ETIOPATHOGENESIS STUDIES OF CANINE IDIOPATHIC MENINGOENCEPHALOMYELITIS

by

RENEE M. BARBER

(Under the Direction of Scott J. Schatzberg)

ABSTRACT

Numerous idiopathic inflammatory disorders of the canine central nervous system (CNS) have been described over the past several decades. These include specific histopathological entities such as granulomatous meningoencephalomyelitis (GME) and necrotizing meningoencephalitis (NME) as well as those that lack a specific histological diagnosis, collectively referred to as meningoencephalomyelitis of unknown etiology (MUE). These idiopathic CNS disorders comprise a group of clinically challenging diseases that frequently carry a poor prognosis despite aggressive treatment. Although the etiopathogeneses of GME, NME and MUE are poorly understood, environmental (e.g., CNS infection) and genetic factors are suspected to contribute to disease development. To elucidate possible infectious and genetic etiopathogenic mechanisms, molecular screening tools were employed for pathogen detection in cases of GME, NME and MUE and genome-wide association (GWA) of single nucleotide polymorphisms was performed on cases of NME. *Mycoplasma canis* and was identified as a candidate etiological agent for GME and NME and La Crosse virus (LACV) as a candidate agent for MUE, while members of the genera *Ehrlichia*, *Anaplasma*, *Rickettsia*, *Bartonella* and *Borrelia* and viral groups adenovirus, alphavirus, bornavirus, bunyavirus, coronavirus, enterovirus, flavivirus, herpesvirus,
paramyxovirus, parechovirus, polyomavirus and rhabdovirus species were determined to be infrequently associated with GME, NME and MUE. Additionally, GWA of Pug dogs with NME identified two loci strongly associated with disease development, including a 4.1 Mb region of dog leukocyte antigen class II. Although further research is needed to validate the role of \textit{M. canis} and LACV in these disorders, these results support previous theories of multifactorial etiopathogeneses, where both environmental triggers and genetic susceptibility play an important role in disease pathogenesis. Moreover, the genetic risk loci identified in cases of NME provide important preliminary data to support in depth genetic analysis of this disease and other idiopathic meningoencephalomyelitides in numerous affected breeds.

INDEX WORDS: Central nervous system, CNS, inflammation, Dog, Granulomatous meningoencephalomyelitis, GME, Necrotizing meningoencephalitis, NME, Meningoencephalomyelitis of unknown etiology, MUE
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To the loves of my life: Jamie, Joyce, E.C., Jack, Jilian, Hanah, Bailey, Bumble,

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CHAPTER 1
INTRODUCTION

Granulomatous meningoencephalomyelitis (GME), necrotizing meningoencephalitis (NME) and meningoencephalomyelitis of unknown etiology (MUE) are commonly recognized idiopathic inflammatory disorders of the canine central nervous system (CNS). Collectively termed canine idiopathic meningoencephalomyelitis, these disorders are associated with significant morbidity and mortality and as the term idiopathic suggests, their etiopathogeneses are poorly understood. Importantly, our lack of knowledge regarding their etiopathogeneses has precluded development of antemortem diagnostic tests, preventing effective treatment trials. Numerous etiopathogenic theories have been suggested for GME, NME and MUE, including autoimmunity, CNS infection and genetic predisposition but ultimately, a multifactorial etiopathogenesis, with contribution of genetic and environmental factors, is considered most likely. The aim of this research is to identify underlying causes of disease development and pathogenesis associated with GME, NME and MUE with the long-term goal of improving diagnosis, treatment and prognosis of these devastating disorders.

Infectious etiologies have long been considered for GME, NME and MUE. Vector-borne pathogens in the genera *Ehrlichia, Anaplasma, Rickettsia, Bartonella* and *Borrelia* all have been implicated in canine CNS infections and despite a lack of concrete evidence that these pathogens play a role in GME, NME or MUE, expensive serological tests for these organisms routinely are performed in patients presenting with clinical signs of idiopathic meningoencephalomyelitis. Additionally, based on strong clinical and histopathological
similarities to viral encephalitides in humans and dogs, veterinary pathologists and neurologists have long speculated about a viral cause for these disorders. Adeno-, borna-, herpes-, parvo-, retro-, West Nile, canine parainfluenza, encephalomyocarditis and La Crosse viruses all have been suggested.\textsuperscript{2,4,10,11,21-23} Although preliminary studies have failed to identify an obvious infectious etiology for GME, NME or MUE,\textsuperscript{10,11} a thorough investigation of these disorders to identify infectious agents has been critically lacking.

One goal of this investigation is to rigorously evaluate the CNS of dogs diagnosed with GME, NME and MUE for a wide variety of microorganisms to identify infectious agents potentially associated with these disorders. The specific hypothesis of the research described is that viral pathogens affecting the canine CNS are associated with the development of GME, NME and / or MUE. Since conventional laboratory diagnostics such as serology and culture are frequently insufficient for the diagnosis of CNS pathogens,\textsuperscript{24} we utilized highly sensitive molecular techniques including consensus, degenerate and consensus-degenerate hybrid PCR\textsuperscript{25} and sequence-independent, single primer amplification\textsuperscript{26} to address the following specific aims:

\textit{Specific Aim 1}: To determine if common canine vector-borne bacterial pathogens can be detected in the CNS of dogs with GME, NME and / or MUE. The working hypothesis is that \textit{Ehrlichia}, \textit{Anaplasma}, Spotted Fever Group \textit{Rickettsia}, \textit{Bartonella} and \textit{Borrelia} species are uncommon causes of idiopathic meningoencephalomyelitis in dogs.

\textit{Specific Aim 2}: To determine if viruses can be detected in the brain tissue of dogs with GME and NME. The working hypothesis is that viral antigens in the CNS are associated with the development of GME and NME.
**Specific Aim 3:** To determine if viruses can be detected in the cerebrospinal fluid of dogs with MUE. The working hypothesis is that viral antigens in the CNS are associated with the development of MUE.

A second goal of this investigation is to identify genetic risk factors associated with the development of NME in Pug dogs. GME, NME and MUE are overrepresented in small breed dogs, indicating that genetic susceptibility contributes to disease development and recently, NME was shown to be associated with certain dog leukocyte antigen class II (DLA II) haplotypes in Pug dogs. However, NME is inherited in a non-Mendelian fashion, suggesting that multiple genetic and/or environmental factors contribute to disease phenotype, and the aforementioned study only utilized 752 markers across the genome, which may have precluded the identification of additional risk loci.

The specific hypothesis of this research is that NME demonstrates polygenic inheritance within the Pug breed with specific genetic variants leading to an altered risk for disease development. To evaluate this hypothesis, we utilized genome-wide association of > 170,000 single nucleotide polymorphisms (SNPs) to address the following specific aim:

**Specific Aim 1:** To identify genetic susceptibility loci in Pug dogs with NME through genome-wide SNP association. The working hypothesis is that multiple genetic loci contribute to NME inheritance in Pug dogs.

These hypotheses and specific aims are designed to further elucidate the etiopathogeneses of canine idiopathic meningoencephalomyelitis (specifically GME, NME and MUE) with the broad goal of improving disease outcome. GME, NME and MUE largely have been assumed to be autoimmune in nature based on steroid-responsiveness, although there are numerous reports suggesting that the prognosis for infectious meningoencephalitis in humans may worsen without
the use of corticosteroids.\textsuperscript{29,30} Determining whether or not infectious pathogens play a role in these disorders is a critical step in understanding disease pathogenesis, developing targeted therapy and working towards preventative measures such as vaccines. Additionally, it is important to validate the hypothesis that vector-transmitted bacterial pathogens are uncommon causes of meningoencephalomyelitis in dogs to allow clinicians to avoid expensive diagnostic tests for these organisms in non-endemic regions. Finally, identification of genetic mutations involved in NME development will increase our ability to identify at risk dogs, improve antemortem diagnosis and allow for development and institution of safe and efficacious treatment modalities. Moreover, this knowledge could help in the identification of genetic factors that are associated with the development of clinically similar, non-prototypical forms of multiple sclerosis in people and will provide the foundation for analogous genetic studies in purebred dogs affected by other variants of idiopathic meningoencephalomyelitis (eg GME, necrotizing leukoencephalitis).

REFERENCES


2.1 CANINE IDIOPATHIC MENINGOENCEPHALOMYELITIS

2.1.1 Background

Central nervous system (CNS) inflammation may affect the brain parenchyma (encephalitis), spinal cord (myelitis), leptomeninges (meningitis) or some combination of these. The inclusive term meningoencephalomyelitis often is used to describe CNS inflammation in one or more of these regions that can arise from infection, toxin exposure, cellular damage, neoplasia or autoimmune disease. When an underlying cause cannot be identified, the terminology idiopathic meningoencephalomyelitis may be used. In dogs, idiopathic meningoencephalomyelitis encompasses numerous inflammatory disorders with unknown etiology that can be broadly classified into three categories: presumptively-diagnosed, histopathologically-diagnosed and clinically-diagnosed. Presumptively-diagnosed idiopathic meningoencephalomyelitides include antemortem cases that lack a definitive clinical, histopathological, cytological, serological or polymerase chain reaction (PCR) diagnosis, collectively referred to as meningoencephalomyelitis of unknown etiology (MUE). Histopathologically-diagnosed idiopathic meningoencephalomyelitides include disorders that are defined by unique histopathological features, such as granulomatous meningoencephalomyelitis (GME), necrotizing meningoencephalitis (NME) and necrotizing leukoencephalitis (NLE). These disorders require postmortem histopathological confirmation for definitive diagnosis and
are classified as MUE until this confirmation is acquired. Finally, clinically-diagnosed idiopathic meningoencephalomyelitides include disorders such as steroid-responsive meningitis-arteritis and idiopathic tremor syndrome that are defined by unique clinical and diagnostic features. These disorders typically can be differentiated from GME, NME, NLE and MUE on an antemortem basis without the requirement of a histopathological diagnosis.

The work presented here focuses on GME, NME and MUE. A brief review of these disorders and what is known about their etiopathogeneses follows. It should be noted that since very little is known about the etiopathogeneses of these disorders, the theories presented rely heavily on speculations from the literature, with supporting information presented when available. Additionally, because MUE represents a spectrum of diseases, including but not limited to GME and NME, much of etiopathogenic information available comes from studies of GME and NME but not MUE.

2.1.2 Granulomatous meningoencephalomyelitis

GME is a disorder defined by distinct patterns of CNS inflammation that occur in the absence of an identifiable underlying disease process. Inflammation associated with GME can have a diffuse, focal or ocular distribution. In diffuse disease, perivascular cuffs of macrophages, lymphocytes and plasma cells are present in the brainstem and frequently extend into the cerebrum and spinal cord. Cerebral lesions most often affect the white matter and less commonly the gray matter and leptomeninges. Epithelioid differentiation of macrophages results in discrete nests of cells within the perivascular cuff. In focal GME, a mass lesion in the cerebrum, cerebellum, brainstem, spinal cord or some combination of these forms as the result of similar, coalescing perivascular infiltrates. The same perivascular
infiltrates occur in ocular GME but primarily are located in the retinal and post-retinal portions of the optic nerve and chiasm.\textsuperscript{14,21}

Although GME is a histopathologically-defined disorder, a fair amount is known about its clinical features. GME is acute in onset, invariably progressive and associated clinical signs reflect the location and severity of inflammation within the CNS.\textsuperscript{7,11} The most common signs seen in diffuse and focal GME include seizures, depression, blindness, head tilt, circling, nystagmus, cervical pain, paresis, ataxia and proprioceptive deficits,\textsuperscript{6,7,11,13,17-19,22-24} while patients with ocular GME typically present with an acute onset of visual impairment\textsuperscript{17} but can develop additional CNS signs as the disease progresses.\textsuperscript{12,19,25-28} Treatment with immunosuppressive therapy is thought to markedly improve GME’s typically grave prognosis, although concrete information regarding GME prognosis is not available.\textsuperscript{5,13,14,19,29-31} Reported survival times range from one to greater than 1,215 days\textsuperscript{19} with disseminated disease having a significantly worse prognosis (median survival of 8 days) than focal disease (median survival of 114 days).\textsuperscript{19}

True epidemiological studies of GME are lacking, but information regarding distribution, prevalence and risk factors can be deduced from the literature. GME has been reported in numerous countries, including the United States, Australia, New Zealand, Japan and Europe\textsuperscript{7,11,24,32-34} and is thought to be relatively common, having been estimated to represent 5% to 25% of all canine neurological disorders\textsuperscript{12,18,35} and 53% of reported GME, NME, NLE and MUE cases.\textsuperscript{36} All ages and breeds can be affected by GME \textsuperscript{3,14} but it is seen most commonly in middle aged,\textsuperscript{19} female dogs\textsuperscript{36} of toy and terrier breeds.\textsuperscript{36}
2.1.3 Necrotizing meningoencephalitis

Similar to GME, NME is defined by unique patterns of CNS inflammation. Perivascular infiltrates of lymphocytes, plasma cells and macrophages predominate in the leptomeninges and extend into superficial cortex with varying degrees of accompanying cerebral necrosis.\(^6,8,14\) Although gray matter can be affected, inflammation and tissue necrosis predominate in the white matter.\(^14\) Reactive astroglialis also is present and extensive.\(^14\) Unlike GME, the brainstem and spinal cord are not commonly affected.\(^14,37\)

Clinically, NME is associated with an acute onset of progressive neurological signs.\(^6,8,14\) Seizures and mentation changes are common, likely because of disease predilection for the cerebrum and thalamus,\(^3,6,36,37\) but signs vary and often reflect the multifocal nature of the inflammatory lesions.\(^3,8,14\) Accurate studies regarding NME prognosis are not available, but it generally is accepted that NME is fatal without aggressive immunosuppressive therapy and carries a guarded to poor prognosis even with treatment.\(^6\) Reported mean survival times in dogs receiving corticosteroid monotherapy versus combination immunosuppressive therapy range from 58 to 97 days and 177 to 306, respectively.\(^38,39\)

Information regarding NME epidemiology can be gained from the literature. Similar to GME, NME can be assumed to have a global distribution, having been reported in the United States, Australia, Japan, Germany, France and Italy.\(^8,16,40-44\) A median age of onset of 18 months (range 4-113 months) has been reported and females typically are overrepresented.\(^39\) Historically referred to as Pug Dog Encephalitis due to its prevalence in this breed, NME has now been recognized in numerous small breeds, including the Pug, Maltese, Chihuahua, Shih Tzu, Lhasa Apso, Boston Terrier, Papillion, Pekingese, Pomeranian, Yorkshire Terrier and West Highland White Terrier\(^6,8,37,43-49\) and in order to avoid confusing breed-specific terminology, it is now
termed NME regardless of the breed in which it is diagnosed. Although the exact prevalence of NME is unknown, it recently was confirmed it to be the most common neurological disorder affecting Pug dogs.

2.1.4 Meningoencephalomyelitis of unknown etiology

The term MUE first was introduced in 2005 to describe dogs with meningoencephalomyelitis that lack a definitive clinical, histological, cytological, serological or PCR diagnosis. Other synonymous terminology has been utilized, including idiopathic meningoencephalomyelitis, meningoencephalomyelitis of unknown origin (MUO), inflammatory brain disease and non-infectious meningoencephalomyelitis (NIME). In some cases, signalment and advanced imaging findings are more suggestive of GME or NME and these patients may be diagnosed with presumptive GME or NME but ultimately, these animals are still considered to have MUE until histopathological confirmation has been obtained.

The clinical diagnosis of MUE is based on compatible cerebrospinal fluid (CSF) and advanced cross-sectional imaging findings in a dog with the appropriate clinical presentation that lacks evidence of infectious, toxic, congenital, metabolic, neoplastic or degenerative diseases. Analysis of CSF typically demonstrates elevated lymphocytes, monocytes or a combination of these as well as elevated total protein concentrations, which are suspected to result from increased permeability of the blood brain barrier, intrathecal antibody production or a combination of these. Advanced cross sectional imaging can be performed by computed tomography (CT) or magnetic resonance (MR) imaging, but MR is more sensitive for diagnosing MUE and is the imaging modality of choice. Imaging with MR typically can demonstrate evidence of CNS inflammation in the brain and spinal cord parenchyma as well as meninges in cases of MUE and also is useful in helping to rule-down other disease processes.
The ideal treatment for MUE is unknown for two reasons. First, MUE represents a spectrum of diseases, making treatment trials difficult.\textsuperscript{6,60} Second, most information regarding MUE treatment comes from retrospective studies.\textsuperscript{5,30,31,51} Regardless, all cases of MUE are treated with some form of immunosuppressive mono- or poly-therapy.\textsuperscript{6} Corticosteroids most commonly are used in combination with one or more additional immunomodulatory agents,\textsuperscript{6} including azathioprine,\textsuperscript{61} cyclophosphamide,\textsuperscript{62} cyclosporine,\textsuperscript{30,38} cytarabine,\textsuperscript{31,51} ketoconazole,\textsuperscript{30} leflunomide,\textsuperscript{63} lomustine,\textsuperscript{64,65} mycophenolate mofetil,\textsuperscript{66} procarbazine\textsuperscript{5} and vincristine.\textsuperscript{62} Additionally, antibiotics frequently are initiated until infectious disease titers for \textit{Ehrlichia canis}, \textit{E. platis}, \textit{E. equi}, \textit{Rickettsia rickettsii}, \textit{Anaplasma phagocytophilum}, \textit{Toxoplasma gondii}, \textit{Neospora caninum} and/or \textit{Borrelia burgdorferi} are determined to be negative.\textsuperscript{30,31,51,62}

2.2 ETIOPATHOGENIC THEORIES

2.2.1 Background

GME, NME and MUE have been recognized for over half a century but little is understood about their underlying etiopathogenesis.\textsuperscript{6-8,11,14,67,68} These disorders share some clinical and pathological similarities with disorders of humans and other species\textsuperscript{11,14,69} but ultimately, they are unique diseases of the domestic dog. Several important etiopathogenic theories have been proposed over the past several decades, including, neoplasia,\textsuperscript{6,68} CNS infection,\textsuperscript{7,8,11,14,70,71} genetic predisposition\textsuperscript{6,69,72} and autoimmunity.\textsuperscript{6,69}

2.2.2 Neoplasia

Although it has long been assumed that GME is a disorder resulting from primary immune dysregulation, there are veterinary neuropathologists that have suggested that it is a lymphoproliferative disorder that has features of both inflammation and neoplasia.\textsuperscript{6} Focal GME,
in particular, shares many similarities with neoplasia with lymphocytes having variable degrees of pleomorphism and mitotic indices. Additionally, lymphoblasts have been identified in CSF from dogs with GME. Whether these cells represent reactive inflammatory cells or a true neoplastic population is unclear. Importantly, the presence of lymphoblasts in the CSF can complicate the antemortem diagnosis, making it difficult to differentiate between MUE and lymphoma.

