A Retrospective Review of Autopsies with Encephalitis from 1998-2018 in Manitoba, Canada

by

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ABSTRACT

Encephalitis morbidity and mortality has been a focus of public and clinical interest, especially with arboviral trends such as West Nile Virus. Worldwide, the majority of encephalitis cases have an unknown etiology. This presents a challenge for diagnosing and treating encephalitis in order to minimize long term neurological deficits or death. A literature review demonstrates a lack of information on common viral etiologies at autopsy, as well as techniques to accurately identify the viral pathogen. In this study, we defined encephalitis as lymphocytic infiltration beyond the glia limitans into brain tissue with associated microglial activation, as demonstrated by immunohistochemistry. We retrospectively reviewed the Manitoba autopsy records from 1998 to 2018 and identified 114 cases of definite or presumed viral encephalitis. Cases with encephalitis at autopsy ranged from stillborn infants to 86 years of age. Males were more affected than females. In 20 cases, a viral entity was identified. The most common proven entities were herpes simplex and polyoma virus followed by West Nile virus. Possible viral encephalitis without definitive cause likely contributed to death in 36 cases. Possible mild viral encephalitis, incidentally, identified at autopsy, was identified in 58 cases with an unrelated cause of death. In most of the severe cases a viral entity was presumed but not identified due to lack of testing or failure of testing methods. There were peaks in August and September of known WNV encephalitis. This suggests an arboviral etiology for cases of possible viral encephalitis contributing to death and incidental mild encephalitis. Developments in PCR technologies may allow increased detection and identification of viruses in cases of encephalitis which present to autopsy without a definite diagnosis, or sometimes even without clinical suspicion of encephalitis.

KEY WORDS
Autopsy; brain; encephalitis; inflammation; virus
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ADEM</td>
<td>Acute disseminated encephalomyelitis</td>
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<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
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<td>CCR</td>
<td>Chemokine receptor</td>
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<td>CM</td>
<td>Cerebral malaria</td>
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<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CSF</td>
<td>Cerebral spinal fluid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>EL</td>
<td>Encephalitis lethargica</td>
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<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin fixed paraffin embedded</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HSC</td>
<td>Health Sciences Centre</td>
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<tr>
<td>HSE</td>
<td>Herpes simplex encephalitis</td>
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<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
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<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IG</td>
<td>Immunoglobulin</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>ISH</td>
<td>In situ hybridization</td>
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<tr>
<td>LFA</td>
<td>Lymphocyte function-associated antigen</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1 (CCL2)</td>
</tr>
<tr>
<td>ME</td>
<td>meningitis/encephalitis</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MR</td>
<td>Magnetic resonance</td>
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</tbody>
</table>
MS  Multiple sclerosis
NBF  Neutral buffered formalin
NK   Natural killer cell
NMDAR N-methyl-D-aspartate receptor
PML  Progressive multifocal leukoencephalopathy
POWV Powassan virus
RNA  Ribonucleic acid
RT-PCR Reverse transcriptase-polymerase chain reaction
SIDS Sudden infant death syndrome
SLEV Saint Louis encephalitis virus
SUDI Sudden unexpected death of an infant
SV   Simian virus
TLR  Toll-like receptor
TNF  Tumor necrosis factor
VCAM Vascular cell adhesion molecule
VLA  Very late antigen
WEEV Western Equine encephalitis virus
WNV  West Nile virus
ZIKV Zika virus
INTRODUCTION

Clinical significance

Encephalitis can be defined in broad pathologic terms as inflammation in the brain, which may affect neurons, blood vessels, and the surrounding glial parenchyma. It is a multifocal syndrome and can be a rare complication of many infections\(^1\). This definition is quite simple when considering the grand scheme of clinical conditions that encompass the term “encephalitis”. The main signs and symptoms include, but are not limited to confusion, fevers, dizziness, seizures, memory deficits, coma, or death. The causative agents include viruses, bacteria, fungi and autoimmune disorders. The causation can differ based on sexually transmitted infections, lack of vaccinations, immunodeficiency or travel to regions with endemic infections\(^2\). Cases of encephalitis accompanied by patchy and scant predominately lymphocytic inflammation in the subarachnoid compartment falls under the classification of meningoencephalitis. Meningoencephalitis exhibits features of both meningitis (inflammation of the meninges) and encephalitis\(^3\).

Viral exposure can be somewhat determined by geography and climate; Manitoba’s climate ranges from subarctic (<\(-30^\circ\text{C}\) in winter) to dry heat (>\(30^\circ\text{C}\) in summer). In the warmer season, there is potential for expansion of arboviral carrying mosquitos to reach previously unaffected regions. One of the difficult tasks clinically, is the ability to recognize symptoms, determine the etiology, and treat efficiently. Currently, there are no accurate statistics for encephalitis in Canada due to many factors including how its reported to the provincial health units\(^4\). The incidence in western countries has been documented to be 7.4 cases/ 100,000 population/ year with children being even higher,
10.5-13.8 cases/100,000 population/year\textsuperscript{5}. Many regions worldwide reported that 37-85% of encephalitis cases are of unknown etiology\textsuperscript{4}. This reveals the challenge of diagnosing encephalitis and treating effectively to minimize long-term neural deficits, or death.

Understanding the normal brain anatomy and physiology including the blood brain barrier, leukocyte trafficking and mechanisms by which pathogens gain access to the brain are important. Also, understanding immune suppression in terms of infection and how non-infectious encephalitis arises will aid diagnosis and treatment.

Histologic features of brain tissue

The central nervous system (CNS) is composed of the brain and spinal cord. The cell types in the brain include neurons and glial support cells. Neurons relay electrical signals through synapses to other neurons by converting the signal to a chemical “message”\textsuperscript{6}. The glial cells include astrocytes, oligodendrocytes and microglial cells. Oligodendrocytes wrap around neurons forming the myelin sheath aiding the speed of chemical transduction through the neuron\textsuperscript{6}. Microglial cells are important immune cells only found in the brain. Astrocytes are star-shaped cells important in almost all aspects of brain function\textsuperscript{6}.

Normal function of neurons demands tight regulation in regard to the passage of molecules into and out of the CNS. There are three sites at which the CNS limits and regulates molecular exchange: the blood-brain barrier, the choroid plexus epithelium and the arachnoid epithelium\textsuperscript{7}. Both the choroid plexus and arachnoid epithelium control exchange between the blood and the ventricular cerebral spinal fluid (CSF) and
subarachnoid CSF, respectively. Blood vessels are required for the essential delivery of oxygen and nutrients to tissues as well as removing metabolic waste and allowing communication with the immune system. The blood-brain barrier (BBB) consists of non-fenestrated capillaries allowing for the most selective route of entry for nutrients and molecules into the CNS. The BBB is formed by endothelial cells that line the cerebral capillaries, pericytes and astrocyte foot processes that form the glia limitans. The BBB allows for proper neuronal function, protects the CNS from pathogens, inflammation and injury.

Pericytes are cells embedded in the vascular basement membrane and extend their cellular processes spanning several endothelial cell bodies within the BBB. They regulate angiogenesis, wound healing, immune cell infiltration and help to maintain a tight barrier from the CNS and external blood.

Astrocytes are derived from the developing neural tube. Astrocytes release a variety of agents such as cytokines like tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), which modulate the brain endothelial permeability allowing immune cells to cross the BBB into the brain. In response to proinflammatory cytokines, astrocytes can promote the downregulation of tight junction components in the BBB. Through production of several diffusible molecules (e.g. Shh and angiotensin II) astrocytes have important functions in up-regulating the BBB and initiating the expression of polarized transporters. Astrocyte foot processes are closely associated with the endothelial cells of the BBB forming the glia limitans. These reactive astrocytes may allow the entrance of pathogens leading to additional inflammation and pathology. A non-specific astrocytic reaction is typically seen at the site of viral-induced damage but, its
distribution in patches is particularly characteristic of encephalitis\textsuperscript{11}. Reactive astrocytic change may be visualized with immunostaining using an antibody specific to glial fibrillary acidic protein (GFAP) demonstrating the enlarged nucleus and the extendend astrocyte processes\textsuperscript{11}.

