First Confirmed Case of Encephalitis caused by Murray Valley Encephalitis Virus Infection in a Horse

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Abstract

A five year old Australian stock horse in Monto, Queensland, developed neurological signs and was euthanized after a six day course of illness. Histological examination of the brain and spinal cord revealed moderate to severe, subacute, non-suppurative encephalomyelitis. Sections of spinal cord stained positively in immunohistochemistry with a flavivirus-specific monoclonal antibody. Reverse transcription-polymerase chain reaction assay targeting the envelope gene of flavivirus yielded positive results from brain, spinal cord, cerebrospinal fluid and facial nerve. A flavivirus was isolated from the cerebrum and spinal cord. Nucleotide sequences obtained from amplicons from both tissues and virus isolated in cell culture were compared with those in GenBank, and had 96-98% identity with Murray Valley encephalitis virus. The partial envelope gene sequence of the viral isolate clustered into Genotype 1, and was most closely related to a previous Queensland isolate. This is the first confirmed case of naturally-occurring equine encephalitis attributable to Murray Valley encephalitis virus infection.

Key words

Encephalitis; flavivirus; horse; *Murray Valley encephalitis virus*

Murray Valley encephalitis virus (MVEV), an arbovirus in the family *Flaviviridae*, genus *Flavivirus*, is an important human pathogen. The principal vector is the fresh water mosquito *Culex annulirostris*[16]. The virus is considered to be endemic in northern Australia, and periodically reactivated or re-introduced into southern and eastern Australia, but the epidemiology is complex, involving the interplay of vertebrate host, vector and environmental factors. Although infections occur in a variety of vertebrate hosts, amplification is thought to occur principally in wild birds (waders), especially the Nankeen night heron (*Nycticorax caledonicus*)[16].

Horses are known to be susceptible to a number of mosquito-borne flaviviral encephalitides, including infections by West Nile virus (WNV) and Japanese encephalitis virus[17]. Kunjin virus, now recognised as a sub-type of WNV[9] has been implicated as the cause of a single case of naturally-occurring encephalomyelitis in a horse from Victoria[1]. MVEV is known to infect horses, based on serological evidence[7], and there is considerable anecdotal evidence to suggest it can cause neurological disease[~~22~~21], however naturally-occurring cases of equine encephalitis due to MVEV have not yet been confirmed.

Experimental infections of horses with MVEV have had varied outcomes, depending on the dose used and route of inoculation. Intracerebral challenge with high doses ~~inoculations of high titres~~ of virus have produced encephalitis in horses[7,18]. Small doses of peripherally inoculated MVEV have either failed to produce infections[7] or have resulted in short-lived viraemia and mild, transient clinical signs[13].This report describes a case of severe, naturally-occurring encephalomyelitis in a horse, attributed to MVEV.

During April 2008, a five-year-old Australian stock horse mare from Monto, Queensland (24°52´S 151°07´E) developed neurological signs. When examined by the veterinarian on day 1, the horse was in a mildly stuporous state, but could be roused by stimulation. Mild ataxia and forelimb propioceptive deficits were noted. Appetite was depressed, but vital signs were normal. Treatment with parenteral[a] and oral[b] non-steroidal anti-inflammatories was instigated. By day 3, there was a degree of facial paralysis, affecting the upper and lower lips, but not the eyelids. By day 6, the horse was laterally recumbent and unable to lift its head. At this stage, it was euthanized with barbiturate[c] overdose, and underwent a field necropsy. The only abnormality noted at necropsy was brain swelling, which decreased after the dura mater was opened.