2.2.3 CNS infection

Numerous investigators have suggested pathogens, particularly viruses, may play a role in the development GME, NME and MUE. Although they have unique histopathological characteristics, GME and NME both are defined by non-suppurative inflammation, which is a common finding in viral CNS disease. In the initial 1989 descriptive report, herpesviruses were speculated to be the causative agent of NME due to similarities between NME in dogs and herpesvirus encephalitis in people; both disorders have an affinity for the cerebral hemispheres and may result in extensive necrosis. In fact, Cordy and Holliday reported isolation of a herpes-like virus in this manuscript, but the virus isolate was not retained and subsequent virus isolation attempts have been unsuccessful. In 1995, Summers and colleagues suggested GME may be caused by a retrovirus, similar to avian reticuloendotheliosis, but no research has been performed to substantiate or refute this speculation. In 1999, a dog in Florida spontaneously died from an undiagnosed neurological disease. Necropsy revealed microscopic lesions similar to GME and immunohistochemistry was positive for La Crosse virus (LACV) antigens, prompting the authors to suggest LACV may be the etiologic agent in GME. However, there have been no additional reports of LACV meningoencephalomyelitis in dogs. In 1998 and 2002, bornaviruses were associated with non-suppurative CNS inflammation in two
individual dogs in Switzerland and Japan, respectively, and proposed as a causative agent of GME. However, additional cases of bornavirus meningoencephalomyelitis have not been reported and bornaviruses typically have a predilection for CNS gray matter, while GME more predominately affects white matter. In a 2005 report, investigators failed to identify nucleic acids in paraffin embedded tissue from 7 GME and 12 NME cases evaluated by PCR for adenoviruses, herpesviruses and canine parvoviruses. Most recently, in a 2007 immunohistochemical study, cases of GME were sporadically positive for West Nile, canine parainfluenza and encephalomyocarditis viruses. Ultimately, however, a definitive viral etiology has not been identified.

2.2.4 Genetic predisposition

Genetic predisposition has been considered likely to contribute to disease development in GME, NME and MUE based on the predilection of these disorders for certain breeds. In particular, these three disorders occur most commonly in small breed dogs: the Miniature Poodle, Maltese, Dachshund, West Highland White Terrier and Chihuahua most commonly are associated with GME; the Pug, Maltese and Chihuahua most commonly are associated with NME; and the Dachshund, West Highland White Terrier and Chihuahua most commonly are associated with MUE. In fact two recent studies have confirmed a genetic basis for NME in Pug dogs. First, in 2009 NME was confirmed to have familial transmission in the Pug breed, although Mendelian inheritance was not identified. Next, a genome-wide association scan of single tandem repeat (STR) markers demonstrated a single strong association on chromosome 12, which was further narrowed by fine mapping to a region of the dog leukocyte antigen (DLA) II complex containing the DLA-DRB1, -DQA1 and -DQB1 genes. Subsequent sequencing of NME-affected dogs identified a single, high-risk
haplotype. The authors of this work concluded that these findings support genetic risk for NME development. More specifically, they suggested the involvement of DLA II supports that NME is an autoimmune disease.69

2.2.5 Autoimmunity

Autoimmune disease is considered by many to be the most likely cause of both GME and NME.3,6,55,69,79,80 However, the evidence for autoimmunity in these diseases is based primarily on a therapeutic response to immunosuppressive therapy and limited other supportive data, including intralesional T lymphocytes, CSF autoantibodies and an overrepresentation of these diseases in females.36,39,79,81

Establishing a role for autoimmunity in disease can be difficult and the criteria necessary to do so are controversial.82,83 Autoimmunity, by definition, is an adaptive immune response against self-antigen.82 Stringent criteria following a modified version of Koch’s postulates can be used and were outlined by Damoiseaux and Tervaert:

1) the specific adaptive immune response is directed to the affected organ or tissue;  
2) autoreactive T cells and/or autoantibodies are present in the affected organ or tissue;  
3) autoreactive T cells and/or autoantibodies can transfer the disease to healthy individuals or animals;  
4) immunisation with the autoantigen induces the disease in animal models;  
5) elimination or suppression of the autoimmune response prevents disease progression or even ameliorates the clinical manifestation.82

Interestingly, in the same manuscript Damoiseaux and Tervaert argue that these criteria are too strict and offer that in vivo induction of disease (as outlined in points 3 and 4) is unnecessary.82 They do, however, insist that there must be clear evidence of an adaptive immune response against self-antigen and that this response must be involved in the development of disease pathology.82 Based on this, the following criteria will be utilized when evaluating the body of evidence supporting that GME and NME are autoimmune diseases: 1) an adaptive
immune response must be directed towards the affected organ / tissue; 2) autoreactive T lymphocytes or autoantibodies must be present in the affected organ / tissue and must be involved in disease pathology; 3) suppression of the autoimmune response should decrease disease pathology and / or ameliorate clinical signs. It is important to note, however, that without the use of in vivo studies, it would be difficult to prove that autoreactive T lymphocytes or autoantibodies play a primary role in disease pathology. Although the supportive evidence for GME and NME being autoimmune diseases is substantial, definitive evidence of a self-directed adaptive immune response is lacking.

Criterion 1: an adaptive immune response must be directed towards the affected organ / tissue. Strong evidence of an adaptive immune response directed towards the CNS exists for both GME and NME. Both diseases are characterized by spontaneous, progressive mononuclear inflammation affecting the brain and spinal cord and importantly, evidence of infectious agents, toxin exposure, ischemia or other underlying causes of cellular damage is lacking. Additionally, immunohistochemistry (IHC) studies further characterizing the inflammatory response in GME and NME support an important role for T lymphocytes in both disorders. In 1998, Kipar and colleagues phenotyped 11 cases of GME with unknown clinical histories and proposed an autoimmune etiology based on a predominance of T lymphocytes and MHC class II positive intralesional inflammatory cells. They concluded that very few B cells were present within the inflammatory lesions. Evaluation of five Chihuahuas with NME in 2008 demonstrated that macrophages and T lymphocytes were consistently present within lesions with a variable presence of B lymphocytes. In 2003, Suzuki and colleagues evaluated four GME and 11 NME cases by IHC and confirmed the presence of T lymphocytes in both disorders but did not evaluate for B lymphocytes. Although an adaptive immune response within the CNS is
clearly present in both GME and NME, further work needs to be done to characterize the nature of this response. It must also be noted that these IHC studies were conducted on small numbers of cases with variable disease progression and treatment histories and that they had a limited scope due to a historical lack of available canine antibodies for paraffin embedded tissue (eg CD4, CD8).

Criterion 2: autoreactive T lymphocytes or autoantibodies must be present in the affected organ / tissue and must be involved in disease pathology. As discussed, verifying that an autoreactive immune response is involved in disease pathology is more difficult to prove. To our knowledge no attempts have been made to identify autoreactive T lymphocytes in cases of GME or NME. On the other hand, autoantibodies (against glial fibrillary acidic protein) have been identified, but their role in disease pathology remains questionable.6 Glial fibrillary acidic protein (GFAP) was identified in 1969 by MS researchers and is now known to be a critical structural component of astrocytes.84 Anti-GFAP antibodies have been identified in the CSF of virtually every case of NME evaluated by a group of researchers at the University of Tokyo85-87 and in three cases of GME also evaluated by these researchers.85 However, these autoantibodies also have been identified in dogs with brain tumors and healthy control Pugs (but interestingly, not in healthy control dogs of other breeds).85,87 It is possible that these autoantibodies represent a breed-specific fragility of astrocytes or are secondary to CNS tissue destruction as opposed to initiating disease pathology.6 To truly fulfill this criterion and label GME and NME as autoimmune diseases, these autoantibodies need to be shown to initiate disease pathology or autoreactive T cells that contribute to disease pathology need to be identified.

Criterion 3: suppression of the autoimmune response should decrease disease pathology and / or ameliorate clinical signs. Positive patient response to immunosuppression has been
considered one of the greatest pieces of evidence supporting an autoimmune etiology for GME and NME. However, a positive response to steroids and other immunotherapies does not necessarily implicate primary autoimmune disease. For example, human studies show that the prognosis for certain viral and bacterial meningoencephalitides may worsen without the use of corticosteroids. It is certainly possible that immune dysregulation in GME and NME secondary to a non-autoimmune etiology could result in a positive response to immunosuppressive treatment.

Significant research is still needed to validate GME and NME as autoimmune disorders. Next steps may include immunophenotyping the inflammatory cell infiltrates (eg. CD4 versus CD8 T lymphocytes), determining what cytokines are involved in the inflammatory response, determining if there is a clonal T cell population present and if so, what the antigenic target is (eg self versus non-self), determining if other autoantibodies are present and what role, if any, autoantibodies play in disease pathology.

2.2.6 Conclusions

The underlying factors that contribute to GME, NME and MUE development are unclear. There is strong evidence that genetic risk contributes to NME, and the fact that certain DLA II haplotypes predispose Pug dogs to disease development may support the commonly held theory that GME and NME are autoimmune diseases. However, a transmission and heritability study of NME in Pug dogs did not identify Mendelian inheritance, suggesting that multiple risk factors contribute to disease development. Schatzberg and colleagues have long speculated that GME and NME are multifactorial disorders that require interplay of multiple genetic and / or environmental risk factors. Unfortunately, environmental risk factors may be difficult to identify. Geographic region, seasonal distribution and recent vaccination were not found to alter
NME development in an epidemiological study.\textsuperscript{39} However, the sporadic reports of viruses associated with GME, NME and MUE support that stimulation with certain antigens may be an important risk factor in disease development.\textsuperscript{6}

Interestingly, although GME and NME are considered distinct diseases based on their differing pathology, it has been suggested that they could represent variants, or a spectrum, of the same disorder.\textsuperscript{i} These disorders share many similarities including a predilection for middle aged, female, small breed dogs; mononuclear cell inflammation confined to the CNS; favorable response to treatment with immunosuppressive therapy; and a guarded prognosis despite aggressive treatment.\textsuperscript{6} This phenomenon has been seen in other disease processes; there are numerous variants of multiple sclerosis (MS), for example, that vary in clinical presentation and pathology but all share the CNS inflammation and demyelination that is characteristic of MS.\textsuperscript{90} Also, Storch and colleagues used a rat model of experimental autoimmune encephalitis (EAE) to demonstrate that minor modifications in major histocompatibility complex (MHC) haplotypes could result in unique, reproducible histopathological patterns of disease following immunization with myelin-oligodendrocyte glycoprotein.\textsuperscript{91} It is possible that the neuropathological differences seen in GME and NME could be the result of variable genetic backgrounds (or environmental triggers).\textsuperscript{6,36}

2.3 REFERENCES


\textsuperscript{i} Schatzberg SJ, Department of Small Animal Medicine and Surgery, University of Georgia College of Veterinary Medicine, Athens, GA: Personal communication, 2010.


CHAPTER 3

EVALUATION OF BRAIN TISSUE OR CEREBROSPINAL FLUID WITH BROADLY REACTIVE POLYMERASE CHAIN REACTION FOR EHRlichia, Anaplasma, SPOTTED FEVER GROUP Rickettsia, Bartonella and Borrelia Species in Canine Neurological Diseases (109) Cases\(^1\)

3.1 ABSTRACT

**Background:** Vector-transmitted microorganisms in the genera *Ehrlichia, Anaplasma, Rickettsia, Bartonella* and *Borrelia* are commonly suspected in dogs with meningoencephalomyelitis (MEM), but the prevalence of these pathogens in brain tissue and cerebrospinal fluid (CSF) of dogs with MEM is unknown.

**Hypothesis/Objectives:** To determine if DNA from these genera is present in brain tissue and CSF of dogs with MEM, including those with meningoencephalitis of unknown etiology (MUE) and histopathologically-confirmed cases of granulomatous (GME) and necrotizing meningoencephalomyelitis (NME).

**Animals:** 109 dogs examined for neurological signs at 3 university referral hospitals.

**Methods:** Brain tissue and CSF were collected prospectively from dogs with neurological disease and evaluated by broadly reactive polymerase chain reaction (PCR) for *Ehrlichia, Anaplasma, Spotted Fever Group Rickettsia, Bartonella* and *Borrelia* species. Medical records were evaluated retrospectively to identify MEM and control cases.

**Results:** 75 cases of MUE, GME or NME, including brain tissue from 31 and CSF from 44 cases, were evaluated. Brain tissue from 4 cases and inflammatory CSF from 30 cases with infectious, neoplastic, compressive, vascular or malformative disease were evaluated as controls. Pathogen nucleic acids were detected in 1 of 109 cases evaluated. Specifically, *Bartonella vinsonii subsp. berkhoffii* DNA was amplified from 1/6 dogs with histopathologically-confirmed GME.

**Conclusion and clinical importance:** The results of this investigation suggest that microorganisms in the genera *Ehrlichia, Anaplasma, Rickettsia* and *Borrelia* are unlikely to be
directly associated with canine MEM in the geographic regions evaluated. The role of *Bartonella* in the pathogenesis of GME warrants further investigation.
3.2 INTRODUCTION

In dogs, central nervous system (CNS) inflammation may affect the brain, spinal cord, leptomeninges or some combination of these. The inclusive term meningoencephalomyelitis (MEM) may be used to describe CNS inflammation that can arise from infection, toxin exposure, cellular damage, neoplasia or autoimmune disease. The underlying cause of MEM in dogs is confirmed rarely on an antemortem basis, and a presumptive diagnosis typically is made based on clinical presentation, neuroanatomic localization, cerebrospinal fluid (CSF) analysis, advanced imaging and infectious disease testing. Postmortem diagnosis also may be challenging because many cases lack a recognized histological pattern. The term meningoencephalomyelitis of unknown etiology (MUE) is used to encompass those inflammatory CNS cases that lack a definitive histological, cytological, serological or PCR diagnosis.1

Granulomatous meningoencephalomyelitis (GME), necrotizing meningoencephalitis (NME), necrotizing leukoencephalitis, steroid-responsive meningitis and arteritis and idiopathic tremor syndrome are important differential diagnoses for MUE. Although these disorders have unique histopathological features, they collectively represent aberrant immune responses in the CNS. Although autoimmune processes have been postulated,2-5 the pathogeneses remain to be fully elucidated and likely are multifactorial, including genetic and environmental factors.6 Coupled with inherited abnormalities in immune regulation and tolerance, antigenic triggers secondary to infection could incite an immune response targeting CNS proteins with resultant inflammation.7

Neoplasia and direct CNS infection comprise additional differential diagnoses for MUE. Although bacterial MEM is rare in dogs,8 Ehrlichia, Anaplasma, Rickettsia, Bartonella and Borrelia species all have been implicated in canine CNS infections.9-15 Central nervous system
Signs have been reported secondary to infection with monocytotropic and granulocytotropic ehrlichiosis, granulocytotropic anaplasmosis and *Rickettsia rickettsii*. A causal relationship has not been established definitively, but *Bartonella* spp. also have been associated with numerous canine neurological disorders including meningoencephalitis, meningoencephalitis, meningitis and myelitis. Similarly, members of *Borrelia burgdorferi* sensu lato (sl) are important causes of neurological disease in humans and have been implicated in naturally-occurring and experimental canine CNS disease. However, a recent investigation suggested *B. burgdorferi* sl is not a common cause of naturally-occurring canine neurological disease.

Although the authors hypothesize that *Ehrlichia, Anaplasma, Rickettsia, Bartonella* and *Borrelia* species are uncommon causes of MEM in dogs, the identification of infectious etiologies in MEM could direct antibiotic therapy and improve clinical outcomes. Broadly reactive polymerase chain reaction (PCR) assays can be used to identify the nucleic acids of many or all species in a particular genus, allowing identification of suspected as well as unsuspected agents. In this investigation, broadly reactive genus PCR for *Ehrlichia, Anaplasma, Spotted Fever Group (SFG) Rickettsia, Bartonella* and *Borrelia* species was applied to DNA extracted from 75 dogs with MEM (including brain tissue from 31 dogs with GME and NME and CSF from 42 dogs with MUE and two dogs with NME) to determine if these pathogens are associated with canine MEM.
3.3 MATERIALS AND METHODS

3.3.1 Case samples

Brain tissue was collected between 2002 and 2008 in accordance with Animal Care and Use guidelines at Texas A&M University College of Veterinary Medicine and Biomedical Sciences (TAMU-CVM) from postmortem cases of MEM, including dogs that presented for routine necropsy and pugs from a concurrent NME study. All cases were evaluated by a single board-certified pathologist (BP) to verify the histopathological diagnosis before inclusion in this investigation. Intralesional tissues from GME, NME and control cases were collected non-aseptically as a part of routine necropsy tissue collection and were stored at -80°C.

Cerebrospinal fluid was collected in routine fashion from the cerebellomedullary or lumbar cistern from dogs that presented with neurological signs to the University of Georgia College of Veterinary Medicine (UGA-CVM), TAMU-CVM and The Royal Veterinary College (RVC), University of London between 2003 and 2008. Cytologic analysis and protein quantification were performed by a board-certified clinical pathologist, and excess CSF was stored at -80°C.