Immune cells and the brain

The two types of immune cells within the brain are perivascular macrophages/pericytes and microglial cells. Perivascular macrophages are derived from a monocyte lineage and are able to cross the BBB in order to phagocytose cellular debris\textsuperscript{8}. Microglial cells are resident CNS parenchymal immune cells that are involved in neuronal development, innate immunity and may also act as antigen presenting cells (APCs) during an adaptive response\textsuperscript{8}. Microglial cells are the first cells to respond to invasion and are attracted to the site of injury through the recognition of Toll-like receptors (TLRs) leading to the release of cytokines including TNF-α\textsuperscript{12}. Their migration and release of cytotoxic mediators are required to kill microbes. The role of microglial cells in CNS disease has been extensively studied including stroke and multiple sclerosis\textsuperscript{12}. Microglial reaction is detected by HLA-DR immunostain as evidence of brain irritation or as a reaction to stimulus/injury. HLA-DR is a type of MHC class II receptor utilized for antigen presentation to the T cell receptor\textsuperscript{13}. Diffuse upregulation is likely reflection of cytokines from the systemic circulation in cases of disease or possibly even illness stress.

As a function of normal surveillance, immune system regulation is required to clear potential infections of the brain. In order to gain access to the CNS, immune cells are
recruited into the microvessels and pass through the BBB. Leukocyte recruitment is a complex process requiring the signalling and coordination of multiple steps: tethering, rolling, arrest and trans-endothelial migration\textsuperscript{14}. The endothelial cells express adhesion molecules (for example, P-selectin or VCAM-1) which tether the immune cell and roll it along the cellular surface with the aid of selectins. Once the cells reach a spot of interest, integrins mediate the arrest and chemokines orchestrate cell passage through cell junctions\textsuperscript{14}. Once inside the perivascular space, inflammatory cells penetrate the glia limitans to enter the brain parenchyma\textsuperscript{15}. The glia limitans is composed of astrocyte foot processes in the basement membrane. The mechanisms concerning migration across this barrier is not well known\textsuperscript{15}. One study has suggested that matrix metalloproteases may be involved in leukocyte migration across the glia limitans\textsuperscript{16}.

Leukocyte influx into the brain is a defining feature of viral encephalitis which warrants viral clearance and resolution of infection. Infiltrating leukocytes may paradoxically contribute to a more severe outcome destructing normal surrounding neuronal cells while clearing the virus\textsuperscript{17}. In ischemic stroke, neutrophils, macrophages and natural killer (NK) cells are the earliest responders in acute inflammation\textsuperscript{18}. Neutrophils outnumber other leukocytes in early acute ischemic lesions reaching a maximum infiltrate after 3 days and may contribute to infarct development\textsuperscript{18}. Higher numbers of neutrophils have been associated with BBB breakdown and brain injury in an ischemic stroke model\textsuperscript{19}.

The immune system has limited access to the brain due to its tight regulation of molecular passage. Under normal conditions, the brain lacks the same degree of surveillance from circulating lymphocytes compared to other extracerebral tissues\textsuperscript{20}. 

This is attributed to the physical nature of the BBB, paucity of dendritic cells, low major histocompatibility complex (MHC) expression and lack of lymphatic drainage in the brain\textsuperscript{15}. Despite the absence of typical immune features and a lymphatic network, life-threatening CNS immune responses can occur during infection with encephalitic pathogens, especially viruses\textsuperscript{17}.

Monocytes are recruited to the CNS in human and animal \textit{in vitro} models of viral encephalitis including Human immunodeficiency virus (HIV), simian immunodeficiency virus and West Nile virus (WNV) encephalitis\textsuperscript{15}. \textit{In vitro} data suggests that the chemokine CCL2 (Monocyte chemoattractant protein-1 (MCP-1)) is essential for monocyte recruitment and enhances peripheral lymphocytes in HIV-infected patients to cross the BBB\textsuperscript{15}. Once in the brain these monocytes will differentiate into perivascular macrophages and help contribute to the neuroimmune response along with microglial cells. CCL2 also attracts T cells and NK cells by binding to CCR2 (chemokine receptor) present on T cells\textsuperscript{15}. Michlmayr et al (2014), showed that the chemokine expression patterns during viral encephalitis are similar regardless of the pathogen\textsuperscript{17}. Also, the more virulent viral strains were associated with more-rapid induction of inflammatory cytokines and chemokines\textsuperscript{17}.

CD3 is an immunoglobulin protein complex expressed on most T cells which acts as a coreceptor to help activate cytotoxic and helper T cells in the presence of foreign antigen\textsuperscript{21}. CD4+ T cells have been suggested to migrate to the CNS crossing the choroid plexus in CSF. The cellular composition of CSF in patients without CNS inflammation is similar, containing CD4+ central-memory T lymphocytes\textsuperscript{22}. B lymphocytes normally migrate across the BBB during normal physiological conditions. In the presence of
intracerebral foreign antigen, specific B cells and plasma cells will accumulate in the brain parenchyma and antibodies are generated for antigen clearance\textsuperscript{23}. B cells are also present in CNS inflammatory states such as Multiple Sclerosis (MS) where they may contribute to CNS injury and/or protection\textsuperscript{23}. Circulating human B cells express the adhesion molecules VLA-4 (Very late antigen-4) and LFA-1 (Lymphocyte function-associated antigen-1). The counterreceptors VCAM-1 (Vascular cell adhesion molecule-1) and ICAM-1 (Intercellular adhesion molecule-1) on the BBB endothelial cells are up-regulated in MS lesions, at the sites of B cell infiltration\textsuperscript{23}. This suggests that B cells use adhesion molecules similar to how T lymphocytes cross the brain endothelium. Alter et al, (2003) showed that B cells have increased migration across the brain endothelium compared to T cells\textsuperscript{23}.

CNS Viral Infection

Pathogens require access to the brain to establish infection. Viruses utilize multiple mechanisms to enter the CNS such as hijacking immune cells, neuronal transport or through the olfactory pathway. HIV infects CD4\textsuperscript{+} T cells and enters the brain through the BBB\textsuperscript{24}. Retrograde axonal transport is a normal cellular process which moves materials from synapses to cell body and neuron to neuron. The best characterized viruses that utilize axonal transport are poliovirus, rabies virus, WNV and influenza H5N1\textsuperscript{24}. Herpes simplex virus (HSV), rabies virus, influenza A and Bornavirus are suggested to utilize the olfactory pathway\textsuperscript{25}. Despite the route of entry, once inside the CNS, viruses will infect various cell types, replicate and eventually viral burden will overwhelm the immune system and encephalitis, may arise.
Viruses that are classically associated with causing viral encephalitis are from the Herpesviridae family, HIV, polyoma virus and the Arboviruses. For the purpose of this study, the Herpesviridae family will include herpes simplex virus 1 and 2 (HSV-1/2) and cytomegalovirus (CMV). The arboviruses of interest are WNV, Saint Louis encephalitis virus (SLEV), Western equine encephalitis virus (WEEV) and Zika virus (ZIKV). Occasionally, common viruses that produce mild flu-like symptoms have the capability to cause encephalitis such as Influenza and Coxsackie viruses.

HSV is a dsDNA virus from the Herpesviridae family. Herpes simplex encephalitis (HSE) is caused by HSV-1 or 2 through human to human transmission and persists in a latent form within the trigeminal ganglion and the olfactory bulb. “HSE is the most frequent cause of viral encephalitis and it is recommended to initiate treatment with acyclovir when patients present with signs and symptoms of encephalitis even before receiving CSF results.” Human Herpesvirus-6 is suggested to utilize the olfactory pathway by infecting olfactory ensheathing cells (a type of glial cell) gaining access to the brain. Upon active infection the virus spreads to the brain through the meninges, extending to the temporal and inferior frontal lobes initially and then spreading to the remainder of the brain as the infection progresses. In advanced HSE, the brain shows diffuse softening and edema with hemorrhagic necrosis of the inferior frontal and temporal lobes. Microscopic examination reveals activated microglia, microglial nodules and intranuclear inclusions which are all diagnostic features of HSE. The inflammatory changes in the brain with HSE are more severe than any other viral encephalitis and can be detected by imaging. Previously, diagnosis of HSE required a brain biopsy with fluorescent antibody staining and microscopic study to detect
inclusion bodies and viral particles. Today HSV can be detected in CSF by reverse transcription polymerase chain reaction (RT-PCR)\textsuperscript{27}. Without treatment, the brain undergoes severe inflammation and necrosis leading to significant risk of morbidity and death\textsuperscript{28}.

