RESEARCH ARTICLE

Pathogen and Antibody Identification in Children with Encephalitis in Myanmar

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Objective: Prospective studies of encephalitis are rare in regions where encephalitis is prevalent, such as low middle-income Southeast Asian countries. We compared the diagnostic yield of local and advanced tests in cases of pediatric encephalitis in Myanmar.

Methods: Children with suspected subacute or acute encephalitis at Yangon Children's Hospital, Yangon, Myanmar, were prospectively recruited from 2016–2018. Cohort 1 (n = 65) had locally available diagnostic testing, whereas cohort 2 (n = 38) had advanced tests for autoantibodies (ie, cell-based assays, tissue immunostaining, studies with cultured neurons) and infections (ie, BioFire FilmArray multiplex Meningitis/Encephalitis multiplex PCR panel, metagenomic sequencing, and pan-viral serologic testing [VirScan] of cerebrospinal fluid).

Results: A total of 20 cases (13 in cohort 1 and 7 in cohort 2) were found to have illnesses other than encephalitis. Of the 52 remaining cases in cohort 1, 43 (83%) had presumed infectious encephalitis, of which 2 cases (4%) had a confirmed infectious etiology. Nine cases (17%) had presumed autoimmune encephalitis. Of the 31 cases in cohort 2, 23 (74%) had presumed infectious encephalitis, of which one (3%) had confirmed infectious etiology using local tests only, whereas 8 (26%) had presumed autoimmune encephalitis. Advanced tests confirmed an additional 10 (32%) infections, 4 (13%) possible infections, and 5 (16%) cases of N-methyl-D-aspartate receptor antibody encephalitis.

Interpretation: Pediatric encephalitis is prevalent in Myanmar, and advanced technologies increase identification of treatable infectious and autoimmune causes. Developing affordable advanced tests to use globally represents a high clinical and research priority to improve the diagnosis and prognosis of encephalitis.

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Encephalitis is a clinical syndrome of brain inflammation with neurological dysfunction that can cause significant long-term morbidity and mortality.[1, 2] Epidemiological studies from the USA, Europe, and Australia suggest that 3.2–10.5 per 100,000 pediatric hospitalizations are due to encephalitis.[1, 3, 4] Successful treatment is dependent on

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timely diagnosis, and accurate identification of infectious and non-infectious etiologies.[4] Although definitive diagnosis may require pathological specimens, risks associated with invasive procedures and limited resources preclude this analysis in many patients. Recognizing these limitations and the need for consistent diagnoses, the International Encephalitis Consortium published a consensus statement in 2013 outlining diagnostic criteria for encephalitis of presumed infectious or autoimmune etiologies in the hope of improving case definitions for prospective studies and facilitating public health surveillance.[2]

Prior studies have attempted to identify encephalitis etiologies using pathogen-specific polymerase chain reaction (PCR) and enzyme immunoassays with variable success.[4–6] Regional variability in infectious diseases, immunization status, genetic backgrounds, referral bias, prospective or retrospective nature of the studies and available resources may influence the observed variability of encephalitides internationally.[5, 7–9] Furthermore, the impact of autoimmune causes of encephalitis has only recently begun to be appreciated, with prevalence studies estimating that they may account for a significant proportion of cases.[7, 10] The largest prospective study to date with testing for all known autoantibodies studied 296 patients with encephalitis other than acute disseminated encephalomyelitis. An infectious etiology was identified in 100 (34%) patients, autoimmune in 64 (22%), and encephalitis of unclear cause was noted in 132 (45%).[11]

Few prospective studies of encephalitis have been performed in Southeast Asia due to limited resources, despite a high prevalence of cases.[12–17] The Republic of the Union of Myanmar represents a unique population, where only a small number of children with encephalitis have been studied as part of a recent larger survey of pediatric encephalitis in the Greater Mekong region.[17] We sought to prospectively identify infectious and non-infectious etiologies of pediatric encephalitis using local tests, as well as advanced testing, and to compare the diagnostic yield of these different approaches. In addition, we attempted to describe the clinical presentation of these children to assist healthcare providers in resource-limited regions in identifying cases of infectious encephalitis (IE) and autoimmune encephalitis (AE).

Methods

Participants

We prospectively recruited children aged <12 years who were admitted to Yangon Children's Hospital, Yangon, Myanmar, with suspected subacute or acute encephalitis (see below for case definitions) from September 2016 to August 2018, into two consecutive cohorts. The hospital is a 500-bed, expandable to 1,000-bed, tertiary care pediatric hospital affiliated with the University of Medicine 1 in Yangon, Myanmar, and accepts children aged <12 years only. Its catchment area encompasses the lower Myanmar region, which accounts for approximately half of the country's population. All identifying patient information was removed prior to analysis. We received institutional review board approval from Yangon Children's Hospital, Washington University in St. Louis, University of Barcelona, and University of California San Francisco (UCSF). Trained research team members obtained informed consent from caregivers to use leftover blood and cerebrospinal fluid (CSF) clinical samples for research. Samples were transported on ice to the main clinical laboratory of Yangon Children's Hospital within 15 min of collection. After using samples for clinical tests, leftover samples were sent to the adjacent research laboratory and stored at −80 °C.

Case Definitions

We recruited children aged <12 years who fulfilled the diagnostic criteria for encephalitis, proposed by the 2013 International Encephalitis Consortium Consensus Statement (Table 1).[2] Patients with prior history of neurological disorders were excluded. We used the same clinical features of suspected or presumed AE (PAE) used by Graus et al.[18] to define PAE (Table 1). As the 2013 International Encephalitis Consortium Consensus Statement for the definition of encephalitis encompasses both IE and AE, children with clinical features different from PAE were classified as presumed IE (PIE).

