-five patients tested positive for anti NMDA receptor antibodies out of all those who were tested for autoimmune encephalitis related antibodies which makes positivity rate of 5%. Out of these 19 were females (76%) and 6 were males (24%). Median age of patients who tested positive for anti NMDA encephalitis was 19 years (range: 1 to 57).

The sensitivity of rodent brain IIF in diagnosing anti NMDR encephalitis taking CBA testing as gold standard was 92.6%, specificity was 98.5%, PPV 78.1% and NPV 99.6%. The overall accuracy of the procedure was 98.2%. The

diagnostic accuracy of rodent brain immunofluorescence (IF) is depicted in Table 1.

Cost per test was calculated for commercial cell-based assays and in house rodent brain Immunofluorescence. Cost per test for CBAs was Rs 6513 (28.88 USD) and for in house rodent brain Immunofluorescence was Rs 366.8 (USD 1.63) (Table 2)

The calculation done is as follows:

(I) Sensitivity = $\frac{True\ Positive}{True\ Positives+False\ Negatives}$ x 100
25/25+2 =92.5%

(II) Specificity = $\frac{True\ Negative}{True\ Negatives+False\ Positives}$ x 100 466/466+7=97.8%
466/466+7=98.5%

(III) Positive Predictive Value = $\frac{True\ Positive}{True\ Positives+False\ Positives}$ x 100
25/25+7 = 78%

(IV) Negative Predictive Value = $\frac{True\ Negative}{False\ Negatives+True\ Negatives}$ x 100
466/466+2 =99.6%

(V) Accuracy = $\frac{True\ Positives+True\ Negatives}{True\ Positives+True\ Negatives+False\ Positives+False\ Negatives}$ x 100
25+466/25+466+2+7 =98%

Table-1: Diagnostic Accuracy

Rodent Brain IF	CBA	
	Anti-NMDAR Ab Positive	Anti-NMDAR Ab Negative
Anti-NMDAR Ab Positive	25	07
Anti-NMDAR Ab Negative	02	466

Table-2: Comparison for cost of Reagents (Commercial Vs in house)

	Reagent name	Total cost	Adjustment for QA and wastage	Cost per test
I	**Commercial**			
1	Anti NMDAR Test kit (Euroimmune Leubeck, Germany)	Rs 250000 (1109 USD) (50 tests)	Rs 75000 (333 USD) (at 30%)	Rs 6500 (28.82 USD)
2	Disposables (Tips, test tubes)	Rs 500 (2.2 USD)	Rs 150 (0.7 USD) (at 30%)	Rs 13 (0.06 USD)
			Total cost per test (commercial)	Rs 6513 (28.88 USD)
II	**In House**			
1.	FITC conjugated anti Human IgG (Jackson Immunoresearch lab)	Rs 60,000 (266 USD) (0.5ml vial) Used at 1:80	Rs 18000 (79.8 USD) (at 30%)	Rs 156 (0.69 USD)
2.	O.C.T. (Compound embedding for cryostat Milano-Italy	Rs 4837 (x2) (21.5 USD)X2	Rs 1934 (at 20%) (8.9 USD)	Rs 23.2 (USD 0.1
3.	Rodent brain	Rs 300 (USD 1.33)	Rs 50 (USD 0.22)	Rs 0.7 (USD 0.003)
4.	Brain transport cost	Rs 1000 (USD 4.43)	-	Rs 2 (USD 0.009)
	Adhesive glass slides (Matsunami, Japan)	Rs 60840 (500 slides) (USD 270)	Rs 3000 (30%) (USD 13.3)	Rs 127.7 (USD 0.57)
6.	Disposables (coverslips)	Rs 2000 (USD 8.87)	Rs 600 (USD 2.66)	Rs 5.2 (USD 0.023)
7.	Phosphate buffer saline (MP Biomedicals IIKirch, France)	Rs 14041 (USD 62.3)	Rs 4212 (USD 18.7)	Rs 36.5 (USD 0.16)
8.	Glycerol	Rs 500 (USD 2.22)	Rs 150 (at 30%) (USD 0.67)	Rs 1.3 (USD 0.006)
9.	Sucrose (inno-train Kronberg im Taunus, Germany)	Rs 461 (40mg) (USD 2.04)	138 (at 30%) (USD 0.61)	Rs 1.2 (USD 0.005)
10.	Disposables (Tips, test tubes)	Rs 500 (USD 2.22)	Rs 150 (at 30%) (USD 0.67)	Rs 13 (USD 0.058)
			Total cost per test (In house)	Rs 366.8 (USD 1.63)