Cytomegalovirus is a dsDNA virus from the Herpesviridae family. All Herpesviridae viruses express two glycoproteins, gB and gH/gL important for viral entry into cells\textsuperscript{29}. CMV almost exclusively affects highly immunocompromised patients with 85\% HIV-infected individuals\textsuperscript{2}. In patients infected with both CMV and HIV it is difficult to distinguish between CMV encephalitis and HIV encephalitis or HIV dementia\textsuperscript{26}. Infected neurons and glial cells enlarge and develop cytoplasmic and intranuclear inclusions and those areas can become necrotic\textsuperscript{27}. A pregnant mother who carries CMV may transmit to the fetus causing fetal CMV infection. If infection occurs before mid-gestation this may obstruct neural migration and cause microcephaly and cortical dysplasia\textsuperscript{27}. Diagnosis relies on CMV positive CSF PCR but, can also be detected using an association between neurologic symptoms, blood, urine or throat culture deemed positive for CMV\textsuperscript{2,30}.

HIV is a ssRNA virus belonging to the Retroviridae family. HIV infects CD4+ T helper cells using gp120 to bind CD4 and a coreceptor (CXCR4 or CCR5)\textsuperscript{31}. By targeting T helper cells, HIV affects the normal function of the immune system and thus the patient is vulnerable to opportunistic infections or various cancers\textsuperscript{32}. HIV is capable of reaching the CNS hidden within infected CD4+ T cells passing through the BBB\textsuperscript{11,32}. HIV infects astrocytes with little or no viral replication. Astrocyte dysfunction causes neighboring cells to die or behave abnormally which may
eventually cause clinical dementia affecting approximately 20% of HIV infected individuals\textsuperscript{32}. HIV targeted drugs do not penetrate the BBB and have no access to the brain thus, HIV survives hidden within the astrocytes. HIV testing is initially done using an enzyme-linked immunosorbent assay (ELISA) to detect viral antibodies and if there is a positive result, the patient is retested to confirm\textsuperscript{32}.

HIV encephalitis causes progressive memory loss, intellectual deterioration, behavioral changes and motor deficits\textsuperscript{27}. There are no hallmark macroscopic features of HIV encephalitis other than the generic symptoms of swelling, edema, diffuse myelin and vascular damage. The microscopic histological hallmark is the presence of multinucleated giant cells, which express macrophage immune markers that can be isolated by IHC\textsuperscript{11}. There is no viral production in either neurons or glial cells therefore brain damage is not directly due to the viral load rather, is caused by cytokines and neurotoxins produced by the activated monocytes and microglial cells\textsuperscript{27}.

Arboviruses utilize an insect vector like the \textit{Culex} species of mosquitos to transmit the virus to humans\textsuperscript{26}. Overall, they are less common in causing encephalitis compared to Herpes and other Enteroviruses. However, when patients recently traveled to an endemic area, certain arboviruses should be considered as a differential etiology.

WNV is a ssRNA virus from the \textit{Flaviviridae} family and is the most common arbovirus causing encephalitis in adults. One out of every 150 infected individuals, typically elderly patients will develop encephalitis during infection\textsuperscript{27}. Before 1999, WNV had never been detected in the Americas. A possible explanation is the increase of world travel and the likelihood that an infected person returns from an endemic country\textsuperscript{33}. In 2003, a total of 1388 cases of WNV and 14 deaths were reported in
Canada. In the same year, clusters of encephalitis were identified in the summer and autumn months suggesting a potential underlying arboviral etiology. In Manitoba, there were 143 cases in 2003, 58 cases in 2005 and 582 cases in 2007. The disease presentation can range from asymptomatic to mild flu-like systems with fever extending to a change in mental status and may progress to coma.

A recent study on Tick-borne flaviviruses suggests there is direct infection of the BBB endothelial cells to allow for viral entrance into the CNS without disrupting the BBB integrity. This mode of entry could be utilized with other flaviviruses to potentially cause encephalitis through an arboviral vector.

Diagnosis of WNV heavily relies on anti-WNV antibodies in the CSF or blood serum. Histopathological examination can show varying degrees of neuronal necrosis in the gray matter, microglial and leukocyte infiltrates and neuronal degeneration. IHC demonstrated viral antigens in neurons, neuronal processes and areas of necrosis within the brain while no viral antigens were present in other major organs.

Saint Louis encephalitis virus was one of the first arboviral infections in the USA before the outbreaks of WNV. SLEV is endemic in both South and North America and shows summer outbreaks in North America. Western Equine encephalitis virus is present on the western half of both North and South America. Like most arboviruses, infection is often asymptomatic and has the potential to cause large outbreaks in horses and humans which has been documented since the 1930s. However, this disease has become rare and no case has been reported in the USA since 1999. Powassan virus (POWV) is an emerging flavivirus transmitted by the blacklegged tick *Ixodes*
scapularis. Ticks harboring POWV have been detected in Nova Scotia, Ontario and Manitoba showing an increased risk for individuals who engage in outdoor activities. Illness can range from fever and flu-like illness to meningoencephalitis. Positive RT-PCR serology for POWV-specific Ig (immunoglobulin) G in serum is diagnostic. One study utilized metagenomic sequencing and detected the virus in CSF four weeks before the diagnosis was made by serology, suggesting the utility for this method for timely pathogen detection in severe encephalitis situations.

Zika virus is an emerging mosquito-borne Flavivirus (+ssRNA genome) initially isolated from a rhesus monkey in the Zika forest in Uganda, 1947. In 2015, there was a dramatic increase of infection in Brazil with 440,000 to 1.3 million cases of ZIKV reported. The viral envelope protein mediates host cell infection by fusing to the host cell membranes causing the release of nucleocapsid and viral ribonucleic acid (RNA) into the cytoplasm. Once inside the cell, genome replication initiates on the surface of the endoplasmic reticulum, rearranging the membranes causing a cytopathic effect. ZIKV replicates in multiple cell types including primary human fetal neural progenitors (immature neurons) as well as radial glial cells and mature neurons. Mlakar et al., (2016) reported a case of a terminated 32-week fetus with microcephaly, multiple calcifications in the brain and gross intrauterine growth retardation. No virus or pathologic changes were observed in any fetal organ except in the brain within this case study, suggesting a strong neurotropism for ZIKV.

The Polyomaviridae family consists of a nonenveloped dsDNA genome with multiple viruses including the BK virus, the JC virus and the simian virus 40 (SV40). Respiratory infections are very common in childhood and are usually asymptomatic or
minimally symptomatic with mild fever and upper respiratory symptoms. The virus remains latent in the kidneys or brain. Within immune compromised/AIDS (acquired immunodeficiency syndrome) patients the virus can undergo reactivation causing encephalitis, pneumonia, hemorrhagic cystitis and tubulointerstitial nephritis. Within a case report of BK virus, mortality occurred between a few days to 10 months of the onset of neurologic disease in patients that were immune compromised. Of those patients, BK virus was detected by CSF PCR analysis and there were detectable lesions in the brain.

Progressive multifocal leukoencephalopathy (PML) is a disease of the CNS caused by the destructive infection of oligodendrocytes by the JC virus. JC virus can localize within the CNS and undergo reactivation, with consequent lytic infection and death of oligodendrocytes. The initial symptoms include neuropsychological deficits, visual deficits and motor problems. The natural disease course is progressive and leads to death within months if the patient remains immunocompromised. JC virus is detected through CSF analysis or biopsied brain tissue. Due to the demyelinating nature of PML, there has been connective investigations with MS however, it is difficult to establish a sound correlation between the two.

