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Editorial note:

A severe form of encephalitis associated with antibodies against NR1–NR2 heteromers of the anti-N-Methyl-D-Aspartate receptor (NMDAR) was recently identified. It is increasingly recognised as a reasonably common and treatable cause of acute psychosis. Dr Richard Wong, a specialist Immunologist in Queensland and his team have provided a detailed analysis of the anti-NMDAR testing for autoimmune encephalitis and have kindly shared their experience in HSSA-Pathology Queensland on the performance of anti-NMDAR kits. We welcome any feedback or suggestions. Please direct them to Dr. Janette Kwok (e-mail: kwoksy@ha.org.hk) of Education Committee, the Hong Kong College of Pathologists. Opinions expressed are those of the authors or named individuals, and are not necessarily those of the Hong Kong College of Pathologists.

Laboratory Testing For Anti-NMDAR In Autoimmune Encephalitis: The HSSA- Pathology Queensland Experience

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Introduction

The spectrum of antibodies against intracellular, cell surface and synaptic neuronal antigens has expanded rapidly in recent years. The antigenic targets include ion channels, receptors involved in neurotransmission across synapses and proteins associated with them. There are now more than twenty anti-neuronal antibodies detected in association with neurological diseases. These antibodies may be associated with underlying malignancies and are commonly referred to as paraneoplastic antibodies (PNAs). Many PNAs have been correlated with neurological manifestations and fall into two groups: those that are cytotoxic for example anti-purkinje cell antibody-1 (PCA-1/Yo) and anti-neuronal nuclear antibody-1 (ANNA-1/Hu); and others that have functional activity, such as anti-N-Methyl-D-Aspartate receptor (NMDAR) and anti-Voltage-gated potassium channel (VGKC). Recently there has been a marked interest in both anti-NMDAR and anti-VGKC antibodies as the presence of these antibodies identify patients with treatable neurological disease.

Anti-NMDAR was initially described as a paraneoplastic antibody associated with ovarian teratoma, with a characteristic clinical picture of

encephalitis with psychiatric features, cognitive dysfunction and seizures.[1 2] Although subsequent case series have confirmed that ovarian teratoma is a frequent association, it has become apparent that many patients who are positive for anti-NMDAR do not have evidence of an associated malignancy.

There is also some evidence supporting the need for rapid identification of anti-NMDAR. Patients who are diagnosed and treated with immuno-suppressive/immunomodulatory therapy within 40 days of disease onset, have been reported to have a better clinical outcome than those treated after 40 days.[2]

Given the common finding of psychiatric features in patients with positive anti-NMDAR, there has been interest in the prevalence of anti-NMDAR in patients presenting with their first episode of psychosis. Zandi et al reported that 6.5% (3 of 46) patients recruited prospectively from a cohort of patients with a first episode of psychosis were positive for anti-NMDAR positive in their serum.[3] As discussed below, if the CSF of these patients was also tested, it is likely that the prevalence of anti-NMDAR would be even higher. However it is clearly difficult to obtain informed consent and also actually perform lumbar punctures in acutely psychotic patients.

Finally, it is likely that some patients suspected of having viral encephalitis (including herpes simplex and enterovirus) actually have anti-NMDAR associated autoimmune encephalitis. In the prospective UK multicentre study[4] and a retrospective study from the California Encephalitis Project,[5] the prevalence of positive anti-NMDAR was 10/203 (4.9%) and 32/761 (4.2%) respectively, compared to 28/203 (13.8%) and 7/761 (0.9%) for herpes simplex virus, and 3/203 (1.5%) and 30/761 (3.9%) for enterovirus respectively.[4 5]

Detection of anti-NMDAR

Initially described as a novel neuropil (grey matter) antibody detected by immunohistochemistry on sagittal sections of paraformaldehyde-fixed rat brain,[6] the target antigen was first characterised in 2007. For the next three years, only two laboratories performed testing for anti-NMDAR, namely those of Josep Dalmau (Philadelphia, USA) and Angela Vincent (Oxford, United Kingdom). The initial 2005 paper described the difficulties of detecting the antibody by immuno-histochemistry, and mentioned that the rat brain had to be fixed in 4% paraformaldehyde at 4^{o}C for 10 days prior to cryosectioning.[6] Our experience is that anti-NMDAR could not be detected on fresh frozen mouse brain sections by indirect immunofluorescence.