Based on a patient's clinical presentation and the results of their diagnostic testing, we classified each case as either having a confirmed infectious etiology (CIE), PIE, confirmed AE, or PAE (Table 2 [cohort 1], Table 3 [cohort 2] and Fig 1). A "confirmed" pathogen was a clinically adjudicated infection identified by a direct detection test including culture, pathogen-specific PCR, research-based metagenomic next-generation sequencing (mNGS) followed by orthogonal validation by pathogen-specific PCR or mNGS in technical replicate, or in the case of Japanese encephalitis virus (JEV), presence of JEV-specific immunoglobulin M (IgM) antibodies in CSF confirmed by neutralizing antibody testing. Pathogens were considered "possible" if there was indirect evidence for a particular infection (ie, positive CSF anti-viral serology by VirScan [see Methods]),[19, 20] and the clinical manifestations were consistent. A "confirmed" autoantibody was a clinically adjudicated antibody in CSF identified by cell-based assay and tissue immunostaining.[21–26]

TABLE 1. Criteria for Encephalitis and Presumed Autoimmune Encephalitis

Encephalitis
Major criteria (required)
• Altered mental status lasting ≥24 h.
Minor criteria (minimum of 2 required)
• Fever ≥38 °C in 72 h
• New onset seizures
• New onset focal neurological findings
• CSF WBC count ≥5/cubic mm
• New/acute MRI brain abnormalities suggestive of encephalitis
• Abnormal EEG consistent with encephalitis
Presumed Autoimmune Encephalitis (=suspected or possible autoimmune encephalitis from Graus et al.)
All 3 features required:
• Subacute onset (rapid progression of less than 3 months) of one of the following: working memory deficits (short-term memory loss), altered mental status, or psychiatric symptoms • At least one of the following: new onset seizures, new onset focal neurological findings, CSF WBC count ≥5/cubic mm, new/acute MRI brain abnormalities suggestive of limbic encephalitis, demyelination or inflammation • Reasonable exclusion of alternative causes

Abbreviations: CSF = cerebrospinal fluid; EEG = electroencephalogram; MRI = magnetic resonance imaging; WBC = white blood cells.

Data Collection and Testing

For each patient suspected of having acute encephalitis, we prospectively collected all laboratory, imaging, and neurophysiological testing at the time of enrollment, throughout his or her hospital stay, and at follow-up. Through chart review and family interviews, we obtained demographic and exposure history, symptom descriptions and timelines, medications administered during the course of illness, and physical examination findings at the time of diagnosis and discharge from the hospital.

Local tests were performed based on the financial status of each patient and the fluctuating availability of local resources. Blood testing typically included complete blood count, electrolytes (sodium, potassium, chloride), blood urea nitrogen, creatinine, aspartate transaminase, alanine transaminase, C-reactive protein, erythrocyte sedimentation rate, and blood cultures. CSF testing typically included cell count, glucose, protein, Gram stain, culture and Ziehl–Neelsen stain, if tuberculosis was suspected. HIV serology was performed per clinician's judgment. CSF and serum serology for JEV, and dengue virus IgM and direct microscopic examination of blood films and immunochromatographic tests for malaria parasites were performed at the national laboratory in Myanmar when clinical suspicion for these entities was high. Radiologic testing could include chest X-ray, head computed tomography (CT), and/or brain magnetic resonance imaging. An electroencephalogram was performed per the discretion of the clinical team.

In addition to basic local tests, all patients in cohort 2 received additional CSF testing with the BioFire FilmArray Meningitis/Encephalitis (ME) panel, which tests for *Escherichia coli* K1, *Haemophilus influenzae*, *Listeria monocytogenes*, *Neisseria meningitidis*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, cytomegalovirus, enterovirus (EV), herpes simplex virus 1 (HSV-1), HSV-2, human herpesvirus 6 (HHV-6), human parechovirus, varicella-zoster virus, and *Cryptococcus neoformans/gattii*.[27, 28] This testing was performed at the national research laboratory in Yangon. All patients in cohort 2, including patients with PAE, were also tested using comprehensive autoimmune encephalopathy panels (Euroimmun Autoimmune Encephalitis Mosaic panel for N-methyl-D-aspartate [NMDA], α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, leucine-rich glioma-inactivated 1, contactin-associated protein-like 2, and gamma aminobutyric acid-B autoantibodies) performed on serum and CSF. Serum and CSF from all PAE cases, as well as FilmArray-negative PIE cases, when sample was sufficient, were sent to Spain and the USA. To confirm the results of Euroimmun tests and to look for novel autoantibodies, cell-based assays, tissue immunostaining, and, when required, studies with cultured neurons were performed at the University of Barcelona.[25, 26] To detect additional pathogens in CSF, we performed mNGS, enhanced by ViroCap at Washington University in St. Louis, USA.[29–31] Two separate infectious disease assays, mNGS and pan-viral serology using VirScan,[8, 19, 20, 32–34] were performed on CSF at UCSF. Infectious disease testing was only carried out on CSF.

ViroCap Sequencing (Washington University, St. Louis). Total nucleic acid was extracted from 26 CSF samples using the Maxwell Viral Total Nucleic Acid Purification Kit on the Maxwell automated instrument (Promega, Madison, WI). Sequencing libraries were prepared from DNA and RNA, as previously described, using the Swift Biosciences Accel-NGS Kit (Swift Biosciences, Ann Arbor, MI).[35] Libraries were pooled and viral nucleic

TABLE 2. Cohort 1 Clinical Characteristics

Characteristic	Presumed Infectious Encephalitis	Presumed Autoimmune Encephalitis	Incidence Difference (%)	LCL (%)	UCL (%)	*p*
Demographics						
Total cases	43	9				
Male, n (%)	19 (44.2)	7 (77.7)	−33.6	−71.3	4.1	0.143
Median age, yr [IQR]	0.9 [0.5–4.0]	11 [7–12]				<0.001
Clinical, n (%)						
Symptoms						
Fever	40 (93.0)	5 (55.5)	37.5	−2.6	77.5	0.014
Diarrhea	6 (14.0)	0 (0)	14	−3.1	31	0.537
Vomiting	17 (39.5)	0 (0)	39.5	18.2	60.9	0.056
Headache	4 (9.3)	1 (11.1)	−1.8	−25.9	22.3	1
Seizure	35 (81.4)	4 (44.4)	37	−4.3	78.2	0.057
Status epilepticus	0 (0)	3 (33.3)	−33.3	−70.8	4.2	0.002
Visual symptoms	1 (2.3)	0 (0)	2.3	−4.5	9.2	1
Speech disorder	5 (11.6)	9 (100)	−88.4	−100	−72.1	<0.001
Movement disorder	5 (11.6)	7 (77.7)	−66.1	−100	−30.6	<0.001
Psychiatric symptoms	0 (0)	9 (100)	−100	−100	−93.3	<0.001
Limb weakness	11 (25.6)	0 (0)	25.6	5.8	45.3	0.208
Poor balance	3 (7)	0 (0)	7	−7.4	21.3	0.976
Death	5 (11.6)	1 (11.1)	0.5	−22.7	23.7	1
Neuroimaging, n (%)						
Head CT obtained	36 (83.7)	9 (100)	−16.3	−34	1.5	0.445
MRI obtained	0 (0)	4 (44.4)	−44.4	−83.6	−5.3	<0.001
Head imaging obtained	36 (83.7)	9 (100)	−16.3	−34	1.5	0.445
Head imaging abnormal	24/36 (66.6)	3/9 (33.3)	33.3	−8	74.7	0.148