Medical records of dogs from which CSF was collected were evaluated retrospectively to identify cases of MUE, confirmed cases of GME and NME and control cases with inflammatory CSF samples. Age, breed, sex, neurological signs, neuroanatomic localization, magnetic resonance imaging findings, serology results, presumptive diagnosis, treatment and necropsy findings were recorded when available. Cases were classified as MUE if they had > 5 white blood cells (WBC)/μl in the CSF, neuroanatomic localization to 1 or more areas of the CNS and no evidence of other neurological disorders. Cases were classified as inflammatory controls if they had > 5 WBC/μl in the CSF, neuroanatomic localization to the central or peripheral nervous
system and evidence of an identifiable disease process (e.g. disk herniation, polyradiculoneuritis). Histopathological diagnoses were used to classify cases as GME or NME.

3.3.2 DNA extraction, quality control and PCR amplification

Total nucleic acids were extracted from freshly frozen brain tissue and CSF samples using commercially available kits. A separate, sterile blade was used to collect 5-10 mg of frozen tissue from each of the previously collected brain lesions for nucleic acid extraction.

PCR for the canine housekeeping genes histone 3.3 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed on all samples. Negative controls containing no DNA template were run in parallel with all PCR reactions. Additionally, mock nucleic acid extraction of sterile water was performed in parallel with all clinical cases and utilized as a negative control in all PCR reactions. Genomic DNA from a healthy dog was used as a PCR negative control in *Ehrlichia*, *Anaplasma*, SFG *Rickettsia* and *Bartonella* PCR reactions.

Oligonucleotide PCR primers were designed manually to amplify DNA from all known *Ehrlichia* and *Anaplasma* spp. targeting an approximately 600 base pair (bp) fragment of the heat shock protein (groEL) gene: groEL-643s 5’- ACT GAT GGT ATG CAR TTT GAY CG - 3’ and groEL-1236as 5’-TCT TTR CGT TCY TTM ACY TCA ACT TC – 3’. Amplification was performed using conventional PCR in a 25 µl final volume reaction containing 1X PCR mix, 12.5 pmol of each primer and 5.0 µl of DNA template. After a single hot-start cycle at 95°C for 30 seconds, PCR cycled 55 times with the following parameters: 10 seconds at 94°C, 15 seconds at 58°C and 15 seconds at 72°C. After a final cycle at 72°C for 1 minute, PCR products were analyzed by 1.5% agarose gel electrophoresis under ultraviolet exposure. To generate positive controls, PCR amplicons from animals naturally infected with *E. canis* (similar to GenBank accession CP000107), *E. chaffeensis* (similar to CP000236), *E. ewingii* (similar to AF195273),
A. platys (similar to AF399916) and A. phagocytophilum (similar to EU860090) each were cloned into plasmid vectors and Escherichia coli was transformed according to the manufacturer’s protocol. Recombinant clones were selected by blue-white screening of bacterial colonies and DNA insertions were sequenced bidirectionally. Clones with partial sequence of groEL of each positive control were quantified by spectrophotometry (average of 5 measurements) and diluted individually 10-fold, ranging from $1.0 \times 10^9$ to 0.1 plasmid(s)/µl. The limit of detection observed in PCR amplifications was 10 copies of target gene per reaction for each control (equivalent to 2 copies of the gene/µl of extracted DNA). These primers were able to amplify a partial sequence of the groEL gene of Bartonella henselae and B. vinsonii subsp. berkhoffii, but the limit of detection for these organisms was not established. Another previously validated PCR assay was used to detect Bartonella spp. DNA (below), but Bartonella groEL sequences generated using these primers were used for genetic characterization.

Bartonella spp. DNA was amplified using conventional PCR targeting a fragment of the RNA polymerase beta subunit (rpoB) gene as described previously. Bartonella henselae (similar to BX897699) was used as positive control for rpoB PCR. Bartonella spp. positive samples were further characterized for multiple genes, including the intergenic transcribed spacer (ITS) and the groEL gene using primers described above. Spotted Fever Group Rickettsia DNA was amplified using real-time PCR targeting a fragment of the outer membrane protein A (ompA) gene as described previously. Rickettsia conorii (similar to DQ518245) was used as a positive control for ompA PCR.

Borrelia spp. DNA was amplified by real-time PCR using primers FLALS and FLARS previously designed to target a fragment of the flagellin (flaB) gene. Amplification was performed in a 20 µl final volume reaction containing 2X PCR mix, 2.0 units of uracil-DNA
glycosylase, 0.2 µM of each primer and 5.0 µl or 2.0 µl of template DNA from CSF or brain tissue, respectively. After a single hot-start cycle at 95°C for 5 minutes, PCR cycled 70 times with the following parameters: 15 seconds at 95°C, 30 seconds at 55°C and 30 seconds at 72°C. B. burgdorferi strain B31 was used as a positive control for flaB PCR. Melting curve analysis was used to evaluate amplification specificity.

3.3.3 Sequencing

All positive amplifications were gel purified and sequenced using the corresponding PCR primers. Chromatogram evaluation and sequence alignment were performed manually using commercially available software. Bacterial species and strain were defined by comparing DNA sequence similarities with other sequences present in the GenBank database before May 2009 using the Basic Local Alignment Search Tool.

3.4 RESULTS

Brain tissue from 35 dogs was evaluated by all PCR methodologies and included 25 NME cases, 6 GME cases and 4 controls. The 4 controls included 3 brain tumors (1 astrocytoma and 2 meningiomas) diagnosed by histopathology and 1 case of canine distemper virus encephalitis diagnosed by histopathology and immunohistochemistry. Histopathology was the only information available on these cases.

Cerebrospinal fluid from 74 dogs with neurological signs was tested by all PCR methodologies. Among CSF samples, evaluation of medical records identified 42 cases of MUE, 2 cases of histopathologically-confirmed NME and 30 control cases with inflammatory CSF samples. Complete medical records could not be identified for 2 MUE cases, and 1 MUE case had only 5 WBC/µl in the CSF. These cases however were included in the MUE category.
because breed, neuroanatomic localization and CSF results were consistent with MUE. The MUE and NME cases included 20 dogs from UGA-CVM, 4 dogs from TAMU-CVM and 20 dogs from RVC. The range of WBCs in the CSF was 5 – 4,400 cells/μl (median, 55 cells/μl; mean, 551 cells/μl) with 21% of cases having a mixed pleocytosis, 43% a lymphocytic pleocytosis, 20% a neutrophilic pleocytosis, 9% a monocytic pleocytosis and 7% an eosinophilic pleocytosis. The range of total protein was 11 – 1,696 mg/dl (median, 49 mg/dl; mean, 143 mg/dl). The dogs ranged in age from 6 months to 12 years (median, 3 years; mean, 3.7 years) and included 21 females and 23 males. Among the dogs with MUE, breeds represented were Beagle (n = 2), Boxer (n = 4), Golden Retriever (n = 2), Jack Russell Terrier (n = 2), Labrador Retriever (n = 4), Maltese (n = 2), mixed breed (n = 6), West Highland White Terrier (n = 4) and 1 each of 16 additional breeds. Both NME cases were Pug dogs. Serology was not performed routinely, and a single (non-paired) antibody titer was available from only a limited number of dogs with MUE: E. canis from 9/44, R. rickettsii from 11/44, B. henselae and B. vinsonii subsp. berkhoffii from 3/44 and B. burgdorferi sl from 9/44. Titers for immunoglobulin G (IgG) were negative (<1:16) in all but 5 cases. These 5 cases had antibodies that reacted to R. rickettsii antigens, in which a titer of 1:64 was present in 3 cases and 1:128 in 2 cases.

Control cases with inflammatory CSF included atlantoaxial subluxation (n = 1), cervical subarachnoid diverticula (n = 1), disk herniation (n = 10), fibrocartilagenous embolic myelopathy (n = 9), lymphoplasmacytic neuritis (n = 1), neoplasia (n = 4), paralumbar abscess and epidural empyema (n = 1), spinal fracture (n = 1), subdural hematoma (n = 1) and syringohydromyelia (n = 1).

Nucleic acids from Ehrlichia, Anaplasma, SFG Rickettsia and Borrelia species were not detected in the 109 samples tested. Bartonella vinsonii subsp. berkhoffii DNA was identified by
rpoB PCR from the brain tissue of a dog with histopathologically-confirmed GME with homology between 624/625 bp with GenBank accession EU29566. Further confirmation was performed by sequencing amplicons generated by the ITS (429 bp of 429 bp similar to GenBank accession AF312503) and groEL (510 bp of 524 bp similar to GenBank accession AF014835) PCR. Additionally, nucleic acids were extracted independently from a second intralesional brain specimen from the same dog and \textit{B. vinsonii} subsp. \textit{berkhoffii} was re-amplified by \textit{Bartonella} rpoB PCR. All positive controls produced the expected PCR results; all negative controls were free of bacterial amplicons. Histone or GAPDH were amplified successfully from all cases.

The \textit{Bartonella} positive GME case was an 8-month-old male intact Labrador retriever seen at TAMU-CVM for rapidly progressive prosencephalic signs. The patient died spontaneously shortly after presentation despite symptomatic treatment with anticonvulsants and mannitol. Due to the severity and rapid progression of this patient’s clinical signs, CSF was not obtained for evaluation. This patient did not receive immunosuppressive therapy.

3.5 DISCUSSION

Evaluation of brain tissue, CSF or both from 109 dogs with neurological signs, including 75 cases of MEM, did not identify nucleic acids from \textit{Ehrlichia}, \textit{Anaplasma}, SFG \textit{Rickettsia} or \textit{Borrelia} species. \textit{Bartonella vinsonii} subsp. \textit{berkhoffii} was identified in brain tissue from 1 dog with histopathologically-diagnosed GME. This is the first large-scale study to evaluate canine brain tissue and CSF by broadly reactive PCR for multiple species in these genera. The primarily negative results are consistent with a recent report in which the authors failed to demonstrate \textit{B. burdorferi} sl or \textit{A. phagocytophilum} DNA in the blood or CSF of dogs with neurological
disease, \(^\text{22}\) and suggest pathogens in the genera evaluated are not commonly detected in brain tissue and CSF from dogs with canine MEM.

*B. vinsonii* subsp. *berkhoffii* has been reported to be the most common *Bartonella* spp. associated with clinical disease in dogs \(^\text{29}\) and was previously implicated on the basis of serology in neutrophilic and granulomatous meningoencephalitis. \(^\text{12}\) Although *Bartonella* seroreactivity has been reported in 4 antemortem cases of presumptive GME, \(^\text{12,17}\) this is the first report of *Bartonella* DNA amplified from the brain from a dog with histopathologically-confirmed GME. The brain tissue for this investigation was not collected aseptically and DNA carryover, although unlikely, cannot be ruled out in this case. \(^\text{30}\) Importantly, as with other cases of *Bartonella*-associated neurological disease in dogs, direct causation was not established. Immunohistochemical assays for *B. vinsonii* subsp. *berkhoffii* are under development and ultimately will be applied to the brain of this GME case to assess for intra- or peri-lesional organisms.

GME remains an enigmatic inflammatory disorder of the canine CNS characterized by mixed mononuclear cell inflammation predominantly in the CNS white matter. \(^\text{31,32}\) Numerous pathogens, including West Nile virus, canine parainfluenza and encephalomyocarditis virus \(^\text{33}\) have been suggested to play a role in individual cases of GME. The clinical relevance of these pathogens in GME is unclear, but they may reflect the possibility that GME is a non-specific inflammatory response to various antigens, of which pathogens comprise an important subset. \(^\text{1}\) Importantly, *Bartonella* spp. may be another antigenic trigger of GME and their role in canine MEM warrants further investigation. Although we detected *Bartonella* DNA in 1/6 (17%) histopathologically-confirmed GME cases, a much larger sample size ( \(\geq 217\) cases) would be
needed to determine whether or not the true prevalence of *Bartonella* spp. DNA in GME brain lesions is 17% (± 5% with a 95% confidence interval).

*Bartonella* DNA was identified in 1/6 GME cases, but the MUE and NME subsets were uniformly PCR negative. It is not surprising that organisms were not identified in NME cases because previous attempts have not identified infectious agents, and the pathology of ehrlichiosis and rickettsiosis typically does not involve cerebral necrosis. Notably, all postmortem cases evaluated had the histopathological patterns for GME or NME, which may have biased the results against the identification of microorganisms from the genera evaluated. The results also may have been biased based on the geographic regions evaluated because regional variation exists for these vector-borne pathogens. For example, in people Lyme disease and *A. phagocytophilum* infections are most common in the northern United States (U.S.) whereas Rocky Mountain spotted fever (RMSF) is most common in the southeast and south-central U.S. In the United Kingdom (U.K.), PCR evidence of *B. burgdorferi* sl and *A. phagocytophilum* and positive serology for *B. henselae* has been uncommonly identified in dogs with clinical illness. However, the majority of pathogens evaluated in this investigation are not considered common causes of disease in dogs in the U.K. (Shaw, personal communication).

The identification of infectious agents associated with idiopathic canine MEM would improve prevention and therapeutic strategies and potentially decrease disease morbidity and mortality. Molecular methodologies have improved the ability to diagnose and treat infectious MEM in people, with pathogens being implicated in 16-50% of cases. In particular, the diagnosis of viral MEM has been dramatically improved by the advent of PCR for viruses including herpes- (herpes simplex virus, human herpes virus-6, cytomegalovirus, varicella-zoster
virus, Epstein-Barr virus), picorna- (poliovirus), polyoma- (JC virus), retro- (human immunodeficiency virus, human T lymphotrophic viruses) and rhabdo- (rabies virus) viruses.41,43

Nucleic acid amplification by PCR now is used routinely in veterinary medicine and is the basis of ongoing studies to identify infectious etiologies in canine MEM (Schatzberg, unpublished). This study focused on the identification of several important vector-borne microorganisms that have been reported to cause MEM in dogs and humans. These pathogens are not considered common causes of primary neurological disease in dogs but remain key differential diagnoses for MUE in endemic areas. The actual incidence of CNS disease in dogs secondary to vector-borne microorganisms is unknown, but CNS signs have been reported in up to 33% of dogs with ehrlichiosis and 43% of dogs with RMSF.11,44 Although less is known about Bartonella and Borrelia spp. in canine neurological disease, Bartonella spp. were 1 of the most common bacterial pathogens associated with human encephalitis in the California encephalitis project,45 and neurological signs are reported in 10-15% of people with Lyme disease.46

Broadly reactive PCR assays, based on conserved genomic regions, were utilized in this investigation to prevent exclusion of unexpected pathogens in the genera of interest. Borrelia flaB PCR primers were designed to identical sequences of 8 Borrelia spp., including all species reported to cause neurological disease in humans.28,47 Ehrlichia/Anaplasma groEL, SFG Rickettsia ompA and Bartonella rpoB PCR assays were designed with degenerate primers, which employ a pool of primers representing all possible sequences in a multiple sequence alignment. Ehrlichia/Anaplasma groEL PCR primers were designed to all known species of these genera including monocytotrophic and granulocytotropic organisms that cause neurological disease in humans and animals.9,10,13,16,36,44 Bartonella rpoB primers also were designed for all known
species in the genus,\textsuperscript{26} and SFG \textit{Rickettsia} ompA primers were designed to 26 \textit{Rickettsia} spp. including SFG members \textit{R. rickettsii}, a documented cause of CNS disease in dogs and humans\textsuperscript{11,27,44} and \textit{R. conorii}, a cause of CNS disease in people.\textsuperscript{27,48}

The ability to identify pathogens in clinical specimens will always be limited by the sensitivity of available methodologies, and sensitivity can vary dramatically among PCR assays. With \textit{Bartonella} spp., for example, evaluation of blood, tissue and CSF by current PCR techniques has a sensitivity of 10-15\% without pre-enrichment culture (Breitschwerdt, unpublished).\textsuperscript{49} Sensitivities of the assays used in this study ranged from 2.5-10 copies of target gene per reaction for \textit{Ehrlichia/Anaplasma} groEL, SFG \textit{Rickettsia} ompA and \textit{Bartonella} rpoB PCR,\textsuperscript{26,27} although assay sensitivity was not assessed on brain tissue and CSF from naturally-occurring infections due to the unavailability of samples. The primers utilized were designed to recognize multiple species within 1 or more genera, but were validated only against available, clinically important pathogens.\textsuperscript{26-28} Also, although SFG \textit{Rickettsia} ompA and \textit{Bartonella} rpoB PCR have been determined to be sensitive in the presence of canine genomic DNA,\textsuperscript{26,27} this is not the case for \textit{Ehrlichia/Anaplasma} groEL and \textit{Borrelia} flaB PCR. Although mispriming of canine genomic DNA was not noted, the presence of host nucleic acids could have decreased \textit{Ehrlichia/Anaplasma} groEL and \textit{Borrelia} flaB PCR sensitivity.

Pathogen tropism, disease pathogenesis and timing of specimen acquisition also may influence PCR results. Infectious agents may initiate an immune response in the CNS from a distant site or may be cleared from the CNS or be undetectable by the time overt MEM develops.\textsuperscript{7,50} In particular, diagnosis of neuroborreliosis by PCR of CSF has variable, often low sensitivity,\textsuperscript{51} which could be the result of low levels of organism in the CNS\textsuperscript{51} or lack of organisms in the CSF despite their presence in the nervous tissue.
In certain cases, such as neuroborreliosis, evaluation of serum and CSF for antibodies and PCR of blood may be more sensitive than PCR of CSF alone for pathogen identification.\textsuperscript{51,52} Unfortunately, CSF and blood were not available in this investigation for further antibody and PCR studies. However, recent evaluation of several dogs with experimental \textit{B. burgdorferi} infections demonstrated that even when immunosuppressed, these dogs did not develop clinical or histopathological meningoencephalitis despite seroconversion and other systemic signs of Lyme disease (Krimer and Schatzberg, manuscript in preparation). A few dogs in this investigation were evaluated for serum antibodies to \textit{E. canis}, \textit{R. rickettsii}, \textit{B. henselae}, \textit{B. vinsonii} subsp. \textit{berkhoffii} and \textit{B. burgdorferi} but paired titers were not evaluated in these cases. Among the available serology results, 3 dogs had RMSF IgG titers of 1:64 and 2 had RMSF IgG titers of 1:128. Although these titers indicate a low concentration of serum antibodies in 5 patients, the clinical relevance cannot be assessed without paired serology. These values were consistent with exposure to \textit{R. rickettsii} or other cross-reactive SFG \textit{Rickettsia} spp. rather than active infection in each case, in accordance with the associated negative PCR data.