In 2010, Euroimmun (Lubeck, Germany) released the first commercial assays for anti-NMDAR testing using transfected HEK (human embryonic kidney)-293 cells (Figure 1). Two different slide configurations are currently available: one configuration being a 4 "Biochip" mosaic of transfected HEK-293 cells, non-transfected HEK-293 cells, rodent cerebellum and rodent hippocampus sections; while the second configuration comprises only of transfected and non-transfected HEK-293 cells.

Issues with testing

There is ongoing controversy as to whether serum or CSF is the best specimen type to test for anti-NMDAR. Dalmau et al reported that none of the 412 paired serum and CSF specimens that were positive of anti-NMDAR, was positive only in the serum specimens.[7] In addition, the level of anti-NMDAR was higher in CSF than serum in 53 patients that were analysed.[8] These findings have also been taken to support the hypothesis that there is intra-thecal production of anti-NMDAR.[7 8] Dalmau has therefore strongly recommended that CSF should always be tested in patients suspected of having anti-NMDAR associated encephalitis, especially if their serum is negative for anti-NMDAR.

In contrast, Irani and Vincent[9] reported that in 7 paired specimens, serum levels of anti-NMDAR were higher or the same as CSF levels. These authors therefore attributed the finding of "isolated" CSF positivity for anti-NMDAR reported by Dalmau's group, to the relatively high dilution of serum (1/200) used by the Dalmau group (compared to 1/20 in Vincent's laboratory).

These findings illustrate the important fact that, as is the case with most other autoantibody assays, there are crucial method-dependent factors to be considered for anti-NMDAR testing. The laboratories of Dalmau and Vincent, both utilize in-house transfected HEK-293 cells as their primary screening assay. However, there are important differences between these two laboratories (see Table 1).[9] Specifically, Dalmau's laboratory uses fixed permeabilized cells with a NR1:NR2B cDNA ratio of 1:1 in the transfected cells, compared to the use of unfixed, un-permeabilized cells with a NR1:NR2B cDNA ratio of 3:1 in Vincent's laboratory. The commercially available transfected HEK-293 cells from Euroimmun, are acetone fixed permeabilized cells that are only transfected with NR1 (see Table 1). These differences are highly likely to affect diagnostic performance, and produce different results with some specimens. The screening serum dilution also varies 10-fold between Dalmau's (1/200 dilution) and Vincent's (1/20 dilution) which is likely to lead to differences in the ability to detect low levels of anti-NMDAR between these two laboratories.

It should also be noted that other autoantibodies are associated with autoimmune encephalitis including anti-VGKC (anti-LGI1 and/or anti-CASPR2), anti-AMPAR 1/2, anti-$GABA_BR$ and anti-glutamic decarboxylase (GAD).[10] [11] It has been reported that anti-GAD antibodies define a form of autoimmune encephalitis that is non-paraneoplastic, chronic and often unresponsive to treatment with immunosuppression and anti-convulsants.[12] Anti-GAD may be more commonly associated with autoimmune encephalitis (3%) than other antibodies such as anti-$GABA_BR$ and anti-AMPAR 1/2.[13]

How should levels of NMDA-R antibodies be determined and reported?

Using an ELISA-based method in which lysates of HEK-293 cells both expressing and not expressing NR1 subunits of NMDA-R, Dalmau et al determined the concentration of anti-NMDA-R in patients' CSF and serum.[8] However, it is important to note that the use of the term "titre" in this paper is misleading. The method described is based on subtracting the absorbance of the non-transfected cell lysate from that of NR1-transfected lysate. Hence the results for anti-NMDA-R are expressed as an absorbance unit, which is not a true serial dilution-based titre.