Abbreviations: CT = computed tomography; IQR = interquartile range; LCL = lower control limit; MRI = magnetic resonance imaging; UCL = upper control limit.

acid was enriched with ViroCap probes according to the manufacturer's specifications (Roche NimbleGen Madison, WI).[31] Samples were sequenced on the Illumina NovaSeq Instrument (Illumina, San Diego, CA). Data were analyzed with the ViroMatch pipeline.[36]

VirScan Protocol. VirScan was performed in duplicate in accordance with the scaled, vacuum-based, phage immunoprecipitation sequencing protocol, as described previously in detail.[37] Briefly, 10 μl of CSF was incubated overnight with 500 μl of phage library in pre-blocked

TABLE 3. Cohort 2 Clinical Characteristics

Characteristic	Presumed Infectious Encephalitis	Presumed Autoimmune Encephalitis	Incidence Difference (%)	LCL (%)	UCL (%)	*p*
Demographics						
Total cases	23	8				
Male, n (%)	13 (56.5)	8 (100)	−43.5	−72.2	−14.8	0.068
Median age in years [IQR]	1 [0.4–3.2]	8.2 [6.6–11.2]				<0.001
Clinical, N *(%)*						
Altered mental status	16 (69.6)	7 (87.5)	−17.9	−56	20.1	0.596
Fever	20 (86.9)	3 (37.5)	49.5	4.8	94.1	0.022
Diarrhea	3 (13.0)	0 (0)	13	−9.1	35.2	0.703
Vomiting	12 (52.1)	0 (0)	52.2	23.3	81	0.029
Headache	3 (13.0)	2 (25)	−12	−53.4	29.5	0.815
Seizure	18 (78.3)	4 (50)	28.3	−18.7	75.2	0.287
Status epilepticus	5 (21.7)	1 (12.5)	9.2	−27.6	46.1	0.96
Speech disorder	1 (4.3)	7 (87.5)	−83.2	−100	−50.3	<0.001
Movement disorder	4 (17.4)	6 (75)	−57.6	−99.8	−15.4	0.01
Psychiatric/behavior symptoms	0 (0)	7 (87.5)	−87.5	−100	−56.2	<0.001
Death	2 (8.7)	0 (0)	8.7	−11.2	28.6	0.979
Modified Rankin score 2 at 18 months follow-up	16 (69.6)	7 (87.5)	−17.9	−56	20.1	0.596
Modified Rankin Score >2 at 18 months follow-up	5 (21.7)	0 (0)	21.7	−3.5	47	0.378
Neuroimaging, n (%)						
Head CT obtained	22 (95.7)	4 (50)	45.7	1.6	89.7	0.014
MRI obtained	0 (0)	2 (25)	−25	−63.4	13.4	0.1
Head imaging obtained	22 (95.7)	6 (75)	20.7	−18.9	60.2	0.314
Head imaging abnormal	14 (63.6)	2 (33.3)	30.3	−23	83.7	0.387

Abbreviations: CT = computed tomography; IQR = interquartile range; LCL = lower control limit; MRI = magnetic resonance imaging; UCL = upper control limit.

plates with continuous rocking. Protein A/G beads were added in the morning for 1 h, followed by 5 washes using a vacuum manifold and custom 3D printed adapters. After the final wash, protein A/G beads were added directly to prepared 500 µl of *E. coli* in LB broth at an OD 0.3–0.6, and incubated in a shaker for 1–2 h until lysis was observed. Filtered lysate from round 1 was incubated with 10 µl of CSF for a second round. Library preparations were also performed as described,[37] using a Beckman acoustic (Echo 525) liquid handler for a 12.5-µl reaction volume. Samples were sequenced on an Illumina NovaSeq 6000 for 147 base pair paired-end sequencing.

VirScan Data Analysis. Alignment was performed as previously described[38] to a reference database of the full pan-viral library using Bowtie2[39] and filtered for a perfect match. Peptide counts were normalized to read depth by dividing by the total reads in a given sample and

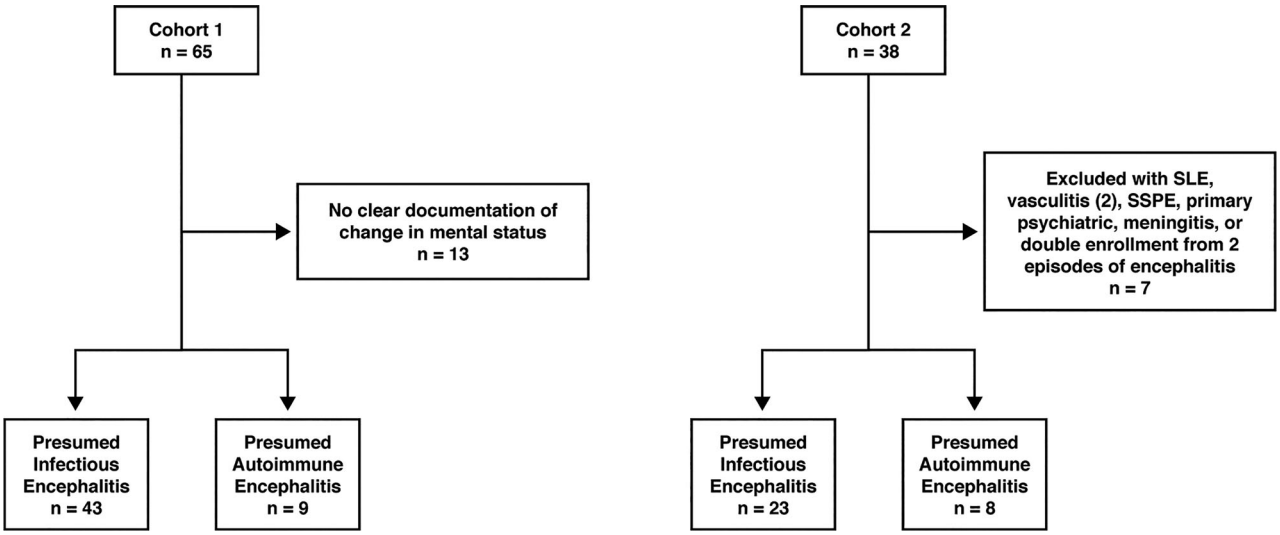

FIGURE 1: Flow diagram showing enrollment, exclusion, and classification details. SLE = systemic lupus erythematous; SSPE = subacute sclerosing panencephalitis