\textit{Ehrlichia}, \textit{Anaplasma}, SFG \textit{Rickettsia}, \textit{Bartonella} and \textit{Borrelia} are unlikely to be detected by PCR in the brain tissue and CSF of dogs with MEM. Although secondary immune-mediated MEM cannot be ruled out, the PCR results for \textit{Ehrlichia}, \textit{Anaplasma}, SFG \textit{Rickettsia} and \textit{Borrelia} species suggest that these pathogens are unlikely to be causes of canine MEM in the southern U.S. and central England by infection of the CNS. The potential role of \textit{Bartonella} spp. in the pathogenesis of GME warrants further investigation. Studies are currently underway to evaluate for other potential etiological agents as well as to investigate the immunological and genetic components of canine MEM.
3.6 FOOTNOTES

a Qiagen MinElute Virus Spin Kit, Qiagen, Chatsworth, CA
b Qiagen DNeasy Blood and Tissue Kit, Qiagen, Chatsworth, CA
c Premix Ex Taq® (Perfect Real Time), Takara® Bio. Inc., Japan
d Integrated DNA Technologies®, Inc., Coralville, IA
e Mastercycler EP, Eppendorf, Hamburg, Germany
f pGem-T easy vector, Promega, Madison, WI
g E. coli DH-5alpha, Invitrogen, Carlsbad, CA
h Eton Bioscience, Research Triangle Park, NC
i LightCycler® 480 SYBR Green I Master, Roche Diagnostics, Indianapolis, IN
j LightCycler® Uracil-DNA Glycosylase, Roche Diagnostics, Indianapolis, IN
k Roche LightCycler, Roche Diagnostics, Indianapolis, IN
l QIAquick PCR purification kit, Qiagen, Valencia, CA
m BigDye Terminators v3.1 and ABI 3730xl, Applied Biosystems, Foster City, CA
n ContigExpress and AlignX, Vector NTI Suite 10.1, Invitrogen Corp., Carlsbad, CA

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3.8 REFERENCES


CHAPTER 4

BROADLY REACTIVE METHODOLOGIES FOR PATHOGEN DETECTION IN GRANULOMATOUS MENINGOENCEPHALOMYELITIS AND NECROTIZING MENINGOENCEPHALITIS

4.1 ABSTRACT

**Background:** Granulomatous meningoencephalomyelitis (GME) and necrotizing meningoencephalitis (NME) are common inflammatory conditions of the canine central nervous system. Infectious pathogens, particularly viruses, have long been suspected to contribute to the etiopathogenesis of GME and NME.

**Hypothesis:** Broadly reactive, molecular-based pathogen detection methods may aid in the identification of infectious agents in GME and NME.

**Animals:** Sixty-eight client-owned dogs evaluated by necropsy at 1 university referral hospital.

**Methods:** Brain tissue prospectively collected at necropsy from GME, NME and control cases was evaluated by broadly reactive polymerase chain reaction (PCR) for 5 viral families, 3 viral genera and 1 viral group. Tissue homogenates from these cases also were inoculated onto rat XC cells, and culture supernatant from 1/9 NME cases exhibiting a cytopathic effect underwent sequence-independent, single-primer amplification (SISPA). Additionally, these tissues were retrospectively evaluated for mycoplasmas by PCR, culture and immunohistochemistry (IHC).

**Results:** Viral nucleic acids associated with GME and NME were not identified by PCR or SISPA. *M. canis* was identified by *Mycoplasma* genus-specific PCR and subsequently was cultured from 4/5 GME and 4/8 NME cases as well as 2/9 controls. IHC did not detect *M. canis* in 11 GME, 27 NME or 10 controls evaluated with strain PG14 polyclonal antiserum.

**Conclusions and clinical importance:** The primarily negative results suggest that viral pathogens are not commonly found in the brain tissue of dogs with GME and NME. Further investigation is warranted to determine the significance of *M. canis* in cases of GME and NME.
4.2 INTRODUCTION

Granulomatous meningoencephalomyelitis (GME) and necrotizing meningoencephalitis (NME) are progressive, often fatal diseases of the canine central nervous system (CNS) named for characteristic patterns of non-suppurative inflammation and accompanying neuropathological changes.¹ Although extensive information exists regarding the clinical and pathological features of these disorders, the underlying factors that contribute to disease development and pathogenesis remain elusive.¹ Numerous etiopathogenic theories have been suggested, including autoimmunity,¹,² direct CNS infection,³-⁶ parainfectious immune dysregulation¹ and genetic predisposition.¹,² Ultimately, a multifactorial etiopathogenesis, with contribution of genetic and environmental factors, is considered likely for both disorders.¹,⁴

Genetic predisposition has been confirmed in Pug dogs with NME²,⁷ and is strongly suspected in GME based on overrepresentation of this disease in small breeds,¹ but contributing environmental factors have not been identified for either disorder. Although CNS infection has been suspected to contribute to the development of GME and NME,¹,³-⁶ routine diagnostic techniques such as culture and microscopy have failed to identify protozoal, fungal or bacterial pathogens.⁸

The investigators evaluated the hypothesis that broadly reactive molecular techniques may aid in finding occult viral and atypical bacterial infections associated with GME and NME. Brain tissue was collected from cases of histopathologically-confirmed GME and NME to evaluate for the presence of known and novel pathogens using broadly reactive polymerase chain reaction (PCR) for 8 viral groups: adenovirus, bunyavirus, coronavirus, enterovirus, flavivirus, herpesvirus, paramyxovirus and parechovirus; unbiased sequence-independent, single-primer amplification (SISPA); and *Mycoplasma* genus-specific PCR.
4.3 MATERIAL AND METHODS

4.3.1 Study population

Cases of GME and NME were identified from dogs presenting for necropsy at the Texas A&M University College of Veterinary Medicine and Biomedical Sciences (TAMU-CVM). Control dogs were identified concurrently and included dogs with non-neurological illness or neurological illness other than GME and NME presenting for necropsy at TAMU-CVM. All GME and NME cases were evaluated by a single board-certified pathologist (BP) to verify the histopathological diagnosis before inclusion. All controls were diagnosed by board-certified pathologists based on complete necropsies. Age at disease onset, breed, gender status, treatment administered and survival time were recorded at time of sample collection.

4.3.2 Sample collection

Brain tissue was collected as a part of routine necropsy between 2002 and 2010 in accordance with Institutional Animal Care and Use guidelines: an approximately 1 cm³ piece of tissue was collected from the frontal lobe and immediately transferred to -80°C until use. Time from patient death to sample acquisition ranged from 0 to 24 hours. Separate, sterile blades and petri dishes were used to collect tissue for downstream applications. Additional tissues were fixed in neutral-buffered, 10% formalin and paraffin-embedded for histopathology and immunohistochemistry (IHC).

4.3.3 Nucleic acid extraction and PCR quality control

Genomic DNA (gDNA) and total RNA were extracted from brain tissuea,b and stored as single-use aliquots at -80°C. A 215 base pair (bp) fragment of the canine histone 3.3 gene was amplified from all samples to confirm DNA integrity.⁹ RNA integrity was confirmed in all samples by reverse transcription PCR (RT-PCR) amplification of superoxide dismutase
(expected product size 440 bp). To avoid contamination, nucleic acid extraction, PCR preparation, PCR and sequencing were carried out in different rooms. Negative controls containing no DNA or RNA template were run in parallel with all PCR reactions. Additionally, mock nucleic acid extraction of sterile water was performed in parallel with all clinical cases and utilized as a negative control for PCR reactions.

4.3.4 Broadly reactive viral PCR

Consensus, degenerate or consensus-degenerate hybrid primers were used for broadly reactive viral PCR (Table 4.1). Adenovirus PCR; b bunyavirus RT-PCR and coronavirus, flavivirus and paramyxovirus semi-nested RT-PCR; d herpesvirus semi-nested PCR (snPCR); e and parechovirus and enterovirus real-time RT-PCR f (rRT-PCR) were performed according to manufacturer's instructions with a final volume of 50 μl and final primer concentration of 1 μM unless otherwise noted. RT-PCR reactions contained 20 U RNase inhibitor g and PCR and snPCR reactions used 200 μM (each) of deoxynucleotide triphosphates (dNPTs). h Initial reactions were performed with 5 μl template DNA or RNA, and semi-nested reactions were performed with 2 μl of template from the initial reaction. Assays were individually optimized and sensitivity was determined by 10-fold serial dilutions of target-containing plasmids.

Pan-adenovirus i and pan-paramyxovirus j primers were used as described previously. 4,12 DNA from canine adenovirus (CAV)-1 and CAV-2 and template RNA from human parainfluenza virus 2 were used as positive controls for pan-adenovirus and pan-paramyxovirus reactions, respectively.

Previously designed bunyavirus primers were used for RT-PCR. 13 After initial reactions at 60°C for 1 minute, 45°C for 30 minutes and 94°C for 2 minutes, RT-PCR cycled 40 times at 94°C for 15 seconds, 50°C for 30 seconds and 72°C for 30 seconds, followed by a final
elongation at 72°C for 7 minutes. RNA from a mutated clone of La Crosse virus (LACV) was used as a positive control. Primer sensitivity was determined to be 100 to 500 copies of target gene per reaction using Cache Valley virus and LACV.

Previously designed pan-coronavirus primers\textsuperscript{14} F2, R3A (0.5 μM) and R3B (0.5 μM) were used for the initial reaction and F2, R2A8 and R2B8 were used for the semi-nested reaction. Reverse transcription began at 60°C for 1 minute, 45°C for 30 minutes and 94°C for 2 minutes, followed by 40 cycles at 94°C for 15 seconds, 50°C for 30 seconds and 72°C for 30 seconds with a final elongation at 72°C for 7 minutes. RNA from human coronavirus OC43 was used as a positive control. Primer sensitivity was determined to be 10 to 100 copies of target gene per reaction using representative viruses from each antigenic group.\textsuperscript{14}

Previously designed flavivirus primers\textsuperscript{15} cFD2 and MAMD were used for the initial reaction and cFD2 and FS778 for the semi-nested reaction. Reverse transcription began at 60°C for 1 minute, 42°C for 30 minutes and 94°C for 2 minutes, followed by 40 cycles at 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 1 minute with a final elongation at 72°C for 7 minutes. RNA from a mutated clone of St. Louis encephalitis (SLE) virus was used as a positive control. Primer sensitivity was determined to be 100 to 500 copies of target gene per reaction using Japanese encephalitis, SLE, Dengue, West Nile and Yellow fever viruses.

Previously designed pan-herpesvirus primers\textsuperscript{16} DFASA and GDTD1B were used for the initial reaction and VYGA and GDTD1B were used for the semi-nested reaction. Both reactions began with an initial hot-start at 94°C for 2.5 minutes, followed by 50 cycles at 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute, with a final elongation at 72°C for 10 minutes. DNA from canine herpesvirus type 1 was used as a positive control.
Previously designed parechovirus and enterovirus primers (0.4 μM each) and probes (0.2 μM each) were used for rRT-PCR.\textsuperscript{17,18} After initial reactions at 50°C for 30 minutes and 95°C for 10 minutes, rRT-PCR cycled 50 times with the following parameters: 95°C for 15 seconds, 58°C for 30 seconds and 72°C for 10 seconds, with probe detection during the 58°C annealing step.\textsuperscript{i} Threshold cycle values were determined using commercially available software.\textsuperscript{i} Template DNA from human parechovirus 1 (Harris strain) and echovirus 30 were used as positive controls for parechovirus and enterovirus rRT-PCR, respectively. Primer sensitivity was determined to be 10-30 copies for human parechovirus 1 and 10-100 copies for echovirus 30.

4.3.5 SISPA

Brain tissue was minced and inoculated onto the rat XC cell line,\textsuperscript{19} and cultures were monitored for cytopathic effect (CPE). Total RNA was extracted from culture supernatants\textsuperscript{l} and subjected to SISPA.\textsuperscript{20} After denaturation at 70°C for 5 minutes, first strand cDNA synthesis was performed using 200 μl of reverse transcriptase\textsuperscript{k} with the primer 5’-GTTTCCAGTCACGATA NNNNNNNNNN-3’ under the following conditions: 25°C for 10 minutes, 50°C for 50 minutes and 85°C for 5 minutes. cDNA was RNase H-treated prior to second-strand synthesis.\textsuperscript{l} cDNA was heated (94°C for 2 minutes) and cooled (10°C for 5 minutes) prior to enzyme addition, followed by incubation at 37°C for 8 minutes and 94°C for 8 minutes. Random PCR amplification\textsuperscript{c} was performed with the primer 5’-GTTTCCAGTCACGATA-3’ and 200 μM (each) of dNPTs.\textsuperscript{g} An initial hot-start at 94°C for 2 minutes was followed by 40 cycles at 94°C for 30 seconds, 40°C for 30 seconds, 50°C for 30 seconds and 72°C for 1 minute. The products were purified\textsuperscript{m} and separated by agarose gel electrophoresis. Bands detected in affected cases but not control samples were excised, extracted\textsuperscript{n} and subcloned\textsuperscript{o} according to the manufacturer’s instructions. Insert containing plasmids were purified\textsuperscript{p} and sequenced.
4.3.6 Mycoplasma isolation and identification

Mycoplasma genus-specific primers designed to amplify an approximately 400 bp region of the 16S ribosomal RNA (rRNA) gene were used for nested PCR with a 50 µl final volume, 1 µM final primer concentration and 200 µM (each) dNTPs. The initial reaction was performed with primers MY-16S-447 (5’-GTCAGAAAGCGATGGCTAACTA-3’) and MY-16S-844 (5’-CGAGCATACTACTCAG GCGGAT-3’) using 2 µl gDNA, and the second amplification with primers MY-16S-483n (5’-CGGTAATACATAGGTCGC-3’) and MY-16S-748n (5’-TATCTAATCCTGTGTTGCTCC-3’) using 2 µl template from the initial reaction. Both reactions began with an initial hot-start at 94°C for 2 minutes, followed by 40 cycles at 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute with a final elongation at 72°C for 7 minutes. DNA from Mycoplasma orale was used as a positive control.

Ciphered, single blinded brain tissues from cases and controls were shipped overnight on dry ice for bacterial culture. Brain tissue was homogenized and incubated at 37 °C under ambient atmospheric conditions (AAC) in 2 mL SP-4 medium containing 0.5% w/v glucose plus 0.21% w/v L-arginine (SP-4 G/A) for 4 hours. The inoculated media were passed through a 0.22 µm filter to remove brain homogenate and potential environmental contaminants. Filtrates were inoculated onto SP-4 G/A agar and passed into 10 mL SP-4 G/A broth and incubated at 37 °C in AAC (broth) or 5% CO2 (agar) until mycoplasmal growth was apparent. Individual colonies were passaged in SP-4 G/A broth prior to isolate identification to segregate mixed infections. Isolates were presumptively identified by PCR-restriction fragment length polymorphism, and the identification was confirmed by direct sequencing of the 16S rRNA gene as described previously. The identity of the samples remained blinded until the end of the analysis.
4.3.7 Sequencing

All viral and *Mycoplasma* PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining under ultraviolet exposure, and amplicons were purified\textsuperscript{mn} or cloned\textsuperscript{o} and purified\textsuperscript{p} for sequencing. Purified amplicons and plasmids from PCR and SISPA were sequenced\textsuperscript{f} using the corresponding amplification primers. Species were defined by comparison of DNA sequences with GenBank database entries using the Basic Local Alignment Search Tool.

4.3.8 Immunohistochemistry

Unconjugated, lyophilized polyclonal antibodies generated in rabbits to *Mycoplasma canis* strain PG14\textsuperscript{a} were used for IHC. *M. canis* strain PG14 colonies on SP-4 agar were formalin-fixed and paraffin-embedded for use as a positive control. Negative controls included SP-4 agar with no bacteria as well as *Mycoplasma edwardsii*\textsuperscript{d} and *Mycoplasma spumans*\textsuperscript{u} grown on SP-4 agar, all formalin-fixed and paraffin-embedded. Brain tissue sections were verified to contain inflammatory lesions in all cases of GME and NME.

Optimal antibody staining was determined to occur at a final dilution\textsuperscript{v} of 1:150,000 with a staining time of 60 minutes. Non-specific binding was blocked\textsuperscript{w} for 5 minutes prior to incubation with the primary diluted antibody or universal negative control.\textsuperscript{x} Two substrate-chromogen systems were used. For the horseradish peroxidase detection system, endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 5 minutes prior to primary antibody incubation followed by addition of biotinylated goat anti-rabbit IgG,\textsuperscript{y} horseradish peroxidase conjugated streptavidin\textsuperscript{x} and 3, 3′-diaminobenzidine.\textsuperscript{x} For the alkaline phosphatase detection system, incubation with primary antibody was followed by addition of
biotinylated anti-Ig, labeled with alkaline phosphatase conjugated streptavidin and fast red. Slides were counterstained with Gills II hematoxylin and bluing.

4.3.9 Statistical analysis

The relationship between GME or NME diagnosis and Mycoplasma PCR or culture positivity was assessed by Fisher's exact tests (when \( n \leq 24 \)) or chi-square analysis with 1 degree of freedom (when \( n > 24 \)). Student's t-tests were performed, with Satterwaite's approximation when necessitated by unequal variance, to assess a relationship between clinical parameters (age at disease onset, breed, gender status, treatment administered and survival time) and Mycoplasma PCR or culture positivity. Commercially available software was used for all analyses, and significance was defined as \( P < .05 \).