True serial dilution-based titres could theoretically be performed with the transfected HEK-293 cells. Indeed, the Euroimmun kit insert for the Glutamate Receptor Mosaic 3 slides recommends an initial screening dilution of 1/10 for serum specimens, followed by two further serial dilutions of 1/100 and 1/1000. However, discerning the end-point dilution on transfected cells is problematic, in terms of defining the criteria for a negative result. This includes the need to define the number of transfected cells per field, the precise magnification to be used, and the minimum visual intensity that indicates a positive transfected cell. There is also the high likelihood of variation between different batches/lots of the transfected cells, which will affect the validity of comparing results obtained on different batches/lots of slides. For these reasons, it is not recommended to attempt to determine visual end-point titration for other transfected antigens, such as for anti-60kD SSA/Ro on the 60kD SSA/Ro transfected HEp-2000 (Immunoconcepts, Sacramento, CA, USA) cells used for anti-nuclear antibody testing.

The cost of serial dilution is also an important consideration for laboratories that are using commercial slides for anti-NMDAR testing. The significant cost involved with routinely performing 3 dilutions (1/10, 1/100 and 1/1000) on all positive specimens will either have to be borne by the laboratory or passed on to the patient or requesting clinical department.

The laboratory of Angela Vincent reports the results of their anti-NMDAR testing as a score between 0 to 4, based on visual assessment of the intensity of immunofluorescent staining at a single serum dilution (1/20).[14] A score of 0 indicates absent/negative staining while a score of 4 reflects very strong staining.

Due to concerns regarding the effect of lot-to-lot variation on single dilution intensity scoring, our laboratory is currently evaluating an alternative approach. This involves concurrent testing of the latest specimen with the original (positive result) specimen at 1/100 dilution. Both specimens are digitally photographed with a 20x objective with identical exposure parameters. The number of positive transfected cells and the immuno-fluorescence staining intensity is compared visually using the digital photographs placed side-by-side on the same digital display. The results are classified and reported as follows:

- No discernable difference in staining intensity between specimens;
- A decline in staining intensity from the original specimen to the current specimen ;
- An increase in staining intensity from the original specimen to the current specimen (see Figure 2 for example).

This approach significantly reduces the cost of testing and also removes the issue of lot-to-lot variation. However, very occasionally the current and original specimen have to be repeated at a higher dilution (1/1,000) if the immuno-fluorescence staining intensity is very strong at 1/100 dilution, and no discernable difference in staining intensity can be demonstrated between the specimens at the 1/100 dilution.

Pathology Queensland Experience:

Pathology Queensland commenced anti-NMDAR testing in November 2010. We currently receive specimens from public and private laboratories from most states in Australia, one centre in Indonesia and one centre in Singapore. We use the Euroimmun "Glutamate Receptor Mosaic 3" slide; each well of this slide comprises a 4 "bio-chip" mosaic of transfected HEK-293 cells, non-transfected HEK-293 cells, rodent cerebellum and rodent hippocampus sections. We have chosen these 4 "Biochip" Mosaic slides over the alternative slides that only contain transfected and non-transfected HEK-293 cells, even though the former 4 "biochip" mosaic slides are more expensive. This is because we have found the additional sections of rodent cerebellum and hippocampus to be very useful in confirming the presence of anti-NMDAR (via the typical staining appearance in the cerebellar granular layer and dentate gyrus of the hippocampus), and also enable the detection of other antibodies including anti-GAD, and other paraneoplastic anti-neuronal antibodies (e.g. anti-PCA-1, ANNA-1).

Serum samples are tested at 1/10 dilution while CSF samples are tested neat/undiluted. All anti-NMDAR results are reported as positive or negative. Testing is performed daily with a typical turn-around-time of 24-48 hours from the time of the specimen arriving in our laboratory. Our immunopathologists (who are also qualified clinical immunologists with experience in the clinical management of patients with autoimmune encephalitis syndromes) routinely contact referring clinicians (if contact details are provided) regarding positive anti-NMDAR results and/or other coincidentally detected anti-neuronal antibodies.