Viruses that typically cause mild flu-like symptoms in a healthy individual still have the potential to develop into encephalitis as a complication. Two viruses that are documented are influenza virus and Coxsackie B virus. Influenza A/H1N1 virus can cause encephalitis lethargica (EL) which was an epidemic in the 1920’s affecting young individuals starting as a flulike illness and progressing into encephalitis. Patients displayed a variety of symptoms including psychosis, obsessive behavior, motor
restlessness, agitation and catatonia. The diagnosis relies on PCR of nasopharyngeal secretions in children and young adults\(^2\). Coxsackie B virus is a non-Polio Enterovirus that manifests as CNS infections in newborns\(^4\). The infection is typically self-limiting in a healthy adult. In newborns, \textit{in utero} transmission is suggested as there is often preceding maternal illness\(^4\). The infant often presents with seizures, lethargy and poor feeding but, interestingly can be followed by rapid improvement and benign outcome\(^4\).

Other pathogens causing encephalitis

When patients do not respond to anti-viral therapy, clinicians must then modify their differential diagnosis to include other infectious pathogens such as bacteria, parasites or fungi. \textit{Mycobacterium tuberculosis} was the second most frequently identified cause, after HSV for the etiology of encephalitis in France during 2007\(^5\). For \textit{Tuberculosis}, patients would ultimately be diagnosed with a Chest X-ray and Serology\(^5\). \textit{Listeria monocytogenes} is the second most common bacterial cause of encephalitis following \textit{Mycobacterium tuberculosis}\(^2\). \textit{Listeria} is not the most common cause of bacterial induced encephalitis but, it is associated with the highest lethality in patients with severe comorbidities\(^2\). The risk of \textit{Listeria} infections exponentially increases with age or occurs in severely immune deficient or pregnant patients causing CNS and brainstem damage\(^2\). \textit{Listeria} infection is validated with positive blood culture\(^5\).

Parasitic causes such as free-living amoeba encephalitis has been reported in both immunocompetent and compromised individuals with concomitant skin lesions\(^2\). The diagnosis is typically hard to establish, and stereotactic brain biopsy is often required
as well as positive serological tests. The typical brain lesion is a granulomatous amoebic encephalitis with the amoeba forming rings in the perivascular space of blood vessels\textsuperscript{2}. Parasites can also cross the BBB gaining access to the brain. \textit{Plasmodium Falciparum} is the most common cause of CNS infection among parasites causing cerebral malaria (CM)\textsuperscript{52}. \textit{P. falciparum} mostly affects children under five years of age and pregnant women\textsuperscript{52}. The infectious hallmark is the sequestration of infected red blood cells adhering to the vascular endothelium within the brain causing immediate hypoxia and ischemic injury ultimately leading to a coma\textsuperscript{52}.

Fungal infections of the CNS most frequently occur in immunocompromised patients with hematological disorders, stem cell transplantation, AIDS or after organ transplant\textsuperscript{53}. In HIV-infected patients, the typical fungus causing infections is \textit{Cryptococcus neoformans} but, is on the decline due to the introduction of HAART( highly active antiretroviral therapy)\textsuperscript{53}. \textit{Aspergillus} species affect severely immune compromised hosts from chemotherapy or corticosteroids\textsuperscript{53}. The diagnosis of fungal infections utilizes the culturing of CSF or cerebral/extracerebral tissue. Dependent on the underlying disease and the site of infection, the mortality of most CNS fungal infections remains in the 70\% range\textsuperscript{53}. It has been suggested that microglial activation and astrocyte proliferation is stronger in fungal encephalitis in comparison to bacterial meningitis\textsuperscript{53}.

Identification of organisms

The definite diagnosis is preferably made in a living patient using either serum or CSF samples for PCR diagnosis. Serology titers of IgG or IgM can be useful to quantify
the virus or to detect the intensity of infection. In the presence of fever and meningeal irritation, no time should be wasted to diagnose a potential life-threatening bacterial meningitis with a lumbar puncture. In the situation where, bacterial meningitis is ruled out, both CT and lumbar puncture should be performed to assess polymorphonuclear cells for presumed viral infection. Detecting levels of specific antibodies could imply that an immune response is in progress. “Thus, CSF PCR has become the diagnostic modality of choice in detecting viral infections.”

Magnetic resonance (MR) imaging can be useful to exclude other disease processes and in some situations, can suggest the type of encephalitis. In HSE, lesions are detected in the frontal-temporal region with stereotypical temporal lobe localization of cytotoxic edema. In WNV, the brain is grossly normal and the inflammatory infiltrate is focal and slight with the brain stem (medulla) and thalamus involved most consistently. In cases with PML, there may be microscopic areas of necrosis however, on MRI lesions do not show mass effects or edema. It is important to use a conjunction of diagnostic tests such as imaging, CSF PCR, or IHC to guarantee a complete workup for encephalitis.

Film Array ME (meningitis/encephalitis) panel is a rather new technique starting to enter clinical laboratories across the united states. With multiplex PCR, it can detect 14 different pathogens from patient CSF within 1 hour including 6 bacterium, 7 viruses and 1 fungi known to cause meningitis or encephalitis.

Electron microscopy (EM) is most commonly used for examination of biopsied tissues and autopsy samples from cases of encephalitis. The purpose of utilizing EM is to isolate specific known viral structures such as HSV, rabies virus or adenovirus.
However, due to small sample size of tissue that can be examined this way and the expensive nature of the test, it is an insensitive technique for demonstrating the presence of virus\textsuperscript{11}.

To identify organisms in the brain post-mortem there has to be a degree of suspicion and the utilization of a targeted test. Immunohistochemistry (IHC) is typically used for confirmation of Hematoxylin and eosin (H&E) findings or when there is clinical suspicion of a viral infection but no viral cytopathic effects are identified\textsuperscript{57}. IHC can be used for common viral entities such as Herpes, CMV, WNV, and polyoma/SV40. Without clinical suspicion at autopsy, only after histological examination shows features suggestive of infectious encephalitis, will viral investigation begin. Even with clinical suspicion, molecular testing can be quite costly and is often limited by resources such as funding or limited specific viral antibodies available. Years ago, antibodies were less specific for viral entities which lead to cross reactions of multiple viruses from a single family. Therefore, an uncertain diagnosis was made for the etiology. In the investigation of the WNV outbreak in New York in 1999, IHC was used initially to detect the virus in neurons, neuronal processes and in areas of necrosis\textsuperscript{38}. However, a recent study suggests that the use of IHC for diagnosis may be unnecessary in situations where clinical or histologic suspicion is absent\textsuperscript{57}. Alternatively, in situ hybridization (ISH) can be utilized to detect DNA in the brain post-mortem\textsuperscript{58}. RT-PCR can be a useful tool on Formalin-fixed paraffin embedded (FFPE) tissues in patients who die relatively soon after disease onset when previous serology may have been negative\textsuperscript{59}. RNA viruses are more transient in nature and their genome is relatively unstable compared to DNA. This can make detection of RNA
viruses’ difficult postmortem. Developments in PCR technologies may allow for increased detection and identification of viruses in cases of encephalitis which present to autopsy without a definite diagnosis, or sometimes even without clinical suspicion of encephalitis.

When a specific agent is targeted there may only be a narrow window of molecular diagnostic tests available. Due to limited resources in many hospitals, a full complete diagnostic workup may not be completed on every suspected case of mild or severe encephalitis. In situations when a full workup is done, conflicting results may indicate multiple viral entities. Hence, the vast majority of cases are diagnosed with an unknown etiology due to the lack of or nonspecific clinical investigation prior to death.