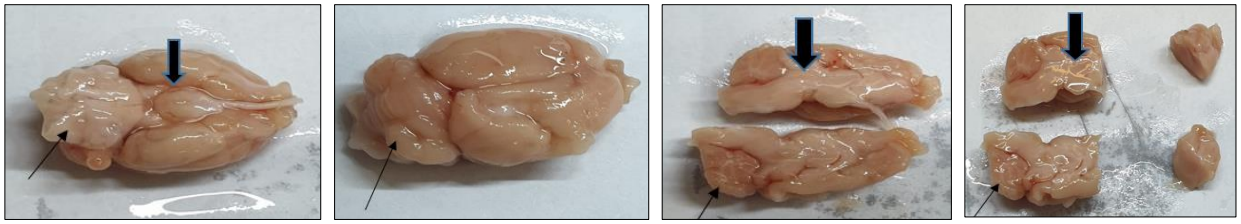

Figure-1. Rat cerebellum (thin arrows) Rat hippocampus (thick arrows)

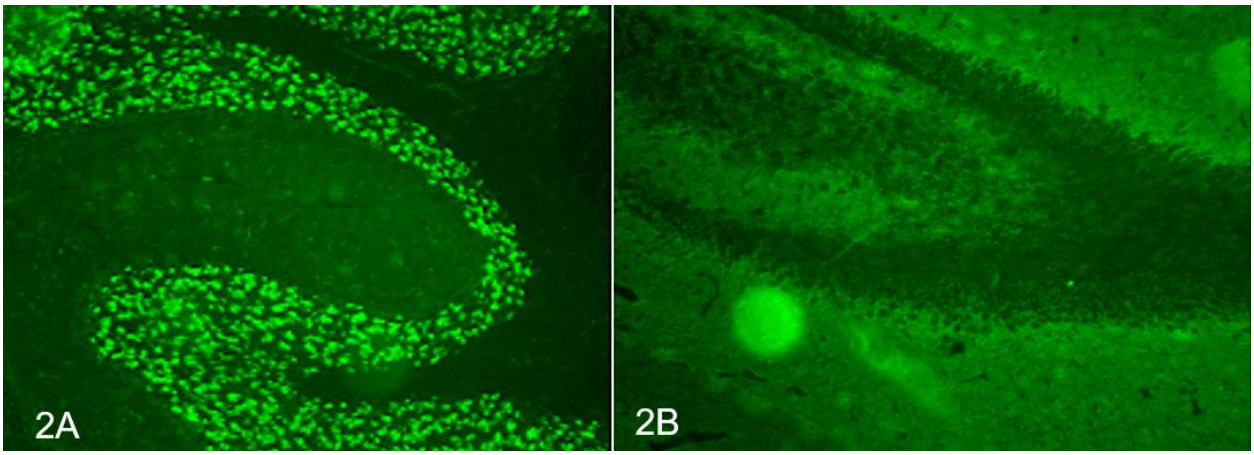

Figure-2: Positive and negative results under florescence microscope. 2A. Anti NMDAR antibodies positive on rat cerebellum. 2B. Anti NMDAR antibodies positive on rat hippocampus.

DISCUSSION

Anti NMDA R encephalitis is a relatively rare disorder with 1 person affected out of 1.5 million population per year.[11] In our study anti-NMDR encephalitis was diagnosed in only 5% of patients who were tested for auto-immune encephalitis related antibodies. Similarly, in a multicentre study in Korea, of the 721 patients with encephalitis of unascertained cause, 40 (6%) were diagnosed with anti-NMDAR encephalitis.[12]

In accordance with published literature our study showed a higher number of female patients (64.6%) who tested positive for anti NMDA encephalitis. According to Kong S who studied anti-NMDA receptor encephalitis patients from 2009-2017, 66% patients were females.[13] Similarly Titulaer MJ *et al* reported that out of 577 patients of anti NMDA R Encephalitis 81% were females.[14]