multiplying by 100,000 to calculate the reads per 100 k (rpK). Fold-change was calculated by dividing the rpK of each peptide by the mean rpK of control samples. Z-scores were calculated for sample peptide fold change relative to the healthy background samples. Conservative cut-offs (rpK > 0, fold change >10, and *z*-score >1) were used to filter out peptides unlikely to be contributory to a patient's profile. Patient samples were analyzed on an individual basis for reactivity to viral peptides relative to the rest of the cohort.

For samples with increased phage reactivity to EV peptides, coverage maps were created in a similar fashion to previously described.[20] The rBLAST package was used to align EV peptides to a reference EV-A71 genome (Genbank Accession AXK59213.1), and rpK was added along the length of the genome for each sample.

Cerebrospinal Fluid mNGS (UCSF). A detailed protocol on mNGS of CSF has been previously published.[40] Total nucleic acid was extracted from 90 µl of CSF using the Zymo Quick-DNA/RNA MagBead via the Integra Viaflo 96, and eluted into 50 µl of nuclease-free water. Half of the extraction was treated with DNAse and prepared for RNA sequencing, whereas the rest was used for DNA sequencing. Library preparation for the RNA and DNA samples was performed using New England Biolabs' NEBNext Ultra II RNA library preparation kit and FS DNA Library Prep Kit, respectively. Sequencing was performed on an Illumina iSeq to calculate pooling volumes and then submitted for sequencing on an Illumina Novaseq 6,000 using 146 base pair paired-end sequencing. Sequencing files were uploaded to CZID (previously known as IDseq), an open source online pathogen detection pipeline, for analysis relative to human CSF background.[41]

Statistical Analysis

Comparisons of demographic and clinical features were performed within cohorts. Categorical data are presented as numbers (total count) and percentages of patients with the variable of interest. Continuous variables are presented as medians with interquartile ranges. Categorical data are presented with *p*-values derived using the χ^2-test. As, in both cohorts, the continuous variable of age violated the assumption of normality, the non-parametric Mann–Whitney *U* test was used for analysis with statistical significance defined as $p < 0.05$.

Results

General Information

We prospectively enrolled 103 patients with suspected acute encephalitis in two consecutive cohorts (65 in cohort 1 and 38 in cohort 2). Children in cohort 1 were recruited between September 2016 and June 2017. Children in cohort 2 were recruited between December 2017 and August 2018. A total of 13 children from cohort 1 were subsequently excluded, because the reason for the change in mental status in these cases was not clear. This left 52 participants for analysis (Fig 1). Of the 52 patients, 9 (17%) were considered to have PAE.[18] The remaining 43 (83%) children were classified as PIE. Cohort 2 included 38 patients, but 7 were excluded due to the following alternative diagnoses: meningitis without clearly meeting diagnostic criteria for encephalitis (n = 1), systemic lupus erythematosus with multi-organ involvement affecting mental status (n = 1), systemic vasculitis (n = 2), subacute sclerosing panencephalitis whose chronic features were not appreciated at the time of enrollment (n = 1), primary psychiatric disorder (n = 1), and

double enrollment of a patient for recurrent admission after initial encephalitis (n = 1; Fig 1). Of the 31 remaining patients, 8 (26%) were considered to have PAE, and the other 23 cases (74%) were classified as PIE.

Results of Testing

Cohort 1 had locally available diagnostic tests only. Two of 43 (5%) PIE cases had a CIE (JEV IgM+ in CSF, n = 2), leaving 41 cases (95%) of PIE with unknown etiology (Fig 2). Among these 41 patients without a CIE, 8 patients had an abnormal chest X-ray suspicious for tuberculosis (TB), but the AFB stain of the CSF was negative. No autoantibody testing was available locally at the time of recruitment.

Cohort 2 had local testing followed by advanced tests. With local testing, one of 23 (4%) PIE cases had a pathogen identified (JEV IgM + in CSF, n = 1) leaving 22 (96%) of PIE cases with an unknown etiology (Fig 2). Of the 8 cases with PAE, testing using the Euroimmun panel showed anti-NMDA receptor antibodies in serum and CSF of 5 (62.5% of PAE, 16% of total cohort 2 cases). None of the PIE cases had positive Euroimmun panel results. The BioFire FilmArray ME Panel identified pathogens in 7 cases of presumed IE (31% of PIE, 23% of total cohort 2 cases) including HSV-1 (n = 3), EV (n = 1), *H. influenzae* (n = 1), cytomegalovirus (n = 1), and *S. agalactiae* (n = 1). The ME panel was negative in all 8 cases of possible AE.

CSF and serum samples from FilmArray-negative PIE cases, as well as all PAE cases, were shipped to laboratories in the USA and Spain. Testing at University of Barcelona did not show any additional or novel autoantibodies, but confirmed the NMDAR antibody results from the Euroimmun panel. mNGS with ViroCap of CSF, carried out at Washington University, St. Louis, detected pathogens of unclear clinical significance in 8 children: Epstein–Barr virus (n = 3), HHV-7 (n = 2), and HHV-6 (n = 3). VirScan detected CSF antibody positivity to respiratory syncytial virus (RSV; n = 1), HSV-1 (n = 1), and EV-B (n = 5). The case with CSF RSV antibodies (case number 8, see Supplementary Tables 2 and 3) did not have RSV RNA detected by mNGS, so this case was classified as a possible RSV CNS infection. The HSV-1 case (case number 17) also had HSV-1 DNA sequences detected by mNGS and confirmatory clinical HSV-1 PCR performed at UCSF. HSV was not detected in this case by either the ME panel or by mNGS with ViroCap, which detected only HHV-6 in this case. The clinical syndrome in this case (see below "Case Highlight") was consistent with HSV encephalitis. Case 7 had weakly enriched EV peptides on VirScan, but was also CSF IgM-positive for JEV, and was clinically adjudicated to be JEV encephalitis. Case 14 enriched EV on VirScan, but had *Mycobacterium tuberculosis* detected by mNGS in technical replicate in CSF. Because this patient also had suspicion for TB based on the chest X-ray and a CSF profile potentially consistent with TB meningitis (Supplementary Table 2), this case was classified as TB meningitis. The remaining EV CSF antibody-positive cases (n = 3) did not have EV RNA (or the RNA of any other pathogen) detected by PCR or mNGS, so these cases were classified as possible EV CNS infections.