Tissues, including brain and cervical spinal cord, were fixed in 10% neutral buffered formalin, and then paraffin embedded, sectioned at 5μm and stained with hematoxylin and eosin, using standard techniques. Histology revealed moderate to severe, subacute, non-suppurative encephalomyelitis. Changes were more severe in the hippocampus, midbrain, medulla and cervical spinal cord than in the cerebrum. The minimum change at all levels of the brain was perivascular cuffs in both gray and white matter. Cuffs were of variable thickness and consisted of a mixture of lymphocytes and histiocytes (Fig. 1). In the spinal cord severe inflammation was largely restricted to the gray matter (Fig. 2). Diffuse and focal gliosis were present throughout brain and spinal cord. In the more severely affected areas of midbrain and medulla swollen, chromatolytic and necrotic neurons were present, with glial satellitosis (Fig 3.). There were occasional small foci of hemorrhage and malacia in

the cerebral white matter, medulla and spinal cord (Fig. 2). There was also mild non-suppurative meningitis. No abnormalities were detected in other tissues.

Sections of cerebrum, brain stem, cerebellum and spinal cord were stained with a mouse monoclonal antibody[d], directed against a conserved epitope of the NS1 protein of MVEV, using methods described elsewhere[2]. Granular and diffuse antigen staining was detected in a small number of degenerate neurons, neuronal processes and the neuropil of spinal cord (Fig. 4). No antigen was detected in cerebrum, cerebellum or brain stem. Most lesions did not contain antigen staining.

Samples of blood, cerebrospinal fluid (CSF), brain, spinal cord and facial nerve were tested by reverse-transcription nested polymerase chain reactions (RT-PCRs) for ~~alphaviruses[20], orbiviruses (Oakey, unpublished) and~~a range of arboviruses, including flaviviruses. For flaviviruses the ~~using~~ primers FU1PM, cFD3PM, FU2 and cFD4PM ~~a modification~~ of a published method[14] were used. FU2 and cFD4PM were modified to include redundancies and enable reaction with a wider range of flaviviruses than the primers described in the original method. The primer sequences used were as follows:

FU1PM: TACAACATGATGGGVAARAGWGARAA

cFD3PM: ARCATGTCTTCYGTBGTCATCCA

FU2 (mod): GCTGATGACACMGCYGGMTGGGAYAC

cFD4PM (mod): AYNACRCARTCRTCYCCRCT

~~All samples were negative for alpha- and orbiviruses.~~ Positive results for flavivirus were obtained from all samples. Brain and spinal cord samples yielded much brighter amplicon bands than samples of CSF or nerve, and the reaction from the blood sample

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was very weak. These flavivirus positive samples were further tested with an RT-PCR specific for Kunjin virus (Oakey, unpublished), and all were negative. All samples were negative for alpha- and orbiviruses.

Three samples of cerebrum, one sample of cervical spinal cord and one sample of facial nerve were inoculated into *Aedes aegypti* (AA) C6/36 cells incubated at 28°C for two weeks. This was followed by subculture into BSR cells, a clone of baby hamster kidney cells (BHK-21), for one week at 34°C with supplementation of 5% CO_2. The AA and BSR cell lines were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum[e] and antimicrobials penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (2ng/ml).~~Three samples of cerebrum, one sample of cervical spinal cord and one sample of facial nerve were inoculated into *Aedes aegypti* (AA) C6/36 cells and incubated at 26-28°C for two weeks in minimum essential medium (MEM) supplemented with 10% fetal bovine serum[e] and antimicrobials penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (2ng/ml)[e]. This was followed by subculture into BSR cells for one week at 34-37°C.~~ Virus was isolated from all samples except facial nerve. Cells showed cytopathic effect (CPE) characterized by severe cytoplasmic vacuolation, stringing, rounding-up and detachment. Supernatants from positive cell culture samples tested PCR-positive for flavivirus.