Autoimmune encephalitis and non-infectious encephalitis

Autoimmune encephalitis and paraneoplastic encephalitis are caused by an antibody specific for CNS antigens and the clinical presentation can mimic infectious encephalitis. Specific antibodies have been characterized to either target intracellular antigens (paraneoplastic) or extracellular epitopes (autoantibodies). Paraneoplastic encephalitis pertains to an antibody specific for an intracellular (onconeural) antigen which is almost always associated with cancer and the antibodies can be used as tumor markers. Paraneoplastic antibodies are not directly pathogenic however, the cytotoxic T cells target neurons causing neuronal death. Paraneoplastic encephalitis generally has a poor prognosis with irreversible neuronal death, has limited response to immunotherapy and relapse often occurs. Autoantibodies are specific for extracellular epitopes of ion channels, receptors (anti-NMDA-receptor) and other
proteins. The antibody is directly pathogenic leading to reversible effects on synaptic function with little neuronal death. Many patients have a good prognosis once the appropriate immunotherapy is initiated but, treatment can often take months. The mechanisms of NMDAR (N-methyl-D-aspartate receptor) antibodies have been extensively studied: the antibodies cross-link and internalize target receptors, depleting NMDAR’s from synapses. Anti-NMDAR encephalitis is the most frequent and best characterized autoimmune encephalitis which combines psychiatric disorders, seizures and consciousness disorders developing later. All antibodies are of the IgG class and can be detected through CSF analysis.

Acute disseminated encephalomyelitis (ADEM) or also referred to as post-infectious or post immunization encephalitis/encephalomyelitis is an immune-mediated inflammatory and demyelinating disorder of the CNS. It is primarily a pediatric disorder between 6-8 years of age. The hallmark is widespread white and gray matter inflammation and demyelination with predominating lymphocytes and monocytes. Patients experience cognitive deficits, ataxia, language disturbances and motor, sensory or visual deficits. Neuroimaging is critical for diagnosis of ADEM showing symmetric bilateral lesions, relative periventricular sparing and deep gray matter involvement.

There is an early manifestation of psychiatric symptoms, abnormal movements and seizures in autoimmune encephalitis compared to infectious causes which could aid etiology investigation. However, in order to exclude infectious causes, CSF is tested for viruses and bacteria, a part of the normal workup for suspected encephalitis. For example, patients with recent transplant, opportunistic infections such as CMV and
HSV should be considered. Patients with cancer could have specific paraneoplastic syndromes based on their tumor type.

Autoantibody testing is critical for the proper diagnosis of autoimmune encephalitis. Dubey et al, (2018) showed the prevalence and incidence of autoimmune encephalitis is comparable to infectious encephalitis and the detection is increased over time with advancing CSF analysis. NMDA-R and other cell surface antibodies are most sensitive and specific with CSF analysis and commercial tests are widely available. Anti-NMDAR encephalitis was identified >4 times more than HSV, WNV or VSV and was the leading cause within the study cohort. This suggests that anti-NMDAR or other autoimmune encephalitides should have a prominent place in the differential diagnosis in order to avoid unnecessary diagnostic and treatment costs. Brain imaging with autoimmune encephalitis may be normal or show an increased signal in the medial temporal lobes however, this pattern is similar to HSV encephalitis. Thus, brain imaging does not accurately distinguish between infectious and autoimmune encephalitis. Furthermore, a normal brain MRI does not exclude these causes indicating the importance for CSF testing. Distinguishing the etiology is essential for patients with autoimmune encephalitis due to their susceptibility for relapse thus, increasing their disease burden.

Role of immune suppression

The combination of immunological suppression associated with a primary disease and medically induced immune suppression after transplant results in a high incidence for opportunistic infections. Transplant associated neurological complications can
involves both the central or peripheral nervous systems and can occur acute post-transplant or even months to years later\textsuperscript{65}. The most characterized syndromes are seizures, meningitis, encephalitis, ischemic stroke and brain abscess\textsuperscript{65}. Infections of the brain or meninges account for 4-29\% of CNS lesions in organ transplant recipients which is associated with higher mortality\textsuperscript{65}. Autopsy studies suggest that there are even higher proportions of transplanted patients with incidental neurological injury which may not have been previously clinically recognized\textsuperscript{65}. Patients post-transplant receive a slew of immune suppressive drugs which may have direct neurotoxic affects or impair the CNS defenses against infectious or neoplastic processes. The characteristic infections are bacterial (\textit{Listeria monocytogenes} and \textit{Mycobacterium Tuberculosis}), viral (CMV, HSV, JC polyoma virus), fungal (\textit{Cryptococcus neoformans}, \textit{Aspergillus fumigatus} and \textit{Candida spp}) and parasitic (\textit{toxoplasma gondii})\textsuperscript{65}. In viral infection, the virus may be acquired from primary infection during immunosuppression, reactivation of previous infection or a transmission from an infected donor. Viral infection may depress immunologic function and has been linked to invasive fungal co-infections\textsuperscript{65}. Toxoplasmosis is seen most frequently in heart transplant recipients occurring three months after transplant with the CNS as the primary site of involvement\textsuperscript{65}. Neurological complications are less frequent in pediatric transplant recipients but, if they do occur, they are more severe and have a higher mortality rate\textsuperscript{65}. Definitive diagnosis is challenging due to low specificity from neuroimaging studies. Management of a post-transplant patient involves mitigating neurological injury by careful consideration of risk versus benefit ratio of immune suppressive drugs\textsuperscript{65}.

In Manitoba, there are approximately 10,000 deaths annually with around 1200
autopsies performed and from those cases, ~400 brains are examined by a Neuropathologist (90% medicolegal). In this study, we defined encephalitis as lymphocytic infiltration beyond the glia limitans into brain tissue with associated microglial activation, as demonstrated by immunohistochemistry.
RESEARCH HYPOTHESIS AND OBJECTIVES

I hypothesize that the majority of cases will be of unknown etiology, similar to the literature. Seasonal trends could suggest an etiology from the groups with no defined cause.

The research design is a retrospective review of pediatric and adult autopsy records with complete neuropathological examinations in Manitoba, Canada between 1998 to 2018. The research objectives are:

1. To identify cases with encephalitis defined by histopathologic and/or clinical criteria.

2. To determine if the encephalitis contributed significantly to the cause of death or was merely an incidental finding.

3. To determine if a causative agent had been identified by conventional means.

4. To establish trends in seasonality among those cases with no defined cause.
MATERIALS AND METHODS

Patient information

Approval for the project was granted by the University of Manitoba Research Ethics Board (protocol # HS23485/H2019:490 with previous protocol # HS12914/ H2011:051).

Autopsy records (~1200 full autopsies per year) at the Health Sciences Centre (HSC), Pathology Department in Winnipeg, MB, Canada were reviewed from 1998 to 2018 inclusive. Patients and subjects from Manitoba as well as Northwestern Ontario were included in this study. Northwestern Ontario is included as per the Memorandum of Understanding between Ontario and Manitoba to provide access to Winnipeg acute care services. The number of brains subjected to comprehensive neuropathological examination per year ranged from 294 (2018) to 608 (1997) with an average of 391 (Figure 1). Using DocFetcher Software (SourceForge.net; open source software license from Eclipse Public License), a search was performed on neurosurgical and neuropathology autopsy records between 1998 and 2018 (7,786 cases). Search words included encephalitis, meningitis, viral and inflammation. Cases were included if there was a clinical or pathological diagnosis of definite encephalitis or meningoencephalitis (Table 1). Cases were excluded if there was a defined fungal or bacterial infection or, if there was granulomatous or neutrophilic inflammation, suggestive of a bacterial or fungal agent. Patient information was recorded: autopsy case number, region of residence, date of symptom onset and other pertinent health history, date of symptom onset and death, age, sex, immune suppression status, neuropathological findings at autopsy and the cause of death in autopsy report. There are five cases where the age is unknown. Seven cases there was no definite cause of death in the preliminary autopsy report.
Figure 1. Total brains saved at autopsy for complete neuropathology assessment in Manitoba from 1992-2018, within this study period 1998-2018 n=7786.
Table 1. *Inclusion and exclusion criteria for the selection of cases.*