4.4 RESULTS

Brain tissue was collected from 11 GME cases, 27 NME cases and 34 controls. The GME cases included 4 Dachshunds, 2 Labrador Retrievers and 1 of each of the following: Airedale Terrier, Bichon Frise, Golden Retriever, Maltese and Shih Tzu. They included 6 females (5 spayed) and 5 males (2 neutered) that ranged in age from 38 to 130 months (median 74 months; mean 67.5 months). The mean survival time from onset of clinical signs was 6.1 days (range 1-30 days). Treatment included antibiotics in 5/11 cases, glucocorticoids in 4/11 cases and anticonvulsant drugs in 3/11 cases. The NME cases included 26 Pug dogs and 1 Chihuahua. There were 20 females (15 spayed) and 7 males (all neutered), ranging in age from 4 to 84 months (median 26 months, mean 18 months). The mean survival time from onset of clinical signs was 131 days (range 1-680 days). Treatment included antibiotics in 6/27 cases, glucocorticoids in 19/27 cases, a non-steroidal immunosuppressive agent in 4/27 cases and
anticonvulsant drugs in 15/27 cases. Controls included 25 dogs of various breeds with non-neurological illness and 5 dogs with neurological illness other than GME and NME: astrocytoma (n = 1), meningioma (n = 1), canine distemper virus (CDV) encephalitis (n = 1), disc herniation (n = 1) and idiopathic meningitis (n = 1).

A total of 6 GME cases, 25 NME cases and 3 controls (1 astrocytoma, 1 meningioma, 1 CDV encephalitis) were evaluated by adenovirus, bunyavirus, coronavirus, enterovirus, flavivirus, herpesvirus, paramyxovirus and parechovirus PCR. No viral nucleic acids were detected other than amplification of CDV nucleic acids with pan-paramyxovirus primers from the control with CDV encephalitis. These same 31 cases and 3 controls were inoculated onto XC cell cultures, and 9 NME cases and 1 control (meningioma) produced a CPE. SISPA was performed on a representative CPE-producing NME case, and 344 clones were sequenced. The only viral nucleic acids identified belonged to a bovine parvovirus that was assumed to be a contaminant of the cell culture media. Additionally, electron microscopy of culture supernatants from these 9 CPE-producing NME cases did not identify viral particles other than those consistent with the previously identified parvovirus (data not shown).

Genus-specific Mycoplasma PCR was performed on 5 GME cases, 25 NME cases and 23 controls. Mycoplasma DNA was amplified from 1 dog with GME, 5 dogs with NME and 1 control (with paroviral enteritis) (Table 4.2). DNA from the 1 GME and 4 NME cases shared 99% sequence identity with *M. canis* (GenBank accessions MCU04654, FJ666136, FJ876261 and AY246564). DNA from the remaining NME case shared 99% sequence identity with *Mycoplasma canimucosale* (GenBank accession EU797451), and DNA from the control shared 97% sequence identity with *M. spumans* (GenBank accession AF538684). Statistical analysis
confirmed that detection of *M. canis* by PCR was significantly associated with a diagnosis of GME ($\chi^2 = 4.77, P = 0.03$) and NME ($\chi^2 = 4.93, P = 0.03$).

Next, 5 GME cases, 8 NME cases and 9 controls (all previously evaluated by *Mycoplasma* genus-specific PCR) were cultured in a single-blind fashion to substantiate the PCR findings. *M. canis* was cultured from 4 dogs with GME, 4 dogs with NME and 2 controls (1 with pulmonary adenocarcinoma and 1 with coagulopathy of undetermined origin) (Table 4.2). Although isolation of *M. canis* was more commonly associated with cases of GME (80%) and NME (50%) than controls (22%), these results were not statistically significant. Additionally, in 1 GME and 3 NME cases a second *Mycoplasma* species was identified: *M. edwardii* in 1 GME and 2 NME cases and *M. spumans* in 1 NME case. *M. spumans* was cultured from the dog with systemic parvoviral infection that was positive for *M. spumans* via *Mycoplasma* genus-specific PCR.

IHC was performed to determine if *M. canis* could be detected in situ in cases of GME and NME, but IHC did not detect *M. canis* in any of the 11 GME, 27 NME or 10 controls evaluated. Additionally, there were no significant associations of clinical parameters in cases of GME or NME with the presence of *Mycoplasma* or *M. canis* detected by PCR, culture or both.

4.5 DISCUSSION

Using broadly reactive PCR for 8 viral groups, as well as unbiased nucleic acid amplification with SISPA, we found no evidence of viral nucleic acids in cases of histopathologically-confirmed GME and NME. In contrast, using PCR and culture we identified *M. canis* in up to 80% of GME and 50% of NME cases. These results suggest viral infections are
not associated commonly with GME and NME but highlight the need to further evaluate the role of *M. canis* in these disorders.

Several investigators have speculated on a possible role for viruses in the pathogenesis of GME and NME, but a comprehensive search for viral pathogens has been lacking. Here, we evaluated fresh-frozen tissue from histopathologically-confirmed GME and NME cases for a diverse collection of viruses, including members of the groups adenovirus, bunyavirus, coronavirus, enterovirus, flavivirus, herpesvirus, paramyxovirus and parechovirus. Additionally, although PCR for the groups alphavirus, bornavirus, polyomavirus and rhabdovirus was not a formal part of this investigation, preliminary evaluation of GME and NME cases for these pathogens also yielded negative results (Barber and Schatzberg, unpublished). Broadly reactive PCR assays were chosen for their ability to identify unknown as well as known pathogens, and SISPA was performed as an unbiased pathogen discovery technique that does not require *a priori* knowledge of target sequences.

While the comprehensive nature of this investigation supports that viruses are not commonly associated with GME and NME, several limitations must be considered, including the small sample size, method and timing of sample collection, lack of standardized virus-infected tissue for PCR control and use of some non-canine viruses as PCR controls. Additionally, the PCR assays utilized ranged in sensitivity from 30-500 copies of target gene per reaction and SISPA often requires $10^5$ to $10^6$ viral copies for pathogen detection, which may have precluded identification of low viral loads. Importantly, however, the PCR assays utilized here have demonstrated utility in identifying viruses associated with canine CNS disease: both the pan-paramyxovirus primers and bunyavirus primers (Barber and Schatzberg, manuscript in preparation) have successfully identified viruses associated with cases of meningoencephalitis of
unknown etiology. Moreover, evaluation of brain tissue from 8 GME cases, 5 NME cases and 11 controls by highly sensitive, unbiased pan-viral microarray\textsuperscript{24} did not identify any viral pathogens, supporting the findings presented here (Kistler, Barber and Schatzberg, unpublished).

To fully utilize the GME and NME tissues collected for this investigation, \textit{Mycoplasma} genus-specific PCR was performed in addition to viral screening. Interestingly, several \textit{Mycoplasma} species regularly associated with the respiratory tract of dogs were identified, and detection of \textit{M. canis} by PCR was significantly associated with a diagnosis of GME and NME. Although \textit{M. canis} has been associated with respiratory and urogenital diseases,\textsuperscript{25} this is the first report of \textit{M. canis} associated with CNS disease or nervous tissue in any species.

The significance of finding \textit{M. canis} associated with GME and NME is unclear. \textit{M. canis} was not detected \textit{in situ}, so contamination due to non-aseptic tissue collection cannot be ruled-out. Alternatively, \textit{M. canis} may be associated with primary disease pathogenesis or may have been present secondary to previously existing disease or patient immunosuppression. Interestingly, \textit{Mycoplasma pneumonia} has been implicated as a common yet elusive etiology in acute childhood encephalitis,\textsuperscript{26} a disease with similar clinical and neuropathological features to that of GME and NME that also has a favorable clinical response to immunosuppressive therapy. Also, 5/11 GME and 6/27 NME cases received antibiotics prior to tissue collection, which may have altered the ability to detect or recover mycoplasmas in these cases. Of note, two \textit{Mycoplasma} species were identified in several cases, which could support contamination with ubiquitous organisms or represent co-infection similar to \textit{Mycoplasma synoviae} and \textit{Mycoplasma meleagridis} in turkeys.\textsuperscript{27} It also is noteworthy that CNS mycoplasmosis involving other species can result in suppurative inflammation,\textsuperscript{28,29} which is not a typical feature of GME and NME
pathology. However, without a better understanding of the pathological processes that drive GME and NME, a role for \textit{M. canis} cannot be excluded.

Mycoplasmas have been infrequently associated with CNS disease\textsuperscript{28-35} and the virulence factors that would allow \textit{M. canis} to invade and colonize the CNS are unknown. Interestingly, neuraminidase activity recently was identified in \textit{M. canis}.\textsuperscript{36} Neuraminidase is a virulence factor possessed by a wide variety of bacterial species that has been shown to promote pathogen colonization, invasion and damage of host tissue\textsuperscript{37} and recently was identified as critical for bacterial entry into the CNS in \textit{Streptococcus pneumoniae} meningitis.\textsuperscript{38}

Genetic susceptibility to \textit{M. canis} infection also must be considered.\textsuperscript{1,2,7} A missense mutation predisposing people to infection-triggered acute necrotizing encephalopathy recently was described\textsuperscript{39} and breed-based susceptibility to certain pathogens also is likely in dogs.\textsuperscript{40} It is possible that genetic immunosusceptibility to \textit{M. canis} infection may contribute to a multifactorial etiopathogenesis in dogs that develop GME and NME. Genetic differences also may explain why \textit{M. canis} infection could be associated with both GME and NME, despite their neuropathological distinctions.

In summary, molecular-based pathogen detection methodologies were used to evaluate histopathologically-confirmed cases of GME and NME for viral pathogens and mycoplasmas. The results support that viruses are not commonly associated with GME and NME but suggest a possible role for \textit{M. canis} in disease pathogenesis. Although contamination cannot be ruled-out based on the results of this investigation, this finding is important because identification of an infectious agent that contributes to the GME and NME pathogenesis could favorably alter disease diagnosis, treatment and outcome. Next steps include a prospective survey for \textit{M. canis}
from aseptically collected GME and NME brain tissue or cerebrospinal fluid and localization of M. canis via in situ hybridization.

4.6 FOOTNOTES

a Qiagen DNeasy Blood and Tissue Kit, Qiagen, Valencia, CA
b TRIzol Reagent, Invitrogen, Carlsbad, CA
c Platinum Taq DNA Polymerase kit, Invitrogen, Carlsbad, CA
d SuperScript III One-Step RT-PCR System, Invitrogen, Carlsbad, CA
e HotStarTag DNA Polymerase kit, Qiagen, Valencia, CA
f SuperScript III One-Step Quantitative RT-PCR System, Invitrogen, Carlsbad, CA
g Roche Diagnostics, Indianapolis, IN
h TaqMan, Applied Biosystems, Foster City, CA
i Roche LightCycler, Roche Diagnostics, Indianapolis, IN
j QIAamp Viral RNA Mini Kit, Qiagen, Valencia, CA
k SuperScript III First-Strand Synthesis System, Invitrogen, Carlsbad, CA
l Sequenase 2.0, United States Biochemical Corporation, Cleveland, OH
m MinElute PCR Purification Kit, Qiagen, Valencia, CA
n QIAquick Gel Extraction Kit, Qiagen, Valencia, CA
o TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA
p QIAprep Spin Miniprep Kit, Qiagen, Valencia, CA
q American Type Culture Collection (ATCC) 988, Rockville, MD
r BigDye Terminators v3.1 and ABI 3730xl, Applied Biosystems, Foster City, CA
4.7 ACKNOWLEDGEMENTS

Adenovirus, coronavirus, paramyxovirus, bunyavirus and flavivirus primers and positive controls were kindly provided by Suxiang Tong at the CDC, Atlanta, GA. Enterovirus primers and probe and enterovirus and parechovirus positive controls were kindly provided by W. Allan Nix at the CDC, Atlanta, GA. The authors thank Suxiang Tong and Susan Ruone at the CDC, Atlanta, GA for their assistance in performing viral PCR assays and SISPA and Deborah Keys for her help with statistical analysis.
Table 4.1: Broadly reactive viral polymerase chain reaction primers and probes

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<th>Target</th>
<th>Primer</th>
<th>Probe</th>
<th>Sequence (5’ → 3’)</th>
<th>Amplicon size (base pairs)</th>
<th>References</th>
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<td>DFASA</td>
<td>GTGTTCAGCTTYGNCAGYNTNTAAYCC</td>
<td></td>
<td>500</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>GDTD1B</td>
<td>CGGCATGCGACAAAAACAGGGATGCNTCNCCRTA</td>
<td></td>
<td>236</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VYGA</td>
<td>ACGTGCAACCGGGTGTAYNKTNACNGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paramyxovirus polymerase L gene</td>
<td>PAR-F1</td>
<td>GAAGGTTATGTCGAAARNTTGGGAC</td>
<td></td>
<td>650</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>PAR-F2</td>
<td>GCTGAAGTTGAGTTACCIICDATTTRTNCC</td>
<td></td>
<td>563</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PAR-R</td>
<td>GTTGCTCTTATGTTGARCGNGMAYAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parechovirus 5’ nontranslated region</td>
<td>AN345</td>
<td>GTAACASWVGGCCTCTGGGSSCAAAG</td>
<td></td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Parechovirus 5’ nontranslated region</td>
<td>AN344</td>
<td>GGCYGCGWGRCTAGATCCAYGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AN257</td>
<td>YY-CCTRYGGGTACCTYCGGCGCATCTTCTTC-BHQ1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

YY, Yakima Yellow; BHQ1, Black Hole Quencher 1; FAM, 6-carboxyfluorescein.
### Table 4.2: Cases and controls positive for mycoplasmas by polymerase chain reaction (PCR) or culture

<table>
<thead>
<tr>
<th>Dog</th>
<th>Breed</th>
<th>Gender</th>
<th>Age at disease onset (months)</th>
<th>Diagnosis</th>
<th>Treatment administered</th>
<th>Survival time (days)</th>
<th>PCR results</th>
<th>Culture results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Airedale terrier</td>
<td>MI</td>
<td>57</td>
<td>GME</td>
<td>A</td>
<td>3</td>
<td>M. canis</td>
<td>M. canis and M. edwardii</td>
</tr>
<tr>
<td>2</td>
<td>Labrador retriever</td>
<td>MI</td>
<td>102</td>
<td>GME</td>
<td>Dz</td>
<td>1</td>
<td>No amplicons</td>
<td>M. canis</td>
</tr>
<tr>
<td>3</td>
<td>Dachshund</td>
<td>FI</td>
<td>69</td>
<td>GME</td>
<td>D, Do</td>
<td>7</td>
<td>No amplicons</td>
<td>M. canis</td>
</tr>
<tr>
<td>4</td>
<td>Bichon frise</td>
<td>FS</td>
<td>90</td>
<td>GME</td>
<td>P</td>
<td>30</td>
<td>No amplicons</td>
<td>M. canis</td>
</tr>
<tr>
<td>5</td>
<td>Pug</td>
<td>FS</td>
<td>15</td>
<td>NME</td>
<td>P, L</td>
<td>497</td>
<td>M. canis</td>
<td>M. canis and M. edwardii</td>
</tr>
<tr>
<td>6</td>
<td>Pug</td>
<td>FI</td>
<td>9</td>
<td>NME</td>
<td>None</td>
<td>1</td>
<td>M. canis</td>
<td>Not cultured</td>
</tr>
<tr>
<td>7</td>
<td>Pug</td>
<td>MN</td>
<td>20</td>
<td>NME</td>
<td>P, C, Ph, Dz, Le</td>
<td>62</td>
<td>M. canis</td>
<td>Not cultured</td>
</tr>
<tr>
<td>8</td>
<td>Pug</td>
<td>FS</td>
<td>6</td>
<td>NME</td>
<td>P</td>
<td>80</td>
<td>M. canis</td>
<td>M. canis and M. spumans</td>
</tr>
<tr>
<td>9</td>
<td>Pug</td>
<td>FS</td>
<td>7</td>
<td>NME</td>
<td>P</td>
<td>43</td>
<td>M. canimucosal</td>
<td>M. canis</td>
</tr>
<tr>
<td>10</td>
<td>Pug</td>
<td>FS</td>
<td>12</td>
<td>NME</td>
<td>P, D</td>
<td>35</td>
<td>No amplicons</td>
<td>M. canis and M. edwardii</td>
</tr>
<tr>
<td>11</td>
<td>Rat terrier</td>
<td>FI</td>
<td>4</td>
<td>Parvoviral enteritis</td>
<td>None</td>
<td>N/A</td>
<td>M. spumans</td>
<td>M. spumans</td>
</tr>
<tr>
<td>12</td>
<td>Miniature Pinscher</td>
<td>MN</td>
<td>132</td>
<td>Pulmonary adenocarcinoma</td>
<td>None</td>
<td>N/A</td>
<td>No amplicons</td>
<td>M. canis</td>
</tr>
<tr>
<td>13</td>
<td>Boxer</td>
<td>FS</td>
<td>108</td>
<td>Coagulopathy</td>
<td>None</td>
<td>N/A</td>
<td>No amplicons</td>
<td>M. canis</td>
</tr>
</tbody>
</table>

MI, male intact; MN, male neutered; FI, female intact; FS, female spayed; A, amoxicillin with clavulanate; C, cyclosporine; D, dexamethasone; Do, doxycycline; Dz, diazepam; L, lomustine; Le, levetiracetam; P, prednisone; Ph, phenobarbital.
4.8 REFERENCES


CHAPTER 5

IDENTIFICATION OF LA CROSSE VIRUS NUCLEIC ACIDS IN THE CEREBROSPINAL FLUID OF DOGS WITH NEUROLOGICAL DISEASE

5.1 ABSTRACT

Background: In dogs, cases of central nervous system (CNS) inflammation that lack a definitive antemortem or postmortem diagnosis collectively are referred to as meningoencephalomyelitis of unknown etiology (MUE). Importantly, the inability to definitively diagnose antemortem cases of MUE may preclude effective treatment.

Hypothesis: Occult viral infections contribute to etiopathogenesis in a subset of MUE cases.