RT-PCR amplicons (415 bp) were purified[f] and used to template dye-termination DNA sequencing reactions using a dideoxynucleotide sequencing kit[g] and the nested flavivirus PCR primers (FU2[mod] and cFD4PM[mod]). Amplicons were sequenced in both the sense and anti-sense direction. The reactions were resolved by Griffith

University DNA Sequencing Facility and resulting chromatograms were proofread and aligned using sequencing software[h]. The flavivirus group amplicon from virus isolated in cell culture was determined to have an identical nucleotide sequence to those obtained directly from tissue specimens. The consensus nucleotide sequence has been recorded as GenBank accession JN206679. This sequence was compared to those in GenBank using the search engine BLAST (http://www.ncbi.nlm.nih.gov/). The highest identity (96%) was with the NS5 gene of MVEV AF161266. BLAST in GenBank of the translated sequence resulted in a 98% similiarity with the NS5 protein of MVEV AAC58777 and AAF05296. No other matches were indicated with the nucleotide search. Matches with lower identities (<90%) were noted with other flaviviruses such as Alfuy virus, Japanese encephalitis virus, Usuto virus, West Nile virus and Kunjin virus.

Further RT-PCR of virus culture extract was performed as previously described[11] to produce a 462 bp amplicon of the envelope gene. The amplicon was sequenced (GenBank accession number JN119766) and aligned with analogous gene regions of reference sequences using Clustal W, as implemented in MEGA 4[24][23]. Subsequent phylogenetic analysis indicated that the equine MVEV isolate belongs to genotype 1 (Fig. 5), with highest levels of nucleotide sequence identity to the Queensland strain 857-2002, isolated from Burketown in 2002[12]. Close genetic relationships also were inferred with other Queensland and Western Australian strains isolated between 1989 and 2002 (98.3-99.6% nucleotide sequence identity). These results suggest that this strain might have been introduced into southeast Queensland from an endemic focus in either northern Queensland or northwestern Australia.

Outside of the endemic area there is a strong association between outbreaks of MVE and certain weather patterns, including excessive rainfall in eastern watersheds[21][20]. Monto, in the north Burnett region of southeast Queensland, is not within the endemic area. Although higher than average rainfall was recorded in Monto during the summer months (January and February 2008) preceding this event (http://www.bom.gov.au/jsp/ncc/cdio/weatherData/, accessed 25/05/11), the factors responsible for the current equine case remain unknown. No human cases of MVE, and a single human case of Kunjin virus infection, were recorded from Queensland during 2008 (National Notifiable Diseases Surveillance System, http://www.health.gov.au/internet/main/Publishing.nsf/Content/cda-surveil-nndss-nndssintro.htm , accessed 25/05/11).

The diagnosis of encephalomyelitis due to MVEV in this case was based on a combination of consistent histopathology, demonstration of flavivirus antigen within neurons and neuropil, virus isolation, positive RT-PCR and nucleotide sequencing. The non-suppurative encephalomyelitis in this horse has the histological hallmarks of a viral infection of the central nervous system: neuronal degeneration, reactivity of the glia, and perivascular cuffing with lymphocytes and histiocytes[17]. Although there are guidelines for differentiating some of the viral encephalitides of horses based on morphology[3,4], there are no published descriptions of the neuropathology of MVEV infections in horses. Moderate to severe non-suppurative encephalomyelitis and meningoencephalitis were reported in two horses which died in southern Australia in 1974 during an epidemic of human MVE[7], but infection with MVEV could not be confirmed in either horse. Horses with naturally-occurring WNV infection have non-suppurative polioencephalomyelitis, with the most severe lesions located in the

brainstem and spinal cord[4]. In the current case of MVE the most severe lesions were also located within brainstem and spinal cord, but preferential involvement of gray matter was evident only in the latter. Other features common to the two infections include petechial and ring haemorrhages[4], and a relative scarcity of flavivirus antigen detectable by immunohistochemistry[3]. In this case, despite widespread distribution of severe lesions, antigen was confined to one section of the spinal cord, and it was sparse within this location. This would indicate that the viral antigen was probably largely cleared or blocked by local CNS antibody at the time of death.