<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
<th>Exclusion Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical diagnosis of viral encephalitis</td>
<td>Bacterial meningitis</td>
</tr>
<tr>
<td>Multiple foci/widespread perivascular inflammation</td>
<td>Fungal infections</td>
</tr>
<tr>
<td>Extravasated lymphocytes on H&amp;E/CD3 IHC stained slides</td>
<td>Neutrophilic or granulomatous inflammation</td>
</tr>
<tr>
<td>Meningoencephalitis</td>
<td>No evidence of inflammation</td>
</tr>
</tbody>
</table>
Tissue fixation and immunohistochemistry

Brains removed at autopsy were fixed intact in 10% neutral buffered formalin (NBF) for 7-21 days. Brains were examined, sliced, and photographed by a neuropathologist. After examination, selected tissue pieces were dehydrated and embedded in paraffin wax. Tissue sections (5 mm thick) were stained with H&E. On cases with meningeal, perivascular, or parenchymal inflammation, Hucker-Conn Gram stain and / or Grocott methenamine silver stain were done to identify bacteria or fungi respectively. If none was identified, and if appropriate based upon the case history or histologic features, immunostains were done to detect CMV, HSV, or JC virus. To characterize the inflammation, immunohistochemistry was performed using antibodies to CD3 (T lymphocytes), CD4 (helper T cells), CD8 (cytotoxic T cells), and HLA-DR (activated microglia) (Table 2). Typically, this would be done on at least 4 sections including medulla oblongata (whether or not inflammation was apparent at that site). After dewaxing and rehydration, antigen retrieval was performed in a Bull’s Eye Decloaking chamber (BioCare Medical, Concord, CA) for 1 minute at 125°C using Dako pH9 retrieval solution. All antibodies were detected using the Dako Envision system (Dako) and diaminobenzidine precipitation solution.

In this study, we defined encephalitis as lymphocytic infiltration beyond the glia limitans into brain tissue with associated microglial activation, as demonstrated by immunohistochemistry.
Table 2.

*Antibodies utilized for Immunohistochemistry staining.*

<table>
<thead>
<tr>
<th>Primary Antibody Target</th>
<th>Host Species</th>
<th>Clone</th>
<th>Manufacturer/Catalogue #</th>
<th>Dilution Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Dako/GA503</td>
<td>1:200</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Mouse</td>
<td>CR3/43</td>
<td>Dako/F0817</td>
<td>1:200</td>
</tr>
<tr>
<td>CD4</td>
<td>Mouse</td>
<td>4B12</td>
<td>Dako/M7310</td>
<td>1:200</td>
</tr>
<tr>
<td>CD8</td>
<td>Mouse</td>
<td>4B11</td>
<td>Leica/PA0183</td>
<td>1:100</td>
</tr>
<tr>
<td>Herpes Simplex</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Cell Marque/ 361A-15-ASR</td>
<td>1:500</td>
</tr>
<tr>
<td>CMV</td>
<td>Mouse</td>
<td>CCH2+DDG9</td>
<td>Dako/IS752</td>
<td>1:100</td>
</tr>
<tr>
<td>SV40</td>
<td>Mouse</td>
<td>PAb416</td>
<td>Calbiochem/DP02-200UG</td>
<td>1:750</td>
</tr>
</tbody>
</table>
Neuropathology assessment

Slides were viewed using a microscope (Olympus BX51 upright microscope, Olympus America Inc. Melville, NY). Cases of encephalitis accompanied by patchy and scant predominately lymphocytic inflammation in the subarachnoid compartment fall under the term of meningoencephalitis. To address research objectives #1 and #2, the following steps were utilized to categorize the cases into their prospective groups (Figure 2). Cases with perivascular or leptomeningeal lymphocyte aggregates on H&E were subjected to IHC (CD3, HLA-DR or specific viral immunostains). If the T lymphocytes were confined to the glia limitans and the HLA-DR showed no evidence of adjacent microglial activation the conclusion was “perivascular inflammation of uncertain significance” and these cases were excluded.

Comparing the normal brain, a brain with extravasated lymphocytes and a brain with encephalitis shows histological differences (Figure 3). Within the normal brain HLA-DR stained cells surround vessels and CD3 lymphocytes are within blood vessel lumens only. Extravasated lymphocytes are confined to the glia limitans usually with diffuse microglial HLA-DR activation. A brain with encephalitis, infiltrating T lymphocytes extend beyond the glia limitans and invade the brain tissue with anatomically corresponding exacerbated HLA-DR activation (Figure 3). Microglial reaction doesn’t always indicate encephalitis, diffuse immunoreactivity simply reflects systemic illness. Focal increase in immunoreactivity corresponding to CD3 suggests local brain irritation at a site of lymphocytic inflammation.

If there are greater than two perivascular collections or the microglia is activated, then multiple slides are examined to determine the anatomical distribution and/or magnitude of the reactive inflammatory response. In this situation, further stains could be used for the
detection of Herpes, CMV or SV40 (polyomavirus). With positive IHC and a specific viral entity identified, the conclusion was “known viral cause of encephalitis”. With negative IHC results, two options were possible. If there is histological encephalitis with negative viral IHC and no obvious non-CNS cause of death, the conclusion was “possible viral encephalitis contributing to death” (Figure 2). If there was a non-CNS cause of death and concurrent mild encephalitis the conclusion was “possible viral encephalitis as an incidental finding”. This anatomical distribution could be associated with CNS dysfunction. In the rare cases where perineuronal microglial nodules was the predominant feature, the conclusion was “cannot be certain if this is viral or autoimmune attack”. A single case of cerebral arteritis in the context of prior cocaine exposure was encountered; it is unclear if this represents autoimmunity triggered by levamisole or primary cerebral vasculitis.
Figure 2. Steps for identifying and categorizing cases with the utilization of IHC.

Autopsy Performed

Brain saved

Exclude:
- No evidence of inflammatory/infectious process
- Fungal or bacterial (including TB) cases
- Neutrophilic or granulomatous inflammation

Perivascular or leptomeningeal lymphocyte aggregates on H&E

CD3 and HLA-DR IHC

CD3 confined to glia limitans and HLA-DR negative

“perivascular inflammation of uncertain significance”

>2 slides of perivascular aggregates or HLA-DR positive

Herpes, CMV, SV40.. etc.

Positive IHC

Clinical diagnosis confirmed-positive viral results

“Known viral cause of encephalitis”

Negative IHC

Obvious non-CNS cause of death

“Possible viral encephalitis as an incidental finding”

Histological encephalitis with negative viral IHC with no obvious non-CNS cause of death

“Possible viral encephalitis contributing to death”
Figure 3. Histology images showing how encephalitis was identified and defined.
The neuropathologist has a significant role in assessing the brain pathology and suggesting a cause of death however, they do not assign the cause or manner of death. This important distinction is ultimately done by the forensic pathologist and the medical examiner.

Data processing
   Data were entered into Microsoft Excel 2018 Version 16.20 (181208) for graphing and analysis.
RESULTS
Neuropathology Findings

Known viral cause of encephalitis cases include cases that are clinically diagnosed prior to death or there is detection of viral DNA through IHC or ISH post-mortem. These cases contain multiple foci or widespread perivascular inflammation. Eight patients had a history of fever or headache in the few days before death suggesting a viral/infectious prodrome. For HSV cases, perivascular lymphoid inflammation was present in the cerebral cortex and white matter and the IHC is positive for HLA-DR, CD3 and CD4/CD8 T cells. IHC is also positive in the neurons and glial cells of the white matter for one or both HSV 1/2. In some cases, ISH was used to detect HSV DNA. The basal nuclei may show perivascular inflammation and the parietal cortex, midbrain and medulla can show focal necrosis, dying neurons or multinucleate giant cells. In one situation, the patients lumbar puncture contained a high lymphocyte count and high protein in conjunction with positive IgM for WNV on blood serology. On histology, there were diffuse activation of microglial cells and dying neurons in the cerebellum and cerebral edema. The diagnosis was deemed mild encephalitis possibly due to WNV, due to negative immunostaining. For WNV, typically there is widespread perivascular cuffing and inflammatory clusters.