Animals: 76 client-owned dogs presented for neurological evaluation to 3 university referral hospitals.

Methods: Cerebrospinal fluid (CSF) was collected prospectively from dogs with neurological disease, and medical records were evaluated retrospectively to identify MUE cases and controls. CSF from MUE cases and controls was evaluated by broadly reactive polymerase chain reaction (PCR) for 8 viral groups: adenovirus, bunyavirus, coronavirus, enterovirus, flavivirus, herpesvirus, paramyxovirus and parechovirus.

Results: CSF from 46 MUE cases and 30 controls was evaluated. Bunyavirus PCR detected La Crosse virus (LACV) nucleic acids in 5/46 MUE cases and 1/30 controls. No additional viral nucleic acids were detected.

Conclusions and clinical importance: Adenovirus, coronavirus, enterovirus, flavivirus, herpesvirus, paramyxovirus and parechovirus nucleic acids were not detected in MUE cases and controls. LACV nucleic acids were detected in a minority of MUE cases and controls. Further investigation is warranted to determine the clinical significance of detecting LACV nucleic acids in the CSF of dogs with neurological disease.
5.2 INTRODUCTION

In dogs, meningoencephalomyelitis of unknown etiology (MUE) collectively represents cases of inflammatory central nervous system (CNS) disease that lack a definitive clinical, histological, cytological, serological or PCR diagnosis.\(^1\) Inflammation in cases of MUE may affect the brain, spinal cord, leptomeninges or some combination of these. Granulomatous meningoencephalomyelitis, necrotizing meningoencephalitis, necrotizing leukoencephalitis, steroid-responsive meningitis and arteritis, infection and neoplasia comprise important differential diagnoses for MUE, but the underlying etiology often remains undetermined, complicating medical management.\(^1,2\)

With the exception of canine distemper encephalitis, tick-borne encephalitis and rabies, viral infection of the CNS rarely is confirmed in dogs.\(^2\) However, viral infection should be considered in all cases of MUE with a purely mononuclear pleocytosis.\(^2\) In the past several decades there have been sporadic reports of flavivirus (West Nile virus),\(^3,4\) bornavirus\(^5,6\) and alphavirus (Eastern equine encephalitis virus)\(^7\) CNS infections in dogs, suggesting that viral etiologies for meningoencephalomyelitis may be more prevalent than previously accepted. The authors hypothesize that occult viral infections contribute to MUE etiopathogenesis in a subset of cases. To identify viruses that may be associated with meningoencephalomyelitis in dogs, we sought to detect viral nucleic acids in the cerebrospinal fluid (CSF) of dogs with MUE. CSF was collected from MUE cases and controls and evaluated by broadly reactive polymerase chain reaction (PCR) assays for pathogens in 8 viral groups: adenovirus, bunyavirus, coronavirus, enterovirus, flavivirus, herpesvirus, paramyxovirus and parechovirus.
5.3 MATERIALS AND METHODS

5.3.1 Case samples

CSF was collected in accordance with Institutional Animal Care and Use guidelines in routine fashion from the cerebellomedullary or lumbar cistern from dogs that presented with neurological signs to the University of Georgia College of Veterinary Medicine (UGA-CVM), Texas A&M University College of Veterinary Medicine and Biomedical Sciences (TAMU-CVM) and The Royal Veterinary College (RVC), University of London between 2003 and 2008. Cytologic analysis and protein quantification were performed by a board-certified clinical pathologist, and excess CSF was stored at -80°C.

Medical records of dogs from which CSF was collected were evaluated retrospectively to identify MUE cases and controls. Age, gender status, breed, clinical history, neurological signs, neuroanatomic localization, diagnostic imaging findings, clinical pathology results, presumptive diagnosis, treatment and necropsy findings were recorded when available. Cases were classified as MUE if they had $> 5$ white blood cells (WBC)/μl in the CSF, neuroanatomic localization to 1 or more areas of the CNS and no evidence of other neurological disorders. Additional cases were classified as MUE if they were small breed dogs with $< 5$ WBC/μl that had neuroanatomic localization to 1 or more areas of the CNS, magnetic resonance imaging (MRI) findings consistent with CNS inflammation and no evidence of an identifiable underlying disease process. MRI findings considered consistent with CNS inflammation were focal or multifocal lesions within the brain or spinal cord parenchyma that appeared hyperintense on T2-weighted (T2W) images and hypointense on T1-weighted images and / or T2W hyperintensity or contrast enhancement of the meninges. Cases were classified as inflammatory controls if they had $> 5$
WBC/μl in the CSF, neuroanatomic localization to the central or peripheral nervous system and evidence of an identifiable disease process (e.g. disk herniation, polyradiculoneuritis).

5.3.2 Nucleic acid extraction and PCR quality control

Total nucleic acids were extracted from CSF and stored as single-use aliquots at -80°C. A 215 base pair (bp) fragment of the canine histone 3.3 gene or 191 bp fragment of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was amplified from all samples to confirm DNA integrity. RNA integrity was confirmed in all samples by reverse transcription PCR (RT-PCR) amplification of superoxide dismutase (SOD) (expected product size 440 bp). To avoid contamination, nucleic acid extraction, PCR preparation (pre-amplification), PCR and sequencing were carried out in different rooms. Negative controls containing no DNA or RNA template were run in parallel with all PCR reactions. Additionally, mock nucleic acid extraction of sterile water was performed in parallel with all clinical cases and utilized as a negative control for PCR reactions.

5.3.3 Broadly reactive viral PCR

Consensus, degenerate or consensus-degenerate hybrid primers were used for broadly reactive viral PCR. Adenovirus PCR, bunyavirus RT-PCR and coronavirus, flavivirus and paramyxovirus semi-nested RT-PCR, herpesvirus semi-nested PCR (snPCR), and parechovirus and enterovirus real-time RT-PCR (rRT-PCR) were performed according to manufacturer's instructions with a final volume of 50 μl and final primer concentration of 1 μM unless otherwise noted. RT-PCR reactions contained 20 U RNase inhibitor and PCR and snPCR reactions used 200 μM (each) of deoxynucleotide triphosphates (dNPTs). Initial reactions were performed with 5 μl template DNA or RNA, and semi-nested reactions were performed with 2 μl of template
from the initial reaction. Assays were individually optimized and sensitivity was determined by
10-fold serial dilutions of target-containing plasmids.

Pan-adenovirus primers previously designed\textsuperscript{11} to an approximately 450 bp region of the
DNA polymerase gene (AdVE2B F and AdVE2B R) and 400 bp region of the hexon gene
(AdVhexon F and AdVhexon R) were used for PCR as previously described.\textsuperscript{12} Canine
adenovirus (CAV)-1 and CAV-2 DNA extracted from purified virus-infected tissue culture
supernatant was used as a positive control.

Pan-bunyavirus primers BCS82C and BCS332V previously designed to an approximately
251 bp region of the small segment were used for RT-PCR.\textsuperscript{13} After initial reactions at 60°C for 1
minute, 45°C for 30 minutes and 94°C for 2 minutes, RT-PCR cycled 40 times at 94°C for 15
seconds, 50°C for 30 seconds and 72°C for 30 seconds, followed by a final elongation step at
72°C for 7 minutes. RNA from a mutated clone of La Crosse virus (LACV) was used as a
positive control. Primer sensitivity was determined to be 100 to 500 copies of target gene per
reaction using Cache Valley virus and LACV.

Pan-coronavirus primers previously designed to an approximately 440 bp region of the
highly conserved polymerase 1b open reading frame were used for snRT-PCR.\textsuperscript{14} Primers F2,
R3A (0.5 μM) and R3B (0.5 μM) were used for the initial reaction and F2, R2A8 and R2B8
were used for the semi-nested reaction. Reverse transcription began at 60°C for 1 minute, 45°C
for 30 minutes and 94°C for 2 minutes, followed by 40 cycles at 94°C for 15 seconds, 50°C for
30 seconds and 72°C for 30 seconds with a final elongation step at 72°C for 7 minutes. RNA
from human coronavirus OC43 was used as a positive control (expected product size 199 bp).
Primer sensitivity was determined to be 10 to 100 copies of target gene per reaction using
representative viruses from each antigenic group.\textsuperscript{14}
Pan-flavivirus primers previously designed to an approximately 250 bp conserved region of the nonstructural protein NS5 gene were utilized for snRT-PCR. Primers cFD2 and MAMD were used for the initial reaction and cFD2 and FS778 were used for the semi-nested reaction. Reverse transcription began at 60°C for 1 minute, 42°C for 30 minutes and 94°C for 2 minutes, followed by 40 cycles at 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 1 minute with a final elongation step at 72°C for 7 minutes. RNA from a mutated clone of St. Louis encephalitis (SLE) virus was used as a positive control (expected product size 214 bp). Primer sensitivity was determined to be 100 to 500 copies of target gene per reaction using Japanese encephalitis, SLE, Dengue, West Nile and Yellow fever viruses.

Pan-herpesvirus primers previously designed to an approximately 500 bp region of the DNA polymerase gene were used for snPCR. Primers DFASA and GDTD1B were used for the initial reaction and VYGA and GDTD1B were used for the semi-nested reaction. Both reactions began with an initial hot-start at 94°C for 2.5 minutes, followed by 50 cycles at 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute, with a final elongation step at 72°C for 10 minutes. DNA from canine herpesvirus type 1 was used as a positive control (expected product size 236 bp).

Pan-paramyxovirus primers PAR-F1, PAR-F2 and PAR-R previously designed to an approximately 650 bp region of the polymerase L gene were used for snRT-PCR as previously described. Template RNA from human parainfluenza virus 2 was used as a positive control (expected product size 563 bp).

Previously designed enterovirus and parechovirus primers (0.4 μM each) and probes (0.2 μM each) previously designed based on highly conserved 5’ nontranslated regions were used for rRT-PCR. After initial reactions at 50°C for 30 minutes and 95°C for 10 minutes, rRT-PCR
cycled 50 times with the following parameters: 95°C for 15 seconds, 58°C for 30 seconds and 72°C for 10 seconds, with probe detection during the 58°C annealing step. Threshold cycle values were determined using commercially available software. Template DNA from echovirus 30 and human parechovirus 1 (Harris strain) were used as positive controls for enterovirus and parechovirus rRT-PCR, respectively. Primer sensitivity was determined to be 10-100 copies for echovirus 30 and 10-30 copies for human parechovirus 1.

5.3.4 Sequencing

All viral PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining under ultraviolet exposure, and amplicons were purified for sequencing. Purified amplicons or plasmids were sequenced using the corresponding amplification primers. Viral species were defined by comparison of DNA sequences with GenBank database entries using the Basic Local Alignment Search Tool (BLAST 2.0).

5.3.5 Statistical analysis

The relationship between MUE diagnosis and LACV PCR positivity was assessed by chi-square analysis with 1 degree of freedom. Commercially available software was used for analysis, and significance was defined as $P < .05$.

5.4 RESULTS

Cerebrospinal fluid from 76 dogs was tested by all PCR methodologies. Evaluation of medical records identified 46 cases of MUE and 30 control cases with inflammatory CSF samples. Cases included 22 dogs from UGA-CVM, 4 dogs from TAMU-CVM and 20 dogs from RVC. The range of WBCs in the CSF was 1 to 4,400 cells/μl (median, 52 cells/μl; mean, 541 cells/μl) with 21% of cases having a mixed pleocytosis, 44% a lymphocytic pleocytosis, 20% a
neutrophilic pleocytosis, 9% a monocytic pleocytosis and 6% an eosinophilic pleocytosis. Only 2 MUE cases had fewer than 5 WBC/μl in the CSF. The range of total protein was 11 to 1,696 mg/dl (median, 48 mg/dl; mean, 146 mg/dl). The dogs ranged in age from 6 months to 12 years (median, 3 years; mean, 3.6 years) and included 23 females and 23 males. Breeds represented were Beagle (n = 2), Boxer (n = 4), Golden Retriever (n = 2), Jack Russell Terrier (n = 2), Labrador Retriever (n = 4), Maltese (n = 2), mixed breed (n = 6), Pug (n = 3), West Highland White Terrier (n = 4) and 1 each of 17 additional breeds.

Control cases with inflammatory CSF included atlantoaxial subluxation (n = 1), cervical subarachnoid diverticula (n = 1), disk herniation (n = 10), fibrocartilagenous embolic myelopathy (n = 9), lymphoplasmacytic neuritis (n = 1), neoplasia (n = 4), paralumbar abscess and epidural empyema (n = 1), spinal fracture (n = 1), subdural hematoma (n = 1) and syringomyelia (n = 1).

All positive controls produced the expected PCR results; all negative controls were free of viral amplicons. Histone or GAPDH and SOD were amplified successfully from all cases. Nucleic acids from adenovirus, coronavirus, enterovirus, flavivirus, herpesvirus, paramyxovirus and parechovirus groups were not detected in the 76 samples evaluated by broadly reactive PCR. Amplicons of the expected size (251 bp) were detected by bunyavirus RT-PCR in 5 MUE cases and 1 control. Direct sequencing of the amplicons from 4/5 LACV-positive MUE cases and 1 control demonstrated > 95% sequence identity to the nucleoprotein gene of several LACV strains, including 65/OH-M (GenBank accession GU206123.1), 97/NC-M (GenBank accession GU206126.1), 93/MO-H (GenBank accession GU206138.1), 74/NY-M (GenBank accession GU206141.1), 00/WV-M (GenBank accession GU206147.1) and 00/NC-M (GenBank accession GU206111.1). Importantly, the clone of LACV used as a positive control used for pan-
bunyavirus PCR contained a 4 bp mutation so we were able to determine that the nucleic acids amplified from MUE cases and controls were not the result of carry-over contamination in the laboratory. Although LACV was detected by PCR in more MUE cases than controls, these findings were not statistically significant ($\chi^2 = 1.54, P = 0.21$).

The LACV-positive cases and control all were evaluated at the UGA-CVM. Case 1 was an 11-year-old male neutered Shih Tzu dog that presented for a 9 month history of generalized seizures. At initial presentation, the seizures were reported to occur 1 to 2 times per month. On presentation, Case 1 had a normal neurological exam, no evidence of systemic disease and no abnormalities on MRI. CSF analysis revealed 1,963 red blood cells (RBC)/μl, 12 WBC/μl, characterized by a mixed pleocytosis, and a total protein concentration of 23.8 mg/dl. No treatment was initiated. 15 months after the initial visit, Case 1 represented to the UGA-CVM for an acute onset of cluster seizures. Anticonvulsant therapy was initiated but the case was lost to follow-up.

Case 2 was an 11-year-old male neutered Boxer dog that presented for a 6 month history of generalized seizures. Neurological examination was consistent with a prosencephalic lesion localization, and MRI revealed a bilaterally symmetrical T2W hyperintensity in the area of the cingulate gyrus. The MRI lesion was not suppressed on fluid attenuated inversion recovery, did not create a mass effect and did not contrast enhance, consistent with cytotoxic edema. CSF analysis revealed 87 RBC/μl, 9 WBC/μl, characterized by a monocytic pleocytosis, and total protein concentration of 17.0 mg/dl. Case 2 also was hypoglycemic and presumptively diagnosed with an insulinoma. Case 2 did not improve with treatment; the owner elected humane euthanasia but did not give consent for necropsy.
Case 3 was a 3-year-old female spayed Pug dog that presented for a 1 year history of pelvic limb weakness and ataxia. Neurological exam was consistent with a thoracolumbar lesion localization, and MRI revealed a focal T2W hyperintensity in the spinal cord parenchyma at the level of the ninth thoracic vertebra. CSF analysis revealed 0 RBC/μl, 3 WBC/μl and a total protein concentration of 38.7 mg/dl. Additional diagnostics did not reveal evidence of concurrent systemic disease. Case 3 did not improve with an anti-inflammatory dosage of prednisone but did show clinical improvement after treatment with combined prednisone and cytosine arabinoside.

Case 4 was a 3-year-old male intact Boston Terrier dog that presented for evaluation of acute-onset blindness and an abnormal gait. Neurological exam was consistent with multifocal CNS disease but no abnormalities were identified on MRI. CSF analysis revealed 69 RBC/μl, 20 WBC/μl, characterized by a lymphocytic pleocytosis, and a total protein concentration of 16.5 mg/dl. Additional diagnostics did not reveal evidence of concurrent systemic disease. Case 5 had complete resolution of clinical signs after treatment with prednisone and cytosine arabinoside.

Case 5 was a 3-year-old female spayed Chihuahua that presented for a 1 year history of vestibular signs that had acutely worsened. Neuroanatomic localization was consistent with right central vestibular disease and MRI revealed multifocal T2W hyperintensities in the cerebrum, thalamus and brainstem. CSF analysis revealed 120 RBC/μl, 1 WBC/μl and a total protein concentration of 19.5 mg/dl. Additional diagnostics did not reveal evidence of concurrent systemic disease. The owner elected humane euthanasia but did not give consent for necropsy.

The control that tested positive for LACV by PCR was a 12-year-old female spayed Weimaraner that presented for an acute onset of seizures. The neurological exam was normal. MRI demonstrated a nasal mass that invaded the left olfactory bulb and frontal lobe through the
cribiform plate. CSF analysis revealed 38 RBC/μl, 26 WBC/μl, characterized by a lymphocytic pleocytosis, and a total protein concentration of 96.1 mg/dl. Biopsy of the nasal mass was consistent with a diagnosis of nasal adenocarcinoma.

Case 5 was the only LACV-positive dog to have received immunosuppressive therapy prior to evaluation and CSF collection.

5.5 DISCUSSION

Using broadly reactive PCR for 8 viral groups we found that the majority of MUE cases and controls evaluated had no evidence of underlying viral infection. However, LACV nucleic acids were detected in 10% of MUE cases and 3% of controls. These results suggest that LACV infection of the CNS of dogs may be more common than previously recognized. Additional work is necessary to determine how frequently LACV infection contributes to clinical disease in dogs.