In addition to case 14, mNGS detected *M. tuberculosis* sequences in 2 additional cases (cases 4 and

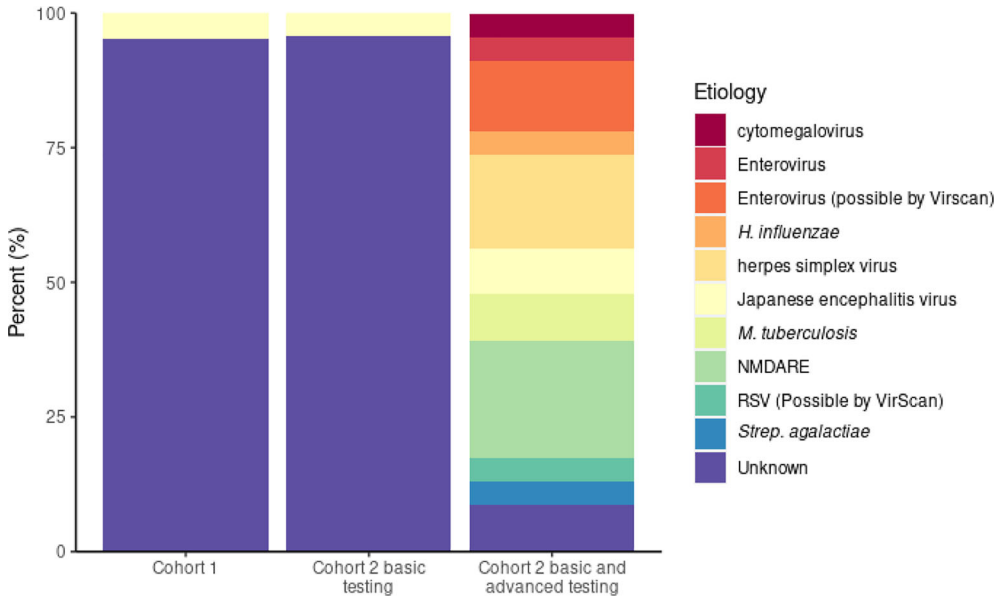

FIGURE 2: Causes of infectious encephalitides. NMDARE = N-methyl-D-aspartate receptor encephalitis; RSV = respiratory syncytial virus. [Color figure can be viewed at www.annalsofneurology.org]

30). Case 4 had chest X-ray abnormalities consistent with pulmonary TB. Although staining for acid-fast bacilli in CSF was negative, the abnormal CSF profile could have been consistent with TB meningitis, and repeat mNGS confirmed the detection of TB. Case 30 was an afebrile patient in the PAE group with paucicellular CSF who was found to have anti-NMDA receptor antibodies in serum and CSF. This patient did not have symptoms typical of TB including fever, headaches, vomiting, cough, or weight loss. He had a normal CXR. He had a speech disorder and movement disorder, typical of NMDA receptor encephalitis. Repeat mNGS on CSF RNA failed to confirm TB detection. Therefore, we categorized case 30 as having a final diagnosis of NMDA receptor antibody encephalitis only. Supplementary Tables 1 and 2 combine demographics, clinical presentation, ancillary test results, and laboratory test results for each individual case in cohorts 1 and 2, respectively. Of note, all cases of PIE that were ultimately diagnosed as bacterial encephalitis had >10 white blood cells in CSF. The results of all infectious disease testing carried out on each participant in cohort 2 are shown in Supplementary Table 3.

Out of the total 31 cases in cohort 2, local tests identified 1 case (3%) with a CIE, and advanced tests identified 10 cases (32%) with a CIE, 4 cases (13%) with a possible pathogen, and 5 cases (16%) with confirmed AE. Figure 2 (third bar) shows etiologies of the IE identified in cohort 2 combining locally available tests, ME panel PCR, mNGS with ViroCap, mNGS, and VirScan. In summary, among 23 cases with PIE, 11 cases had a CIE (1 case was identified by local testing, 7 cases by FilmArray ME panel PCR, 0 cases by mNGS with ViroCap, and 3 cases by mNGS at UCSF with corroborating evidence by VirScan in one of the cases [i.e., enrichment of HSV peptides in a case of herpes simplex encephalitis]), and 4 cases had a possible pathogen identified using VirScan. Figure 3 shows cases of IE with confirmed and possible pathogens in cohort 2, classified by diagnostic tests used to identify them.

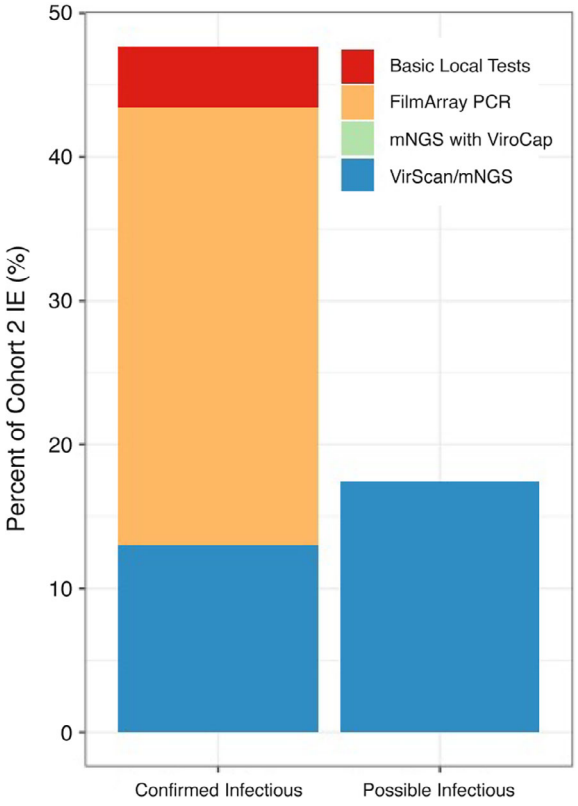

FIGURE 3: Cases of infectious encephalitides with confirmed and possible pathogens in cohort 2, classified by diagnostic tests used to identify them. mNGS = metagenomic next-generation sequencing; PCR = polymerase chain reaction. [Color figure can be viewed at www.annalsofneurology.org]