Possible viral encephalitis contributing to death is a group of patients with no known cause and in most situations is assumed viral. In general, there are multiple foci or widespread perivascular inflammation and microglial nodule formation. IHC is positive for HLA-DR and CD3 for widespread microglial and T cell activation. Often the bacterial and fungal stains are negative and thus, the “cause” is assumed viral. However, these cases all test negative for IHC viruses.
Possible mild encephalitis as an incidental finding has similar histology findings, only milder. There may only be scattered foci of perivascular cuffing and only minimal areas of microglial activation. This degree of inflammation is enough to suggest the encephalitis was incidental and not the contributing cause of death.

Histological examination revealed that in nine cases the lymphocytic infiltrate and microglial reaction was identified only within the brainstem or within the basal ganglia. In two of the cases the inflammation was significant enough within the brainstem to be the probable cause of death. This highlights the importance of multiple samples being taken from cases where no other cause of death is identified at autopsy. Limited sampling would likely have missed the inflammation; with more extensive sampling, including brainstem and cerebellum at brain cutting, the cause of death may be identified.

There were no cases of cortex predominant inflammation and perineuronal microglial clusters that would be strongly suggested of an autoimmune attack on neurons. However, autoimmune remains a possibility in some cases.

Demographics

Within the 21-year period (1998-2018), the total number of brains examined was 7,786 of which 114 cases were included in this study of encephalitis (Figure 4). The ages range from neonatal (0 years) to 86 years old (Figure 5). For specific viral cause 7/18 and 4/18 cases were from the 0-10 and 60-70 age groups, respectively. Two cases had an unknown age from the known viral encephalitis group. For possible viral encephalitis contributing to death there was a peak in both the 0-10 and 40-50 age groups with 9 and 7 cases,
respectively. For incidental encephalitis there are similar peaks in the 0-10, 40-50 and 60-70 age groups with 13, 7 and 7 cases, respectively (Figure 5).

For all groups, males are more affected than females. For yearly distribution, there were peaks in 1999 and 2010 with 12 cases in each year. Between 2013 and 2016 there were up to two cases per year, with zero cases in 2015 (Figure 6). The geographic breakdown of patient residence within this study was primarily from Winnipeg with a minority of cases from Thompson, Steinbach, The Pas and Flin Flon.
Figure 4. Total case distribution, n=114.

- Known Viral Cause of Encephalitis: 20/114 (17%)
- Possible Viral Encephalitis Contributing Death: 58/114 (51%)
- Possible Viral Encephalitis as an Incidental Finding: 36/114 (32%)
Figure 5. Age distribution for total cases, n=109.
Figure 6. Yearly distribution of total cases from 1998-2018, n=114.

- Possible viral incidental encephalitis
- Possible viral encephalitis contributing to death
- Known Viral
Definite viral cases

Twenty cases were of definite viral etiology. The two most common entities were HSV and Polyoma virus causing PML both with 6 cases each. WNV was the next common with 3 out of 20 cases (Table 3). The WNV cases occurred in September 2003, August 2005, 2007; this period corresponded to the early spread of WNV across North America. Four patients had immune deficiencies including HIV, multiple myeloma diagnosed with HSV, esophageal cancer and one with an unknown immune deficiency later infected with CMV.

The monthly distribution of the cases with known viral cause of encephalitis including WNV, Coxsackie B virus and Echovirus were clustered in the late summer to fall (Table 3).

Three out of the six HSV cases were diagnosed pre-mortem with positive CSF-PCR and/or brain culture. The two cases of CMV were diagnosed ante-mortem in an immunocompromised patient. The three cases of WNV were diagnosed pre-mortem with IgM positivity although, in these cases the clinical suspicion was high prior to death.

There were 8 neonatal cases with encephalitis, one case with Coxsackie B virus and one with HSV. In the remainder of the cases, non-specific probable / possible viral changes were observed on microscopic examination.
Table 3.
Specific viral causes of encephalitis identified post or ante-mortem, n=20.

<table>
<thead>
<tr>
<th>Virus</th>
<th># of cases</th>
<th>Age</th>
<th>Month of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV</td>
<td>6</td>
<td>39</td>
<td>Feb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>Feb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>Feb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>85</td>
<td>May</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19</td>
<td>Jul</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>Oct</td>
</tr>
<tr>
<td>PML</td>
<td>6</td>
<td>79</td>
<td>Feb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75</td>
<td>Apr</td>
</tr>
<tr>
<td></td>
<td></td>
<td>67</td>
<td>Jun</td>
</tr>
<tr>
<td></td>
<td></td>
<td>64</td>
<td>Sept</td>
</tr>
<tr>
<td></td>
<td></td>
<td>67</td>
<td>Nov</td>
</tr>
<tr>
<td></td>
<td></td>
<td>?</td>
<td>Dec</td>
</tr>
<tr>
<td>WNV</td>
<td>3</td>
<td>?</td>
<td>Aug</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>Aug</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68</td>
<td>Sept</td>
</tr>
<tr>
<td>CMV</td>
<td>2</td>
<td>&lt;1</td>
<td>Feb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;1</td>
<td>Mar</td>
</tr>
<tr>
<td>Coxsackie</td>
<td>1</td>
<td>&lt;1</td>
<td>Oct</td>
</tr>
<tr>
<td>HIV</td>
<td>1</td>
<td>31</td>
<td>July</td>
</tr>
<tr>
<td>Echovirus</td>
<td>1</td>
<td>9</td>
<td>Aug</td>
</tr>
</tbody>
</table>
Suspected viral cases

In 36 cases there was possible viral encephalitis severe enough to contribute to the death with no definite organism identified. In these cases, patients displayed a range of symptoms including headaches, neurologic decline, seizures, various infections or confusion prior to death. An additional 58 incidental cases of encephalitis were found at autopsy with mild or moderate inflammation thought not to be significant enough to cause death or in cases with an obvious cause of death. In these cases, examples of death were trauma, seizure disorders, hanging, pneumonia, and coronary artery disease. Since 2010, cases of sudden infant death syndrome (SIDS) are labeled as SUDI (sudden unexpected death of an infant) with an undetermined manner of death. Possible viral infection is always a consideration, especially if inflammation is identified in the brain tissue.

Of the 94 cases with suspected viral encephalitis, 10 patients had documented prodromal symptoms prior to death including headache, unwell the evening before, seizures, confusion and neurological decline. From the encephalitis contributing to death group, there were co-existing neoplastic conditions such as a grade 4 glioblastoma and a gangliocytoma with epilepsy. Two patients from the incidental encephalitis group had diffuse grade 3 astrocytoma’s.

The monthly distribution for possible viral encephalitis contributing to death shows a bimodal distribution with a peak in May and a second peak extending from July to October (Figure 7). Sixty-one percent of possible viral encephalitis contributing to death cases occurred in May and from July to October. Incidental cases of possible mild viral encephalitis were similar with a peak in May and a larger peak extending from July to
November. Seventy-one percent of incidental encephalitis cases occurring during May and July to November (Figure 7).
Figure 7. Monthly distribution of possible viral encephalitis, n=93.
DISCUSSION

Our analysis of encephalitis in Manitoba, Canada is the first study of its kind to utilize provincial autopsy-derived data. This study is reliant on the brain tissue saved at the time of autopsy and the retrospective review of cases between 1998 and 2018. Of the 114 cases between the ages of 0 and 86 years, 20 were of known viral cause, 36 were of possible viral encephalitis contributing to death and 58 were of possible mild viral encephalitis as an incidental finding. Overall, males were affected more than females, between the ages of 0-10, 40-50 and 60-70 years of age. However, we do not have the specific data on total numbers of autopsies performed on males/females in the study period. It is unclear whether males are truly more affected than females without absolute autopsy numbers. There were peaks of encephalitis cases in 1999 and 2010. Temporal distribution amongst the three groups showed bimodal distribution with peaks in May and from July to October which could suggest an arboviral etiology.