The differential diagnosis of viral encephalitides in Australian horses has, until recently, been a short list. Many of the recognised viral equine encephalitides are exotic to Australia; these include the alphaviruses Eastern, Western and Venezuelan encephalomyelitis; rabies; equine encephalosis virus; Borna disease; louping ill and other tick-borne encephalitides[8]. For Australian horses there are infrequent records of encephalomyelitis attributable to equine herpesvirus-1[2322], Hendra virus[6] and Kunjin virus[1]. Japanese encephalitis has caused sub-clinical infections in horses in Torres Strait, north of the Australian mainland[10]. This virus is considered to be spreading into previously non-endemic areas[5], and should be considered in future outbreaks of equine neurological disease.

At the time the current case was diagnosed with encephalitis, Hendra virus infection was considered to be a cause of predominantly fulminating respiratory disease in horses[19]. In June 2008 the tenth recognised outbreak of Hendra virus infection manifested primarily as neurological disease[6]. The serious zoonotic risk of Hendra virus infection now dictates a much more cautious approach to post-mortem sampling

in horses with neurological disease (Queensland Government: 2011, Guidelines for veterinarians handling potential Hendra virus infection in horses, Version 4.1. http://www.dpi.qld.gov.au/4790_13371.htm accessed 26/05/11). This complicates the future diagnosis of flaviviral, and indeed any infectious encephalomyelitis in Queensland horses.

There is heightened awareness of the potential for flaviviruses to spread, emerge, and appear in new geographic locations[15]. This first confirmed equine case of MVE is likely to be the forerunner of further such cases.

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Sources and Manufacturers

a. Flunixil (Flunixin meglumine 50mg/ml). Troy Laboratories Pty Ltd, Smithfield, NSW, Australia.

b. Equibutazone (Phenylbutazone 1g/sachet). Virbac (Australia) Pty Ltd, Milpera, NSW, Australia.

c. Lethabarb (Pentobarbitione sodium 325mg/ml). Troy Laboratories Pty Ltd, Smithfield, NSW, Australia.

d. 4G4, kindly donated by Associate Professor Roy Hall, University of Queensland, St Lucia, QLD, Australia.

e. Sigma-Aldrich, St Louis, MO, USA.

f. QIAquick kit, QIAgen, Doncaster, VIC, Australia.

g. Big Dye v3.1, Applied Biosystems. Foster City, CA, USA.

h. Sequencher v4.8 software. Gene Codes, Ann Arbor, MI, USA.

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Figure captions

Figure 1. Cerebral white matter. Dense perivascular cuff of lymphocytes and histiocytes. HE. Scale bar = 50µm

Figure 2. Cervical spinal cord. Perivascular cuffing, diffuse and focal gliosis, and focal hemorrhage (arrow), largely confined to the gray matter. White matter (WM) is relatively unaffected. HE. Scale bar = 500µm

Figure 3. Medulla oblongata. Satellitosis of a chromatolytic neuron with pyknotic nucleus (arrow); diffuse gliosis. HE. Scale bar = 50µm

Figure 4. Cervical spinal cord, showing viral antigen labelling of a degenerate neuronal cell body and process. Peroxidase immunohistochemistry using 4G4 antibody, counterstained with hematoxylin. Scale bar = 100 µm.

Figure 5. Phylogenetic relationships of the equine isolate of Murray Valley encephalitis virus (highlighted) with reference MVEV strains. The tree was constructed from a 462 nucleotide region of the envelope gene using the neighbour joining method and the maximum composite likelihood model of evolution (MEGA software version 4.0). Japanese encephalitis virus is shown as the outgroup. Percentage bootstrap values from 1000 replicates are indicated, with a cut-off value of 50%. The scale bar represents 0.05 nucleotide substitutions per site. Details of MVEV strains are shown as strain name/species of origin (H, human; M, mosquito; E, equine)/place and state of origin/year of isolation. Genbank accession numbers are

bracketed and genotype designation is indicated. NSW, New South Wales; QLD, Queensland; VIC, Victoria; WA, Western Australia.