LACV is an RNA virus of the California serogroup in the genus Bunyavirus, family Bunyaviridae. LACV is maintained between mosquito and vertebrate hosts in deciduous forest habitat and is thought to primarily be transmitted by the bite of the eastern treehole mosquito, Aedes triseriatus.20,21 Importantly, dogs are not thought to act as reservoir hosts.22 Prior to the detection of West Nile virus in 1999,23 LACV was the most commonly reported causative agent of arboviral disease in the United States.24,25 The majority of infections with LACV in people are thought to be asymptomatic or result in mild, flu-like illness, making it difficult to determine the true incidence of LACV infection.26-29 However, neuroinvasive disease can occur and is reportable nationwide.24 Neuroinvasive disease most commonly occurs in children less than 15 years of age26,30 and typically manifests as meningitis, encephalitis or meningoencephalitis.30 Common clinical signs include headache, fever, vomiting, seizures and disorientation.31 Cerebral
edema and inflammation may be seen on advanced imaging, and brain biopsy has demonstrated perivascular infiltrates of mononuclear cells.\textsuperscript{31}

There are two previous reports associating LACV with CNS disease in dogs. In 1994, 5 puppies submitted for necropsy to a diagnostic laboratory in south Georgia were diagnosed with necrotizing panencephalitis secondary to LACV infection.\textsuperscript{32} The puppies were from 2 different litters and were less than 3 weeks of age at the time of death; 3 of the puppies were reported to have had seizures while 2 were found dead. The 5 puppies had similar neuropathological lesions, which included meningeal and perivascular infiltrates of mononuclear cells with focally extensive areas of randomly distributed necrosis. The cerebral cortex was most severely affected. LACV was isolated from brain tissue homogenates. In 1999, La Crosse virus meningoencephalomyelitis diagnosed by immunohistochemistry was reported in a single dog in south Florida.\textsuperscript{33} The dog was a 4-year-old male neutered mixed breed with an acute onset of neurological signs consistent with multifocal CNS disease. The condition initially responded to treatment with glucocorticoids and broad-spectrum antibiotics but ultimately the dog developed cluster seizures prior to spontaneous death. Neuropathological examination of the tissue revealed perivascular infiltrates of mononuclear cells and necrosis of the gray and white matter in the brain and cervical spinal cord.

Unfortunately, it cannot be determined whether detection of LACV nucleic acids in the CSF of the dogs in this investigation represents active or previous infection with LACV. Identification of active infection would require virus isolation from the CNS or serological confirmation of active disease. Laboratory diagnosis of LACV infections in people typically is accomplished by evaluation of serum or CSF for virus-specific immunoglobulin (Ig) M and neutralizing antibodies.\textsuperscript{34-36} Currently there are no established diagnostic criteria for LACV
infection in dogs and there are no readily available serological tests. Virus isolation from CSF was not attempted in this investigation. Additionally, serum was not collected from the cases used in this investigation. However, a significant amount of work was performed to develop an assay for serological evaluation of CSF (Calisher and Bowen, unpublished). CSF from MUE cases and controls was evaluated by enzyme-linked immunosorbent assay for LACV-specific IgM and IgG antibodies and by immunofluorescence assay (IFA) for LACV-specific IgG antibodies but an appropriate positive control was not available so these assays could not be optimized and the results were difficult to interpret. To obtain a positive control for serology, 2 dogs were inoculated with LACV in accordance with Animal Care and Use guidelines and serum and CSF were obtained on days 7, 15, 21, 35 and 49. Unfortunately, however, several additional attempts to develop ELISA and IFA assays using these experimental samples were unsuccessful. A previously developed neutralization assay may aid in further serological evaluation of dogs for LACV antibodies.22

Clinical features also are important in the diagnosis of active LACV infection in people, but the clinical signs of LACV infection in dogs are not well understood and the frequency of asymptomatic and symptomatic infections is unknown. The LACV-positive dogs in this investigation had variable clinical signs, including seizures, blindness, pelvic limb weakness, ataxia and vestibulocerebellar signs. Of these, seizures are the clinical sign most commonly associated with LACV infection. Approximately 50% of people diagnosed with LACV neuroinvasive disease present with seizures31 and 4 dogs previously reported with LACV infection had seizures as a clinical sign.32,33 However, it is impossible to determine if LACV detection in any of the cases presented here was associated with clinical disease. Importantly, the control that was LACV-positive by PCR presented for seizures but a CNS invasive nasal
adenocarcinoma was identified. Screening of healthy and clinically diseased dogs in endemic regions is necessary to determine the incidence and clinical significance of LACV infection in dogs.

Not surprisingly, all of the dogs that were positive for LACV by PCR in this investigation were from Georgia. LACV infection in people most commonly is reported in upper Midwestern, mid-Atlantic and southeastern states, it is likely that LACV infection in dogs also would predominate in these regions.

Finally, although no viruses were detected from the 7 other viral groups evaluated here, it is possible that viruses other than LACV were missed due to study limitations, including small sample size, timing of sample collection, lack of standardized virus-infected CSF for PCR control and use of some non-canine viruses as PCR controls. Additionally, the PCR assays utilized ranged in sensitivity from 30-500 copies of target gene per reaction, which may have precluded identification of low viral loads. Highly sensitive assays such as panviral microarray and deep sequencing would be ideal for screening CSF from MUE cases for viruses but at this time those assays remain cost prohibitive for evaluation of large numbers of samples.

Overall, this investigation utilized broadly reactive PCR to evaluate for a wide variety of viral pathogens in cases of MUE. Although the findings were primarily negative, LACV nucleic acids were detected in the CSF of 6 dogs with neurological signs. These findings, combined with previous reports of CNS invasive LACV infection in dogs suggest that this pathogen may be more common in dogs than previously reported. Importantly, if clinical disease can definitively be associated with LACV infection, more directed therapeutic intervention could be instituted.
Critical next steps include additional screening of dogs for LACV and development of a reliable serological assay to test for LACV antibodies in dogs.

5.6 FOOTNOTES

a Qiagen MinElute Virus Spin Kit, Qiagen, Valencia, CA

b Platinum Taq DNA Polymerase kit, Invitrogen, Carlsbad, CA

c SuperScript III One-Step RT-PCR System, Invitrogen, Carlsbad, CA

d HotStarTaq DNA Polymerase kit, Qiagen, Valencia, CA

e SuperScript III One-Step Quantitative RT-PCR System, Invitrogen, Carlsbad, CA

f Roche Diagnostics, Indianapolis, IN

g TaqMan, Applied Biosystems, Foster City, CA

h Roche LightCycler, Roche Diagnostics, Indianapolis, IN

i MinElute PCR Purification Kit, Qiagen, Valencia, CA

j QIAquick Gel Extraction Kit, Qiagen, Valencia, CA

k BigDye Terminators v3.1 and ABI 3730xl, Applied Biosystems, Foster City, CA

l SAS V 9.2, Cary, NC

5.7 ACKNOWLEDGEMENTS

The authors give their sincerest thanks to C.H. Calisher and R.A. Bowen at the Colorado State University College of Veterinary Medicine and Biomedical Sciences for their work to develop a La Crosse virus serology assay for dogs. This study was funded by grants from the Morris Animal Foundation and American Kennel Club Canine Health Foundation. Preliminary
results of this study were presented at the 27th Annual American College of Veterinary Internal Medicine Forum, Montréal, Québec, Canada, 2009.

5.8 REFERENCES


CHAPTER 6
IDENTIFICATION OF RISK LOCI FOR NECROTIZING MENINGOENCEPHALITIS IN PUG DOGS

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6.1 ABSTRACT

Due to their unique population structure, purebred dogs have emerged as a key model for the study of complex genetic disorders. To evaluate the utility of a newly available high density canine whole genome array with > 170,000 SNPs, genome-wide association was performed on a small number of case and control dogs to determine disease susceptibility loci in canine necrotizing meningoencephalitis (NME), a disorder with known non-Mendelian inheritance that shares clinical similarities with atypical variants of multiple sclerosis in humans. Genotyping of 30 NME-affected Pug dogs and 68 healthy, control Pugs identified two loci associated with NME, including a region within dog leukocyte antigen class II on chromosome 12 that remained significant after Bonferroni correction. Our results support the utility of this high density SNP array, confirm that dogs are a powerful model for mapping complex genetic disorders and provide important preliminary data to support in depth genetic analysis of NME in numerous affected breeds.
6.2 INTRODUCTION

Necrotizing meningoencephalitis (NME) is an idiopathic inflammatory disorder of the central nervous system (CNS) that primarily affects young to middle aged toy breed dogs.\textsuperscript{1-3} Inflammation in NME is characterized by mixed mononuclear cell infiltrates within the cerebral hemispheres and cortical leptomeninges with common clinical signs including seizures, depression, behavior change, circling and visual deficits.\textsuperscript{1} Similar to severe non-prototypical forms of multiple sclerosis (MS) such as Marburg variant,\textsuperscript{4,5} NME is overrepresented in females, is rapidly progressive and often carries a grave prognosis despite aggressive immunosuppressive treatment.\textsuperscript{6,7} NME initially was identified in Pug dogs in the late 1960s\textsuperscript{1} and is known to have a strong familial association in this breed.\textsuperscript{1,6} Studies of Pugs with NME suggest there are likely multiple genes that contribute to disease phenotype\textsuperscript{6} and a recent genome-wide study of simple tandem repeat markers in this breed identified regions of disease susceptibility within DLA II, similar to major histocompatibility complex (MHC) loci previously identified in MS and other proposed autoimmune diseases.\textsuperscript{7}

Purebred dog populations provide a unique opportunity for mapping genetic traits and recent technological developments have made it possible to leverage dogs as a model for the study of human genetic disease.\textsuperscript{8} The extensive linkage disequilibrium within breeds allows successful genome-wide mapping of traits and disease risk using smaller numbers of cases and controls than typically are required in a human-based study,\textsuperscript{9,10} and dogs and humans share similar physiology with over half of the known canine diseases having a similar phenotype to analogous human diseases.\textsuperscript{11} Significant advances in canine genomics, including publication of a high quality draft genome sequence and identification of $> 2.5$ million SNPs, have facilitated mapping of simple and complex canine genetic traits,\textsuperscript{8,9,12} and genome-wide SNP arrays with
coverage of up to 50,000 SNPs have been used successfully to map canine traits.\textsuperscript{8,12,13} The objective of this investigation was to employ a newly available, high density array to evaluate canine NME, a disorder with a presumed autoimmune etiology and complex mode of inheritance that has clinical similarities to atypical, fulminant variants of MS. The identification of genetic risk factors should improve our understanding of NME pathophysiology, increase our ability to identify at risk and affected dogs, allow institution of targeted therapy and ultimately may help in the identification of similar genetic factors that are associated with the development of rapidly progressive MS in people.

6.3 MATERIALS AND METHODS

6.3.1 Study population

Purebred Pug dogs were used for the case-control genome-wide association study. Cases were verified to have NME based on signalment, clinical history and independent evaluation of hematoxylin and eosin brain sections by a veterinary neuropathologist. Cases ranged in age from 4 to 84 months (mean = 18 months, median = 26 months) and consisted of 11 males and 19 females. Control dogs had no evidence of neurological or autoimmune disease, ranged in age from 5 to 204 months (mean = 60 months, median = 48 months) at the time of sample collection and consisted of 30 males and 38 females. Control dogs were followed for 18 months after sample collection to verify that they did not develop neurological or autoimmune disease.

6.3.2 SNP genotyping

Genomic DNA was isolated using the Qiagen (Valencia, CA) Gentra Puregene Tissue Kit or Qiagen DNeasy Blood and Tissue Kit. SNP genotyping was performed with the Illumina (San
6.3.3 Statistical analysis

Genotyping was performed on 98 dogs, including 30 NME cases and 68 controls. Genome-wide analysis was performed with PLINK. Concordance on duplicate samples was 99.96%. Only samples with a call rate of > 95% were included, resulting in analysis of 28 NME cases and 66 controls. A total of 172,115 SNPs were genotyped. Classic multidimensional scaling using a call rate of > 97% and MAF of > 0.10 was performed on 85,366 SNPs to determine population stratification, and 21 controls that were not clustered with the main population of dogs were excluded resulting in a final population of 28 NME cases and 45 controls for analysis. These 45 control dogs ranged in age from 5 to 204 months (mean = 80 months, median = 48 months). Prior to analysis, 7,324 SNPs were excluded for failure to reach the call rate threshold (> 95%) and 81,001 SNPs were excluded for failure to reach the MAF threshold (> 0.05). In total, 86,692 SNPs were used for analysis. Bonferroni correction was applied to account for multiple hypothesis testing with a resulting P value of $5.77 \times 10^{-7}$ across 86,692 SNPs for genome-wide significance. To further evaluate genome-wide significance MaxT permutation testing of 100,000 permutations was applied.

6.4 RESULTS

Initial genotyping was performed on 30 NME cases and 68 controls across 172,115 SNPs. After quality filtering and exclusion of population outliers (Figure 6.1), analysis of 28 NME cases and 45 controls across 86,692 SNPs identified two disease-associated loci that reached genome-wide significance with correction for multiple hypothesis testing. The strongest
association was on chromosome 12 where 35 SNPs within the DLA class II region reached genome-wide significance after Bonferroni correction (raw P value for Bonferroni genome-wide significance < $5.77 \times 10^{-7}$) with the highest SNP having an odds ratio of 16.1 (95% CI: 4.7 - 55.5) (Figure 6.2 and Table 6.1). Permutation testing using 100,000 permutations identified an additional four SNPs that reached genome-wide permuted significance within the DLA II locus and a second region of significance within the STYX gene on chromosome 8 ($P_{raw} = 2.11 \times 10^{-6}$, $P_{permuted} = 0.045$) with an odds ratio of 5.9 (95% CI: 2.7 - 12.5) (Figure 6.2 and Table 6.2). To account for the fact that several of the control dogs were younger than the mean age of disease onset at the time of sample acquisition, the data was re-analyzed excluding all control dogs less than 24 months of age. Both the DLA and chromosome 8 regions remained significant with Bonferroni correction and permutation testing, respectively, but the significance was not improved by this exclusion (data not shown).

Haplotype analysis using Haploview\textsuperscript{16} identified 19 haplotype blocks across a 4.1 Mb region of DLA II on chromosome 12, all of which were associated with an increased risk for developing NME with P values ranging from $2.1 \times 10^{-3}$ to $1.13 \times 10^{-8}$ (Figure 6.3 and Table 6.3). Manually forcing all of these haplotype blocks into a single haplotype resulted in the creation of a 4.1 Mb haplotype containing 241 SNPs. This haplotype was common and strongly associated with an increased risk of developing NME (case frequency 85.1%, control frequency 38.4%, $P = 7.97 \times 10^{-7}$). Haplotype analysis of the STYX region of chromosome 8 identified four haplotypes (Figure 6.4). The most significantly associated and common haplotype spanned the STYX and GNPNAT1 genes and was protective based on phenotype ($P = 1.43 \times 10^{-6}$) (Table 6.4). This block also contained two additional haplotypes significantly associated with NME risk ($p \sim 0.005$, data not shown).
6.5 DISCUSSION

Genome-wide analysis of NME in Pug dogs identified two disease-associated loci, including a strong association with DLA II. Although recognition of self antigen has not been demonstrated definitively as a mechanism of pathogenesis in NME, CNS anti-astrocytic antibodies have been identified\(^{17}\) and the strong DLA II association further supports an autoimmune etiology. Similar to our findings in NME, most autoimmune diseases are polygenic with MHC II polymorphisms having the strongest disease association.\(^{18}\) Haplotype analysis of the DLA II region identified a large, common block strongly associated with altered disease risk. The large number of genes present within this haplotype precludes the precise identification of the associated gene without additional fine mapping and sequencing of this region. Although initially described in the Pug, NME has now been described in numerous other breeds including the Maltese\(^2\) and Chihuahua\(^3\) with identical clinical presentation and pathology suggesting a similar etiopathogenesis among these breeds.\(^2,3\) Fine mapping across breeds should allow identification of smaller disease-associated haplotypes in this region.\(^{12,19}\)

The role of STYX and GNPNAT1 in NME also require further characterization. STYX, serine / threonine / tyrosine interacting protein, is a pseudophosphatase that lacks intrinsic catalytic activity and is structurally similar to members of the dual-specificity phosphatase subfamily of protein tyrosine phosphatases.\(^20\) The only documented role of STYX is in normal sperm formation in mice.\(^21\) The STYX protein has been found in numerous tissues in mice including brain,\(^20\) but its presence and role in immune cells has not been determined. Protein tyrosine phosphatases play a key role in immune system function including lymphocyte activation, with mutations in \(PTPN22\) having been documented in association with autoimmunity.\(^22\) Less is known about the role of pseudophosphatases in immune and
inflammatory responses, although a mutation in the pseudophosphatase MTMR13 has been implicated in a form of Charcot–Marie–Tooth neuropathy,\textsuperscript{23} documenting a role for these proteins in development and maintenance of nervous tissue. STYX also is known to bind to the calcineurin substrate CRHSP-24.\textsuperscript{21} Although calcineurin plays an important documented role in T cell activation, the role of CRHSP-24 is less clear. CRHSP-24 has been found ubiquitously in rat tissue and its dephosphorylation is prevented by administration of the immunomodulatory drugs cyclosporine and FK506.\textsuperscript{24} Interestingly, CRHSP-24 has a brain specific paralog, PIPPin, although interactions between STYX and PIPPin have not been documented.\textsuperscript{21}

**GNPNAT1**, glucosamine-phosphate \(N\)-acyetyltransferase 1, is involved in amino sugar metabolism including the formation of uridine diphospho-\(N\)-acyetylglucosamine (UDP-GlcNAc). UDP-GlcNAc is an important cellular metabolite necessary for the synthesis of chitin, glycosylphosphatidylinositol protein anchors and \(N\)-linked and \(O\)-linked glycans.\textsuperscript{25} \(N\)- and \(O\)-glycans play a documented role in normal thymic T-cell apoptosis,\textsuperscript{26} disruption of which could be speculated to lead to aberrant immune responses in NME.