Since November 2010, anti-NMDAR testing has been performed on 1,578 requests from a total of 1,253 patients. From these 1,253 patients, there have been 37 positive anti-NMDAR results, corresponding to a prevalence of 3.0% (see Table 2). Of these 37 positive patients, both serum and CSF were received from 25 patients, only serum was received from 9 patients, and only CSF was received from 3 patients. The age range of the patients with positive anti-NMDAR ranges from 4 to 77 years of age, with a median age of 25 years. This relatively young median age is comparable to that obtained by Dalmau et al[8] and the prospective UK encephalitis study,[4] in which the median age was 23 years (range 5 to 76 years) and 30 years (range 0 to 87 years) respectively. These findings emphasize the fact that anti-NMDAR encephalitis is a disease that typically affects children and young adults, in comparison to autoimmune encephalitis associated with other antibodies (anti-AMPAR 1/2, anti-$GABA_BR$, anti-LGI1 and anti-CASP2) which typically affect older adults (median age 60 to 62 years).[10]

To date, we have received paired serum and CSF samples from 174 patients. Of these 174 patients, 11 patients (6.3%) were only anti-NMDAR positive in CSF while 13 patients (7.5%) were anti-NMDAR positive in both serum and CSF.

These findings would suggest that even at a serum dilution of 1/10, approximately two fifths (40%) of patients with anti-NMDAR autoimmune encephalitis would be incorrectly considered as anti-NMDAR negative if only their serum was tested. ***We therefore recommend to our clients that if the serum is negative and there is strong clinical evidence for NMDAR-encephalitis, a CSF sample should be submitted for testing.***

Interestingly, we have detected one patient that was only anti-NMDAR positive in serum and negative in CSF. However, the clinical presentation (progressive memory loss and confusion, with diffuse white matter changes on brain MRI) of this older (68 year old) female patient was not typical for encephalitis, and she was subsequently diagnosed with a cerebral diffuse large B-cell lymphoma on brain biopsy. It could therefore be argued that this represents a false positive anti-NMDAR result, possibly induced by augmented exposure of NMDAR to her immune system in the setting of her cerebral lymphoma.

Conclusion:

Anti-NMDAR associated encephalitis is an important and treatable autoimmune disease, which is underappreciated due to the variable spectrum of clinical presentation. It is likely that there are also cases with milder clinical presentations than the original case series. A prompt diagnosis is crucial as early immuno-suppressive/immunomodulatory treatment appears to be associated with better clinical outcomes than delayed treatment.

Testing for anti-NMDAR is becoming increasingly available. However, there are important differences in the assays currently utilized to detect anti-NMDAR, including the serum dilutions employed. Testing for anti-NMDAR in CSF should always be performed if there is strong clinical evidence for NMDAR-encephalitis and the serum specimen is negative for anti-NMDAR.

To maintain expertise and shorter turnaround times, anti-NMDAR testing should be limited to one specialized laboratory per region, and this laboratory should have established experience with the detection of other neuroimmunological antibodies. This is important as all patients with suspected autoimmune encephalitis should also be tested for the other autoantibodies (including anti-VGKC (anti-LGI1 and anti-Caspr2) and anti-GAD) reported to be associated with autoimmune encephalitis.