Demographics, Clinical Characteristics and Course

Cohort 1. The median age of children in cohort 1 was 1.4 years (IQR 0.5–5), and 26 were male (50%; Table 2). There was a statistically significant difference in ages between those diagnosed with PIE and those with PAE, with median ages of 0.9 years (IQR 0.5–4) and 11 years (IQR 7–12), respectively ($p < 0.001$). There was no statistically significant difference in sex between those classified as PIE or PAE, although the proportion of males in the AE category was higher.

Some clinical features were found to be significantly different between groups: fever (40/43 [93%] vs 5/9 [55%]; 95% CI −2.6%, 77.5%; $p = 0.014$) was more prevalent in the IE group, whereas status epilepticus (0/42 [0%] vs 3/9 [33.3%]; 95% CI −70.8%, 4.2%; $p = 0.002$), speech disorders (5/43 [11.6%] vs 9/9 [100%]; 95% CI −100%, −72.1%; $p < 0.001$), movement disorders (5/43 [11.6%] vs 7/9 (77.8%); 95% CI −100%, −30.6%; $p < 0.001$), and psychiatric symptoms (0/43 [0%] vs 9/9 [100%]; 95% CI −100%, −93.3%; $p < 0.001$) were more prevalent in patients with AE. No significant differences in the rate of abnormal head CTs, ICU admission, or rate of case progression to death were identified between groups. Of the 9 patients with PAE in cohort 1, 8 were treated with immunotherapy; 5 with IV methylprednisolone, 1 with IV immunoglobulin, and 2 with a combination of both of these treatments (Table 4). One patient died before therapy could be initiated. All patients that received immunotherapy in the PAE group had improvements in their neurological examinations at discharge.

Cohort 2. The median age of children in cohort 2 was 1.5 years (IQR 0.5–3.8), and 21 were male (68%;

TABLE 4. Treatment of Pesumed Autoimmune Encephalitis in Cohorts 1 and 2

	Steroid	IVIG	Both
Treatment cohort 1 (n = 8)	**5 (62.5%)**	**1 (12.5%)**	**2 (25%)**
Outcome	Improved (100%)	Improved (100%)	Improved (100%)
Cohort 2, (n = 9)	7 (87.5%)	0 (0%)	1 (12.5%)
Outcome	Improved (100%)	–	Improved (100%)

Abbreviation: IVIG = intravenous immunoglobulin.

Table 3). There was a statistically significant difference in age between diagnoses of PIE and PAE in cohort 2, with median ages of 1.4 years (IQR 0.5–3.4) and 8.2 years (IQR 6.6–11.3), respectively ($p < 0.001$). There was no statistically significant difference in sex between those diagnosed with PIE and those with PAE in cohort 2.

Clinical features of all encephalitis cases, as well as treatment outcome of patients with AE from cohort 2, are summarized in Tables 3 and 4. Significant differences noted in cohort 2 were similar to what was observed in cohort 1. Additionally, vomiting (12/23 [52.2%] vs 0/8 [0%]; 95% CI 23.3%, 81%; $p = 0.029$) was significantly more prevalent in patients with PIE.

Case Highlight

One patient from cohort 2 had HSV encephalitis, which was missed on initial FilmArray PCR testing, but testing of other sample aliquots led to diagnosis with VirScan and mNGS (Fig 4A, B) and confirmation by a clinical CSF HSV PCR. This patient was a 1-year-old boy who presented with altered mental status, fever, and left-sided focal seizures followed by generalized tonic clinic seizures with status epilepticus. He also had a left hemiparesis. His CSF examination showed 0 red blood cells/μl, 100 white blood cells/μl (100% lymphocytes), and normal glucose and total protein. His head CT showed bilateral frontotemporal hypodensities. He was treated with antibiotics and mannitol for increased intracranial pressure. He did not receive IV acyclovir, as IV acyclovir is very expensive locally, and it is given only when clinical suspicion is very high. The patient survived with a modified Rankin Scale of 3.

Discussion

The majority of prior encephalitis studies in Southeast Asia have focused on JEV, given its notable permanent neurological morbidity (30–50%) and mortality (30%).[42, 43] Literature regarding encephalitis etiologies in Myanmar is limited to a handful of articles about JEV, and more recently, a subset of children within a larger survey of pediatric encephalitis in the Greater Mekong region.[17, 44, 45] Previous studies have raised questions regarding quality and access to diagnostic testing available at specific centers, the presence of non-infectious etiologies, or unknown infectious agents at the time the studies were carried out.[46] In the present study, we enrolled a total of 103 children comprising two cohorts. A total of 83 children that fulfilled clinical criteria for acute encephalitis were included in the study, 66 (80%) with PIE and 17 (20%) with PAE. Using basic local testing, a CIE was identified in just 3 cases (4%). This rate is lower than the ~15% identification rate cited worldwide,[5, 6] presumably because pathogen-specific PCR testing and/or serological testing was not available locally, except for JEV and dengue virus. By adding advanced testing for pathogens and autoantibodies, 52% of cases from cohort 2 had confirmed etiologies (local n = 1, FilmArray ME panel n = 7, VirScan/mNGS n = 3, AE tests n = 5), and 13% had possible infectious etiologies (VirScan n = 4). Notably, the causes identified with advanced testing in cohort 2 included treatable etiologies, such as HSV, TB, *H. influenzae*, *S. agalactiae*, and anti-NMDA receptor encephalitis.