Previous encephalitis epidemiology

The epidemiology of encephalitis has previously been studied in other geographic regions using different types of data. Encephalitis in Ontario, Canada spanning the years 2002 to 2013\(^4\) was studied using an administrative hospital discharge abstract database. The estimated incidence of all cases of encephalitis was ~4.3 cases/100,000 persons/year. “The incidence rates for infants <1 years of age and adults ≥65 years were 3.9 and 3.0 times than adults between 20-44 years of age, respectively”\(^4\). In the Ontario study, there were peaks during August and September from proven viral encephalitis and suspected viral
encephalitis with unknown cause. Males were more frequently affected than females in all age groups except the 1-4 age group\(^4\).

Another study, conducted in England from 2005-2009 investigated the incidence of infectious and non-infectious encephalitis using hospitalization data\(^68\). The incidence was estimated 4.32-8.66 cases/100,000/ year. Similar to the Ontario study, incidence was highest in patients less than 1 years of age and \(\geq 65\) years. Possible misclassification of encephalitis is a major limitation while using administrative databases. For example, some bacterial causes are classified as meningoencephalitis which could be coded as meningitis in the database rather than encephalitis\(^68\). This could represent the underdiagnoses of encephalitis when there is a bacterial cause.

As well as overall incidence studies of encephalitis, there are data concerning specific encephalitis etiologies. HSV incidence, morbidity and mortality in Sweden between 1990-2001\(^69\) was studied using national inpatient register and diagnostic data from the virus laboratories. 638 hospitalized patients were given a primary diagnosis with HSE. Of those 638 patients, 236 were lab verified HSV-1 infection. There was a higher incidence in the summer months and approximately equal distribution amongst males and females. The highest incidence was in the 75 year age group; the median age for males and females was 62 and 66, respectively\(^69\). Morbidities after HSE included epilepsy, other infections and neuropsychiatric conditions.

Interpretation of findings

In this study, we defined encephalitis as lymphocytic infiltration beyond the glia limitans into brain tissue with associated microglial activation, as demonstrated by
immunohistochemistry. The utilization of IHC was essential in our study to demonstrate a definite difference in severity and extent of inflammation, which is not always obvious on H&E stained slides.

Seasonal variation suggestive of environmental factors

Manitoba’s climate ranges from subarctic temperatures (<-30°C in winter) to dry heat (>30°C in summer). With these seasonal changes, disease carrying insect vectors are a prominent problem. In Canada, the summer and early fall months is the opportune time for WNV transmission during mosquito reproduction. WNV is the only arbovirus under national surveillance, raising the potential for the underdiagnosis of other arboviral infections, which may contribute to an unrecognized burden of encephalitis in Canada.

In our study, the similar trends for monthly distribution of known viral and possible viral encephalitis contributing to death may suggest an arboviral etiology, supporting our hypothesis. The peaks in August and September of known WNV encephalitis, could be correlated to the suspected viral cases. Classifying these temporal patterns may aid in future efforts to determine etiology, which is by far one of the most difficult aspects of investigating encephalitis. If clinicians suspect an arboviral entity during the summer to fall months, they can quickly diagnose and treat the patient before morbidities occur.

Limitations

There was no access to patient health records especially, the pre-mortem blood leukocyte levels. This could be a hint at whether or not an infection was elicited prior to death and could give clinical indication to save the brain. It would be helpful to have patient
records versus pertinent patient history provided within the autopsy report alone. A major limitation in this study is the decline of brain examination post-mortem as compared to the previous years. Typically, brains are saved during an autopsy at the Forensic Pathologist’s discretion. Saving the brain can be hindered by normal decomposition, forensic evidence or simply no clinical indication for saving the brain. Fixing the brain intact is typically warranted by clinical suspicion at the time of death, such as symptoms that could indicate encephalitis. However, if there is the lack of clinical suspicion or the cause of the death is unrelated, many incidental cases of encephalitis are undetected.

In terms of testing brain tissue for IHC viral antibodies, this study is limited to the antibodies available for certain pathogens. We can only detect the viruses that have known detectable antibodies and have been clinically tested with adequate controls. For example, one of the cases displayed diffuse lymphocytes in the brain tissue with no positive bacterial stains. This patient had a clinical diagnosis of herpes zoster involving the ophthalmic and maxillary divisions of the left trigeminal nerve. In this case, there were no reliable IHC antibodies for Varicella zoster virus due to the lack of a positive control. The patient had clinical and histological signs of encephalitis caused by a known virus however, only a clinical diagnosis could be made. The pathological diagnosis was deemed “severe encephalitis likely caused by Varicella zoster virus”.

The cost of advanced tests required to isolate an etiology can be quite substantial and is not included in most budgets. The mean cost for laboratory tests are 210 USD ± standard deviation of 244 dollars with individual tests up to 359 dollars for viral detection70. Unless, the extensive testing is warranted for forensic analysis, priority often is low after the patient is deceased.
The interpretation of death can also be a limitation. For example, there could be a presumed cause of death but, concurrent severe encephalitis found at neuropathological assessment. This encephalitis could be the sole cause of death, a factor to the death or unrelated. This assessment could be interpreted differently amongst individual Pathologists and Medical Examiner investigators when determining the cause of the death for the final autopsy report.

We do not know the absolute numbers of autopsies performed on each sex (male or female) within the study period. Our data suggesting that males are more affected than females cannot be validated without absolute numbers of autopsies for each sex.

The absolute incidence of encephalitis is poorly represented when focusing on autopsy-based data alone. Many patients survive this debilitating infection, of which those cases are not included in this data set. Similarly, not all patients who die from encephalitis or meningitis receive an autopsy. The data is strictly based on autopsy extracted data from saved brain tissue.

Future Directions

As the cause of encephalitis is diverse including viral, autoimmune, paraneoplastic and others, all clinically suspected encephalitis should have thorough serological/CSF investigation to determine the possible cause. If the serology/CSF is negative for virus, further clinical investigations should be performed to rule out autoimmune or paraneoplastic causes of encephalitis.

The limitations of this study highlight the possibility for many future directions. On the technical side, the utilization of IHC for viral detection could have increased sensitivity
and a wider variety of antibodies to detect more viruses. Film Array ME panel could potentially be performed for research purposes to obtain an etiology post-mortem. If a patient is suspected to have encephalitis, a lumbar puncture is performed, and excess CSF could be saved for frozen for biobanking at -80°C. In the situation where the patient succumbs to the disease, an etiology could be identified post-mortem. However, this method only detects select bacteria, fungi and DNA viruses, excluding the RNA viruses entirely.

The data from this study is provincial; a similar study could expand to include every province or an overall federal database of encephalitis at autopsy, which currently does not exist. It could be useful to correlate in-patient data from hospital discharge databases with our autopsy-based data. This would allow the patient to be followed through diagnosis, treatment and then potentially death. This would help to establish trends in symptoms, treatments and etiology of suspected viral cases. The goal would be to group the data and allow clinicians to learn and hopefully, apply it to their treatment regimens with future patients.

Conclusions

To conclude, our study of encephalitis in Manitoba, Canada is the first study of its kind to utilize autopsy-based data. 114 cases out of the total 7,786 brains assessed within the study period were of known or possible viral encephalitis. Males are more affected than females in all groups between the ages of 0 to 86 years old. 58/114 (51%) cases are of incidental finding, which supports our hypothesis. This group displays mild inflammation that may be overlooked with inadequate sampling of the brain tissue. The temporal distribution with peaks in the late summer and early fall months is suggestive of a specific
transmission mode such as an arboviral vector, supporting our hypothesis. Patients with severe encephalitis contributing to death often have a history of viral infection. In spite of this history, definite diagnosis and identification of a viral etiology is uncommon, unless rigorous testing is performed. Histological examination of H&E and CD3 stained slides shows a definite difference in the apparent severity and extent of inflammation, highlighting the usefulness of IHC for diagnosing encephalitis. The lack of information in the literature regarding viral identification and encephalitis incidence at autopsy is quite apparent. The frequency and severity of encephalitis in this study highlights the importance of detecting and clinically treating encephalitis. The hope is that the information presented in this study will aid a clinician’s diagnosis leading to rapid and effective treatment ultimately, reducing encephalitis caused morbidity and mortality.
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