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Table 1: Comparison of available methods for anti-NMDAR testing (modified from Irani and Vincent 2011)[9]

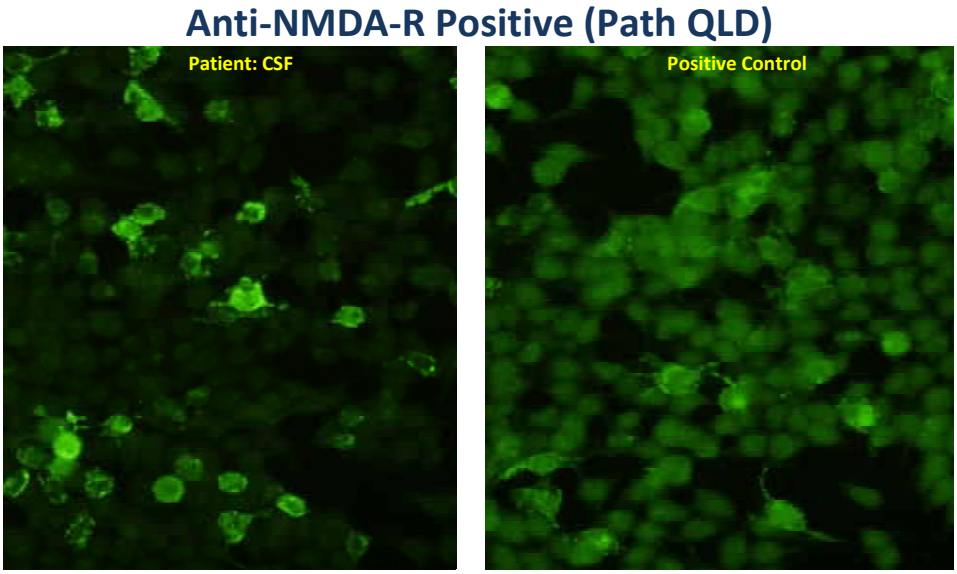
	HSSA-Pathology Queensland (Euroimmun Glutamate Receptor Mosaic 3 slide)	Dalmau et al (2008)[8]	Irani et al (2010)[2]
Serum Dilution	1/10	1/200	1/20
CSF Dilution	Neat/undiluted	1/10	1/1
NR1:NR2B composition used to transfect HEK-293 cells	NR1 only	NR1:NR2B ratio of 1.1	NR1:NR2B ratio of 3:1
Permeabilization/fixation	Permeabilized/Acetone fixed	Permeabilized/fixed	Non-permeabilized /non-fixed

Table 2: HSSA-Pathology Queensland Anti-NMDAR Testing (November 2010 to December 2012)

	HSSA-PATHOLOGY QLD	DALMAU J ET AL, 2008[8]
Positive Anti-NMDAR	37/1253 (3.0%)	n/a
Number of Paired Serum and CSF specimens = 174		
Positive in serum and/or CSF	25/174 (14.4%)#	58
Positive in serum only	1/174 (0.6%)*	0/58 (0%)
Positive in both serum and CSF	13/174 (7.5%)	53/58 (91%)
Positive in CSF only	11/174 (6.3%)#	5/58 (9%)
Age range of patients with positive anti-NMDAR	4 to 77 years (median 25 years)	5 to 76 years (median 23 years)

* This 68 year old female patient had an atypical clinical presentation (progressive memory loss and confusion), diffuse white matter changes on brain MRI, and was subsequently diagnosed with cerebral diffuse large B-cell lymphoma on brain biopsy.
# Of the 25 anti-NMDAR positive patients in whom paired serum and CSF samples were received, 11 patients (44%) had detectable anti-NMDAR only in CSF

Figure 1: Positive anti-NMDAR in CSF (left) and positive serum control (right) on NR1 transfected HEK-293 cells on “Euroimmun Glutamate Receptor Mosaic 3” slides (40x Objective)



Note: Background staining from serum sometimes makes immunofluorescence difficult to read.

Figure 2: Comparison of anti-NMDAR testing on serum specimens from an 18 year old female diagnosed with autoimmune encephalitis. The original serum was collected on September 2011 (left image). The patient then received treatment with rituximab with clinical improvement, but experienced a relapse of symptoms thus prompting the repeat test in September 2012 (right image). Both specimens were tested in parallel at 1/100 dilution on the same run, and digitally photographed using a x20 microscope objective. There is a discernable increase in staining intensity and number of positive NR1-transfected HEK-293 cells in the 2012 specimen (right image) compared to the 2011 specimen (left image)