mNGS can directly detect the full range of neuroinvasive pathogens—viral, bacterial, fungal, and parasitic—by using a single assay,[47, 48] and a version of the test has been clinically validated.[33] ViroCap can enhance the sensitivity of mNGS by enriching for the nucleic acid of all vertebrate viruses (both DNA and RNA genomes) before deep sequencing.[30, 31] Using two different mNGS protocols in separate laboratories, clinically significant pathogens were detected in one case of HSV encephalitis and two cases that were clinically adjudicated to be TB meningitis. Interestingly, the BioFire FilmArray ME assay was negative in HSV encephalitis case. False negatives have been previously reported for HSV and other pathogens included in the ME panel.[49] mNGS with ViroCap detected viruses of uncertain clinical significance in six CSFs (Epstein–Barr virus, HHV-7, HHV-6), possibly because of its enhanced sensitivity for the detection of vertebrate viruses. Overall, mNGS only contributed a

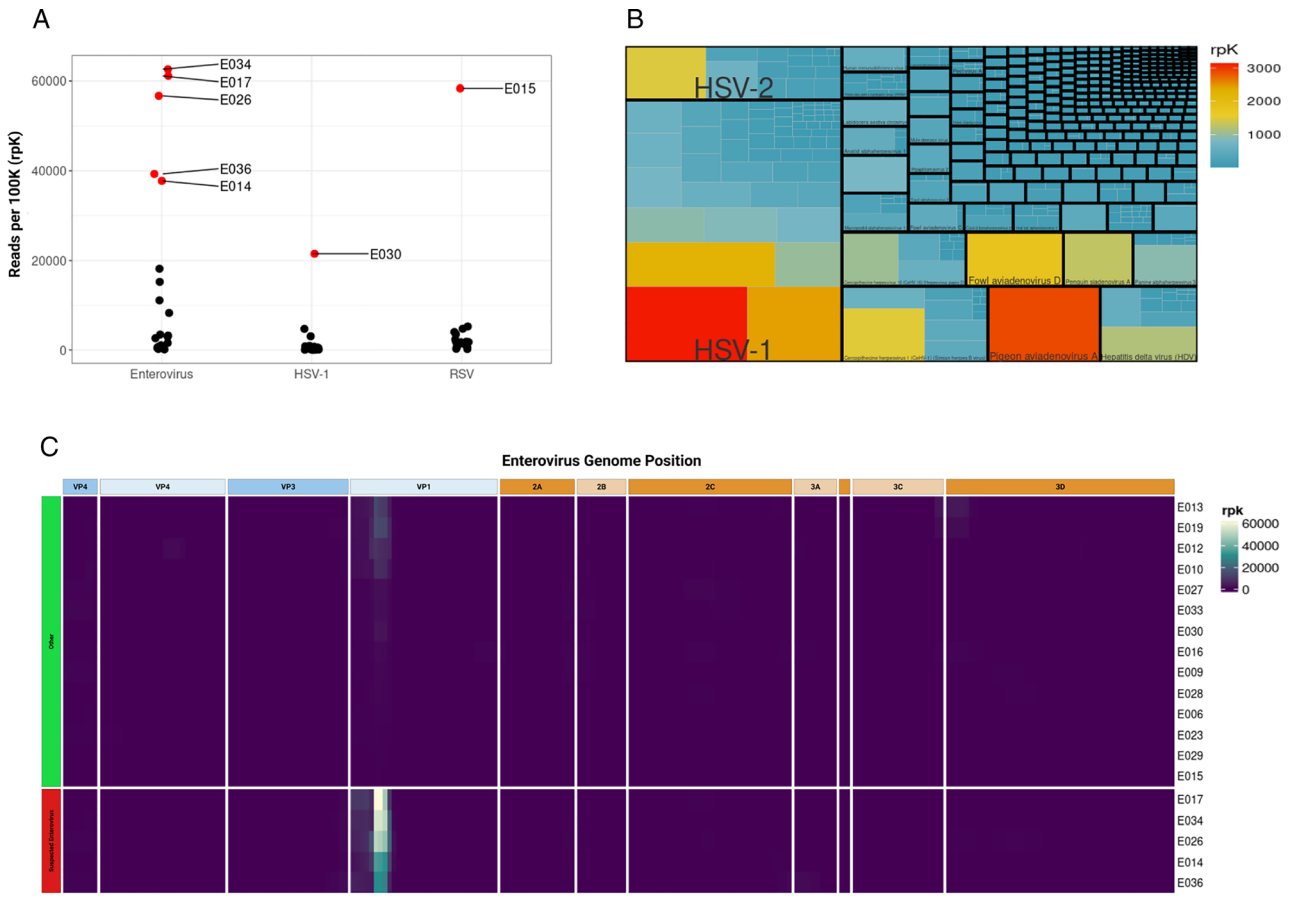

FIGURE 4: VirScan Results. (A) For each peptide in the library, the number of sequencing reads per 100,000 reads in a sample (rpk) was calculated and collapsed on viral species. This identified elevated enrichment above background in a subset of samples to enterovirus (EV), Herpes simplex virus type 1 (HSV-1), and respiratory syncytial virus (RSV). (B) Treeplot of a single sample with high levels of enriched peptides belonging to herpesviruses. Each small tile represents a viral peptide with an area and color proportional to its rpk. Small tiles are clustered together with other tiles representing peptides from the same viral species. (C) Enriched EV peptides were aligned to a reference EV genome (Genbank Accession AXK59213.1) using BLASTP. The rpk was summed at each amino acid position to create a coverage map along the whole viral genome. [Color figure can be viewed at www.annalsofneurology.org]

limited number of additional IE diagnoses in cohort 2, albeit 3 treatable ones. Because only cases that lacked a diagnosis were tested with mNGS, the present study was not designed to make a head-to-head comparison between mNGS test performance and other direct detection tests, such as the ME panel. These findings highlight that the yield of mNGS is dependent on the population being studied with the highest diagnostic yields coming from testing more immunosuppressed populations, including patients with advanced HIV infection and organ transplant patients.[33, 40, 50] Nevertheless, mNGS also has the key advantage of being able to detect emerging or re-emerging pathogens, including in Southeast Asia.[8, 51–53] As sequencing costs continue to drop precipitously and cloud-based, bioinformatics pipelines become more available, obviating the need for extensive computational server infrastructure, mNGS is likely to become increasingly available in lower resource settings.