Literature shows that around 80% autoimmune encephalitis patients present initially with psychiatric symptoms and around 60% are admitted in psychiatric units.[15] According to Wang W et al majority of anti NMDA Encephalitis patients are diagnosed late ranging from 3-83 days and also the misdiagnosis rate is as high as 56.86%. Furthermore around 59% of patients of anti NMDA encephalitis suffer from at least one complication.[16] Similarly a case series published from our centre revealed 2 patients out of 8 suffering from complications including cognitive deficit and hearing loss and in both diagnosis was delayed due to which timely immunosuppressive therapy consisting of PLEX and IVIg was not given.[17] According to studies early treatment is one of the most important predictors of good outcome.[14] This indicates the importance of timely testing for these antibodies which followed by prompt treatment may help lower down the current mortality of this disorder which ranges from 5–11.5%.[18]

In our region reasons for delayed diagnosis are lack of awareness about this disorder and also the unavailability of tests for detection of these antibodies. Laboratory services for the anti NMDA R antibody detection are available at only three centres across the country. The main reason for the restricted availability is that Cell based assays are quite expensive with each test costing around Rs 6513 or USD 28.88 (reagent cost only). Whereas, in comparison each Indirect Immunofluorescence test using in house prepared rat brain costs around Rs 366.8 or USD 1.63 (reagent cost only) which is around seventeen times less, than CBAs (Table 2). The need to develop a cost-effective screening test that may help in establishing the diagnosis for these patients is highlighted by the low prevalence of anti NMDA receptor antibodies in those suspected to be suffering from autoimmune encephalitis, i.e., 5% which means around 95% of patients test would turn out to be negative for these antibodies. According to Qun Deng's data as well the positivity rate for AIE related antibodies is 9.9% and the overall cost for AIE related testing is USD 439.3 per patient.[19] This indicates that if a test with good sensitivity is available, we can use it to exclude anti NMDA encephalitis in most cases thereby sparing the use of CBAs only for those patients who have been 'ruled in' for testing anti NMDA encephalitis. This will also help reduce the overall cost in testing for autoimmune encephalitis.

Another advantage of testing on rodent brain compared to CBA is that it can also detect other antibodies including anti LGI, anti CASPR, anti GABA b, DPPX and some other yet undefined autoantibodies against neuronal receptors. These antibodies can be identified on the basis of different patterns of immunofluorescence produced as described by Glatter *et al*.[20] However in our study we did not come across other autoantibodies patterns which are associated with autoimmune encephalitis. Further studies with a higher

sample size are needed on a diverse range of samples to demonstrate these antibodies as the prevalence of these other types of antibodies is quite low compared to anti NMDA antibodies.

In the present study, we determined the diagnostic accuracy of rodent brain IF in detecting anti-NMDAR antibodies and found that rodent brain is has a sensitivity of 92.6% and is quite specific (98.5%) for detection of anti-NMDAR antibodies and therefore may prove useful for diagnosing anti NMDA R encephalitis in cost restraint set ups.

To our knowledge, this is the first study that has been conducted on the diagnostic accuracy of rodent brain IIF for diagnosis of anti-NMDA receptor encephalitis. There is a need to conduct more studies on this subject to establish the diagnostic accuracy of rodent brain IF for the diagnosis of anti-NMDA receptor encephalitis at national and international level.

CONCLUSION

Indirect Immunofluorescence (IIF) on in house prepared rodent brain tissue sections is recommended to be used for detection of anti NMDA receptor antibodies in patients with suspected NMDA R encephalitis in cost restrained set ups as it is approximately seventeen times less in cost than commercially available CBAs. It has a sensitivity of 92.6%, specificity 98.5% and diagnostic accuracy of 98.2%. This may facilitate timely diagnosis and management of anti NMDA encephalitis and may change the plight of patients suffering from this treatable yet potentially lethal disorder.

AUTHORS' CONTRIBUTION

SS: Data collection, data analysis, test performance, write-up. AA: Literature search, write-up, proof reading. TAA: Conceptualization of the study design, literature search, write-up, proof reading.

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Submitted: December 30, 2022	*Revised: March 20, 2024*	*Accepted: April 29, 2024*

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