Viral nucleic acid can be absent in CSF samples from patients with viral encephalitis depending on when the sample was acquired relative to the onset of symptoms. As a result, detection of anti-viral antibodies in CSF is the gold standard for diagnosis of many neuroinvasive viruses, especially arboviruses, such as JEV. Thus, we also used VirScan[19] to provide a comprehensive profile of CSF anti-viral antibodies.[20, 28, 54] In addition to the case of HSV-1 encephalitis detected by a combination of VirScan and direct detection of HSV-1 DNA by mNGS and clinical HSV PCR, we found high levels of antibodies to EV in 3 cases without another identified infectious agent and to RSV in 1. Consistent with previous publications, patients with EV meningoencephalitis and/or myelitis can have high levels of CSF EV antibodies in the absence of EV RNA detection in the CSF.[20, 32, 55] As in those cases, we saw a similar pattern of regions of the EV genome that were the most immunogenic, including to VP1 (Fig 4C). RSV has rarely been associated with infectious

encephalitis.[56] However, without detection of viral RNA and/or positive CSF viral cultures in these cases, detection of predominantly anti-viral immunoglobulin G antibodies alone in the CSF cannot confirm that these viruses were the cause of the patient's illness.

The present results also suggest that AE accounts for a significant proportion of patients presenting with features of encephalitis without identified pathogens. As 65% of PAE cases had NMDA receptor antibodies in cohort 2, it is essential to carry out autoantibody testing of CSF alongside comprehensive testing of local common pathogens to guide clinical management and perhaps shed light on additional AE triggers that might be geographically restricted, including viral causes of encephalitis other than HSV.[57, 58] Our use of brain tissue and neuronal cell culture immunostaining along with cell-based overexpression assays to screen comprehensively for autoantibodies confirmed the NMDAR antibody results performed by commercial testing by Euroimmun panel locally.[21–26, 59] We did not identify additional autoantibodies.

The time from symptom onset to presentation at Yangon Children's Hospital varied widely, ranging up to 56 days, making more subtle symptoms difficult to ascertain, and placing further importance on comprehensive testing being made available at first presentation. Most patients evaluated in an inpatient setting had a head CT (71/83, 85.5%), but additional diagnostic testing, including brain magnetic resonance imaging and CSF pathogen-specific PCRs, was extremely limited. Children with demyelinating diseases, such as acute disseminated encephalomyelitis, and other rheumatological diseases were likely underestimated, as magnetic resonance imaging was not routinely available, and comprehensive rheumatological evaluations were not performed. Of note, 18 of the 71 cases (25.4%) evaluated with head CTs identified symmetric basal ganglia and/or thalamic hypodensities. Of these, just two developed movement disorders. We were unable to discern any relationship between an underlying IE and the likelihood for development of these imaging abnormalities or development of a movement disorder. Given that thalamic involvement is very common in JEV encephalitis,[60] it is quite possible that some of these patients had undiagnosed infections with JEV or a different arbovirus.

The present study had several limitations generally inherent to studies carried out in resource-limited locations: local testing, particularly in cohort 1, was very limited, but this reflected the reality of the situation on the ground. PCR testing was not available in Myanmar at the time that cohort 1 patients presented, which greatly limited the etiology detection in this cohort. We did not include testing for gamma aminobutyric acid type A receptors and myelin oligodendrocyte glycoprotein antibodies, which are also common antibodies causing pediatric AE.[11] In addition, the deployment of more broad-based diagnostic testing platforms, such as mNGS, introduces the potential for detecting incidental, non-pathogenic viruses and false positive results.[33, 61] Larger prospective studies will have to weigh the benefits of agnostic testing platforms against these potential drawbacks. Other factors affecting the clinical outcomes in the present study are extrinsic factors, which were not controlled for in the analysis, that can account for the relatively poor prognosis of children with encephalitis in any resource-limited setting. These include delays in presentation of children to the hospital, lack of advanced intensive care, as well as the prohibitive cost of treatment options. Finally, the COVID-19 pandemic, and the political unrest and conflicts in Myanmar added an extra layer of complexity, severely limiting our ability to follow up and collect additional information and samples. The limitations of the present study increased our awareness that it might be impossible for this type of study to be carried out again in the near future.

Despite these limitations, there are very few studies identifying etiology of encephalitis in resource-challenged countries of Southeast Asia region, most of which focus on specific encephalitis etiologies or CNS infection, including meningitis as well as encephalitis. A 1-year prospective study carried out at Angkor Hospital for Children, Siem Reap, Cambodia, identified the etiology of CNS infection in 20% of cases and most of them were diagnosed by PCR.[62] A similar detection rate was noted in a prospective study in Thailand.[63] Combined local and advanced testing of cohort 2 in the present study detected an etiology in 64%. Therefore, advanced testing still seems to have a significantly higher etiology detection rate compared with the results from other regional LMIC.

The present study overall adds value to the existing literature by providing a prospective analysis of a large number of patients from a geographic region with limited reporting on the prevalent causes of IE and AE. We have identified specific demographic factors and clinical findings, which may help providers in differentiating between IE and AE in children in Myanmar. The present study also raises awareness that NMDA receptor encephalitis is common in an environment where IE is also common. Thus, consideration of empiric treatment for both types of conditions may be necessary, as immunotherapy for patients with PAE could be lifesaving in some instances, even where autoantibody testing is not available. Indeed, all patients found to have anti-NMDA receptor encephalitis were classified as PAE based on clinical features alone. For healthcare providers in resource-limited environments who

care for children with neurological diseases, awareness and understanding of key clinical differences in encephalitis of varying etiology will be beneficial in providing the most appropriate care for their patients and maximizing opportunities for positive clinical outcomes. Ultimately, however, given the regional variability of testing availability, affordable universal comprehensive diagnostic testing would have a substantial impact on the care of these patients by identifying treatable causes of encephalitis, and we hope that continued work to increase access to multiplexed pathogen-specific PCR, multiplexed autoantibody assays, and even more agnostic technologies that can enhance public health surveillance, such as mNGS and pan-viral antibody testing, in resource-limited settings will continue.[8, 40, 41, 51, 64–67]

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Potential Conflicts of Interest

M.R.W. receives unrelated research grant funding from Roche/Genentech, and received speaking honoraria from Genentech, Takeda, WebMD and Novartis.

K.M.W., T.N.W, and G.A.S. hold a patent for ViroCap (U.S. Patent No. 10,597,736).

J.D. holds patents for the use of Ma2, NMDAR, GABAbR, GABAaR, DPPX, and IgLON5 as autoantibody tests, and receives royalties related to autoantibody tests from Euroimmun.

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