The utility of dogs for the study of genetic traits has been recognized in recent years.\textsuperscript{11} Many common human diseases have a complex mode of inheritance, but responsible genes have remained elusive despite advances in the field of genomics.\textsuperscript{27} The striking similarity of many naturally occurring canine diseases with specific human diseases suggests that risk genes and mechanistic pathways identified in dogs could be applied to advance our knowledge of human disease. The availability of ever-improving technologies for the study of canine genomics makes such comparative studies possible. This investigation evaluated a new genome-wide SNP array with a significantly greater density than previously available arrays (averaging greater than 70 SNPs per Mb). The study confirmed the utility of this array, identifying disease-associated loci in
less than 50 cases and 50 controls. Importantly, an unexpected amount of genetic variability existed within the purebred population of dogs evaluated here, requiring a large number of control dogs to be excluded from the study. These results highlight the importance of testing for population stratification even among canine breed populations that are assumed to be relatively homogeneous.28

In conclusion, evaluation of a canine CNS inflammatory disease that shares similarities with atypical, severe forms of MS identified two important loci associated with disease development. Identification of these loci is an important step in elucidating the pathogenesis of NME. Further structural and functional analysis of the identified loci should improve the global understanding of idiopathic CNS inflammation and may aid in the development of improved diagnostics and treatments for dogs that suffer from this devastating disorder. Moreover, this information may help identify key genetic risk factors in atypical variants of MS.

6.6 ACKNOWLEDGEMENTS

The authors thank the Pug owners, breeders, veterinarians and breed clubs that contributed samples and supported completion of this investigation. This work was supported by Illumina, Inc.; Morris Animal Foundation; the American Kennel Club Canine Health Foundation; and the Pug Dog Club of America. This work was presented in part at the 5th International Conference: Advances in Canine and Feline Genomics and Inherited Diseases, Baltimore, MD, September 22-25, 2010.
Figure 6.1: Multidimensional scaling (MDS) analysis of population stratification. MDS using a call rate of > 97% and MAF of > 0.01 was performed on 85,366 SNPs to determine population stratification on the initial 28 NME cases and 66 controls with sample call rates > 95%, and 21 controls were excluded based on population variance. MDS component 1 (x-axis) was plotted against MDS component 2 (y-axis) with filled circles representing individual Pug dogs.
Figure 6.2: Genome-wide association results for 28 NME cases and 45 controls. (a) Fisher’s exact tests were performed to compare SNP allele frequencies and negative log P values were plotted across the genome. The horizontal dotted line represents the threshold for significant association after Bonferroni correction of \(-\log(P) > 6.24\) with a strong peak on chromosome 12 maintaining genome-wide significance. (b) MaxT 100,000 permutation testing was performed and negative log P values were plotted across the genome. The horizontal dotted line represents the threshold for significant association after permutation testing of \(-\log(P) > 1.3\) with one SNP on chromosome 8 maintaining permuted significance.
Table 6.1: SNPs with genome-wide significance after Bonferroni correction

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<th>FA / FU</th>
<th>Praw</th>
<th>Pgenome</th>
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<td>0.79 / 0.33</td>
<td>8.07 x 10^-10</td>
<td>0.0070</td>
<td>7.5 [3.4 - 16.2]</td>
<td>SPDEF</td>
</tr>
</tbody>
</table>

Abbreviations: Chr, chromosome; Pos, physical position; AR, risk allele; ANR, non-risk allele; FA, allele frequency in cases; FU, allele frequency in controls.
Table 6.2: SNPs with genome-wide significance after permutation testing

<table>
<thead>
<tr>
<th>Canine SNP</th>
<th>Chr</th>
<th>Pos</th>
<th>AR / ANR</th>
<th>FA / FU</th>
<th>Praw</th>
<th>Ppermuted</th>
<th>OR [95% CI]</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>BICF2P3516667*</td>
<td>8</td>
<td>31971609</td>
<td>A / G</td>
<td>0.73 / 0.32</td>
<td>2.11 × 10⁻⁶</td>
<td>0.0452</td>
<td>5.9 [2.8 - 12.5]</td>
<td>STYX</td>
</tr>
<tr>
<td>BICF2P78662*</td>
<td>12</td>
<td>5166878</td>
<td>A / G</td>
<td>0.95 / 0.51</td>
<td>1.62 × 10⁻⁸</td>
<td>0.0089</td>
<td>16.8 [4.8 - 58.3]</td>
<td>RT1-Db2</td>
</tr>
<tr>
<td>BICF2P3225431*</td>
<td>12</td>
<td>5217389</td>
<td>G / A</td>
<td>0.86 / 0.35</td>
<td>3.43 × 10⁻⁹</td>
<td>0.0022</td>
<td>11 [4.6 - 26.3]</td>
<td>RT1-Db2</td>
</tr>
<tr>
<td>BICF2P292492*</td>
<td>12</td>
<td>5227499</td>
<td>G / A</td>
<td>0.86 / 0.35</td>
<td>3.43 × 10⁻⁹</td>
<td>0.0022</td>
<td>11 [4.6 - 26.3]</td>
<td>RT1-Db2</td>
</tr>
<tr>
<td>rs8856588*</td>
<td>12</td>
<td>5710832</td>
<td>G / A</td>
<td>0.79 / 0.28</td>
<td>5.02 × 10⁻⁹</td>
<td>0.0018</td>
<td>9.4 [4.2 - 20.9]</td>
<td>COL11A2</td>
</tr>
<tr>
<td>BICF2P74765*</td>
<td>12</td>
<td>5734305</td>
<td>A / G</td>
<td>0.79 / 0.28</td>
<td>5.02 × 10⁻⁹</td>
<td>0.0018</td>
<td>9.4 [4.2 - 20.9]</td>
<td>TAPBP</td>
</tr>
<tr>
<td>BICF2P185629*</td>
<td>12</td>
<td>5791672</td>
<td>G / A</td>
<td>0.79 / 0.28</td>
<td>5.02 × 10⁻⁹</td>
<td>0.0018</td>
<td>9.4 [4.2 - 20.9]</td>
<td>KIFC1</td>
</tr>
<tr>
<td>BICF2P540937*</td>
<td>12</td>
<td>5829667</td>
<td>G / A</td>
<td>0.79 / 0.30</td>
<td>5.02 × 10⁻⁹</td>
<td>0.0018</td>
<td>9.4 [4.2 - 20.9]</td>
<td>COLMA1</td>
</tr>
<tr>
<td>BICF2P32322760*</td>
<td>12</td>
<td>5992526</td>
<td>A / G</td>
<td>0.79 / 0.28</td>
<td>5.02 × 10⁻⁹</td>
<td>0.0018</td>
<td>9.4 [4.2 - 20.9]</td>
<td>COLMA1</td>
</tr>
<tr>
<td>BICF2P1200278*</td>
<td>12</td>
<td>6024841</td>
<td>A / C</td>
<td>0.79 / 0.28</td>
<td>5.02 × 10⁻⁹</td>
<td>0.0018</td>
<td>9.4 [4.2 - 20.9]</td>
<td>COLMA1</td>
</tr>
<tr>
<td>BICF2P863589*</td>
<td>12</td>
<td>6059850</td>
<td>A / G</td>
<td>0.88 / 0.44</td>
<td>1.40 × 10⁻⁷</td>
<td>0.0329</td>
<td>8.9 [3.6 - 22.1]</td>
<td>COLMA1</td>
</tr>
<tr>
<td>BICF2P1115728*</td>
<td>12</td>
<td>6160615</td>
<td>A / C</td>
<td>0.79 / 0.28</td>
<td>5.02 × 10⁻⁹</td>
<td>0.0018</td>
<td>9.4 [4.2 - 20.9]</td>
<td>COLMA1</td>
</tr>
<tr>
<td>BICF2P1254053*</td>
<td>12</td>
<td>6164202</td>
<td>A / C</td>
<td>0.79 / 0.28</td>
<td>5.02 × 10⁻⁹</td>
<td>0.0018</td>
<td>9.4 [4.2 - 20.9]</td>
<td>COLMA1</td>
</tr>
<tr>
<td>BICF2P1340012*</td>
<td>12</td>
<td>6184107</td>
<td>A / C</td>
<td>0.79 / 0.28</td>
<td>5.02 × 10⁻⁹</td>
<td>0.0018</td>
<td>9.4 [4.2 - 20.9]</td>
<td>COLMA1</td>
</tr>
<tr>
<td>BICF2P1211546*</td>
<td>12</td>
<td>6202865</td>
<td>G / A</td>
<td>0.79 / 0.28</td>
<td>5.02 × 10⁻⁹</td>
<td>0.0018</td>
<td>9.4 [4.2 - 20.9]</td>
<td>COLMA1</td>
</tr>
<tr>
<td>BICF2P863589*</td>
<td>12</td>
<td>6311277</td>
<td>C / A</td>
<td>0.86 / 0.35</td>
<td>3.43 × 10⁻⁹</td>
<td>0.0022</td>
<td>11 [4.6 - 26.3]</td>
<td>COLMA1</td>
</tr>
<tr>
<td>BICF2P1300012*</td>
<td>12</td>
<td>6320910</td>
<td>A / G</td>
<td>0.79 / 0.28</td>
<td>5.02 × 10⁻⁹</td>
<td>0.0018</td>
<td>9.4 [4.2 - 20.9]</td>
<td>COLMA1</td>
</tr>
<tr>
<td>BICF2P1211546*</td>
<td>12</td>
<td>6342204</td>
<td>A / C</td>
<td>0.79 / 0.28</td>
<td>5.02 × 10⁻⁹</td>
<td>0.0018</td>
<td>9.4 [4.2 - 20.9]</td>
<td>LOC1127664</td>
</tr>
<tr>
<td>BICF2P738738*</td>
<td>12</td>
<td>6653816</td>
<td>A / G</td>
<td>0.79 / 0.33</td>
<td>1.30 × 10⁻³</td>
<td>0.0187</td>
<td>7.5 [3.4 - 16.4]</td>
<td>NUDT3</td>
</tr>
<tr>
<td>BICF2P639740</td>
<td>12</td>
<td>6686088</td>
<td>A / G</td>
<td>0.79 / 0.33</td>
<td>1.30 × 10⁻³</td>
<td>0.0283</td>
<td>7.5 [3.4 - 16.4]</td>
<td>NUDT3</td>
</tr>
<tr>
<td>BICF2P535495</td>
<td>12</td>
<td>6793393</td>
<td>G / A</td>
<td>0.79 / 0.33</td>
<td>1.30 × 10⁻³</td>
<td>0.0453</td>
<td>7.5 [3.4 - 16.4]</td>
<td>MGC8455</td>
</tr>
<tr>
<td>BICF2P1306021*</td>
<td>12</td>
<td>6809061</td>
<td>A / G</td>
<td>0.79 / 0.32</td>
<td>5.39 × 10⁻⁸</td>
<td>0.0142</td>
<td>7.9 [3.6 - 17.4]</td>
<td>SPDEF</td>
</tr>
<tr>
<td>BICF2P1462392*</td>
<td>12</td>
<td>6832252</td>
<td>A / G</td>
<td>0.79 / 0.32</td>
<td>5.39 × 10⁻⁸</td>
<td>0.0142</td>
<td>7.9 [3.6 - 17.4]</td>
<td>SPDEF</td>
</tr>
<tr>
<td>rs957837</td>
<td>12</td>
<td>8822596</td>
<td>C / G</td>
<td>0.77 / 0.33</td>
<td>3.94 × 10⁻¹²</td>
<td>0.0356</td>
<td>6.7 [3.1 - 14.6]</td>
<td>LOC112577</td>
</tr>
</tbody>
</table>

Chr, chromosome; Pos, position; AR, risk allele; ANR, non-risk allele; FA, allele frequency in cases; FU, allele frequency in controls; *SNP also significant after Bonferroni correction.
Figure 6.3: Haplotype analysis of NME-associated DLA II locus on chromosome 12. A 4.1 Mb region located from positions 4,713,392 to 8,834,652 is shown with 19 haplotype blocks generated in Haploview. Negative log P values from single SNP associations were derived from genome-wide analysis after removal of population outliers and individual SNPs were plotted as red circles. The horizontal dotted line represents the threshold for significant association after Bonferroni correction of -log(P) > 6.24.
<table>
<thead>
<tr>
<th>Block</th>
<th>Haplotype</th>
<th>Population frequency</th>
<th>Case frequency</th>
<th>Control frequency</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AAGGAGAAA</td>
<td>0.59</td>
<td>0.84</td>
<td>0.43</td>
<td>1.25 x 10^{-6}</td>
</tr>
<tr>
<td>2</td>
<td>AGGCAAG</td>
<td>0.60</td>
<td>0.80</td>
<td>0.47</td>
<td>5.49 x 10^{-5}</td>
</tr>
<tr>
<td>3</td>
<td>GCAGGAGGGGA</td>
<td>0.49</td>
<td>0.79</td>
<td>0.30</td>
<td>1.13 x 10^{-8}</td>
</tr>
<tr>
<td>4</td>
<td>AAAACAGGAAGAG</td>
<td>0.49</td>
<td>0.79</td>
<td>0.30</td>
<td>1.13 x 10^{-8}</td>
</tr>
<tr>
<td>5</td>
<td>AGAAAGCGA</td>
<td>0.49</td>
<td>0.79</td>
<td>0.30</td>
<td>1.13 x 10^{-8}</td>
</tr>
<tr>
<td>6</td>
<td>AAGGAGAAAGAGAGAGAATGGACA</td>
<td>0.49</td>
<td>0.79</td>
<td>0.30</td>
<td>1.13 x 10^{-8}</td>
</tr>
<tr>
<td>7</td>
<td>GAAGAGAAGGACAGA</td>
<td>0.49</td>
<td>0.79</td>
<td>0.30</td>
<td>1.13 x 10^{-8}</td>
</tr>
<tr>
<td>8</td>
<td>GGAACAGGAAAAAGAGAGGAGGCGAA</td>
<td>0.51</td>
<td>0.79</td>
<td>0.33</td>
<td>1.06 x 10^{-7}</td>
</tr>
<tr>
<td>9</td>
<td>CGGGAAAAGGGAGGAGGC</td>
<td>0.67</td>
<td>0.88</td>
<td>0.53</td>
<td>2.20 x 10^{-5}</td>
</tr>
<tr>
<td>10</td>
<td>AG</td>
<td>0.73</td>
<td>0.88</td>
<td>0.64</td>
<td>2.10 x 10^{-3}</td>
</tr>
<tr>
<td>11</td>
<td>AAAAAG</td>
<td>0.50</td>
<td>0.75</td>
<td>0.34</td>
<td>1.88 x 10^{-5}</td>
</tr>
<tr>
<td>12</td>
<td>AGGAGA</td>
<td>0.70</td>
<td>0.89</td>
<td>0.58</td>
<td>5.47 x 10^{-5}</td>
</tr>
<tr>
<td>13</td>
<td>AG</td>
<td>0.50</td>
<td>0.75</td>
<td>0.34</td>
<td>1.88 x 10^{-6}</td>
</tr>
<tr>
<td>14</td>
<td>AATAAAAGAGACGA</td>
<td>0.50</td>
<td>0.75</td>
<td>0.34</td>
<td>1.88 x 10^{-6}</td>
</tr>
<tr>
<td>15</td>
<td>GGAGG</td>
<td>0.50</td>
<td>0.75</td>
<td>0.34</td>
<td>1.88 x 10^{-6}</td>
</tr>
<tr>
<td>16</td>
<td>GCAAG</td>
<td>0.50</td>
<td>0.75</td>
<td>0.34</td>
<td>1.88 x 10^{-6}</td>
</tr>
<tr>
<td>17</td>
<td>GGCGAGCAAAA</td>
<td>0.50</td>
<td>0.75</td>
<td>0.34</td>
<td>1.88 x 10^{-6}</td>
</tr>
<tr>
<td>18</td>
<td>ATGAACCG</td>
<td>0.51</td>
<td>0.77</td>
<td>0.34</td>
<td>6.49 x 10^{-7}</td>
</tr>
<tr>
<td>19</td>
<td>GGCA</td>
<td>0.51</td>
<td>0.77</td>
<td>0.34</td>
<td>6.49 x 10^{-7}</td>
</tr>
</tbody>
</table>
Figure 6.4: Haplotype analysis of NME-associated locus on chromosome 8. A 488 kb region located from positions 31,736,206 to 32,225,068 is shown with 4 haplotype blocks generated in Haploview. Negative log P values from single SNP associations were derived from genome-wide analysis after removal of population outliers and individual SNPs were plotted as red circles. The horizontal dotted line represents the threshold for significant association after Bonferroni correction of -log(P) > 6.24. The additional blue region within each haplotype block represents MaxT 100,000 permuted haplotypes.
Table 6.4: Haplotype blocks generated by Haploview for STYX region of chromosome 8

<table>
<thead>
<tr>
<th>Block</th>
<th>Haplotype</th>
<th>Population frequency</th>
<th>Case frequency</th>
<th>Control frequency</th>
<th>$X^2$</th>
<th>$P_{case}$</th>
<th>$P_{permut}$</th>
<th>Start position</th>
<th>End Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GAAGGG</td>
<td>0.74</td>
<td>0.59</td>
<td>0.83</td>
<td>10.28</td>
<td>0.0013</td>
<td>0.0092</td>
<td>31736206</td>
<td>31795128</td>
</tr>
<tr>
<td>2</td>
<td>AG</td>
<td>0.47</td>
<td>0.27</td>
<td>0.59</td>
<td>14.30</td>
<td>2.00 x 10^{-4}</td>
<td>0.0008</td>
<td>31866373</td>
<td>31883390</td>
</tr>
<tr>
<td>3</td>
<td>GGAG</td>
<td>0.52</td>
<td>0.27</td>
<td>0.68</td>
<td>23.24</td>
<td>1.43 x 10^{-6}</td>
<td>1.00 x 10^{-5}</td>
<td>31971609</td>
<td>32009283</td>
</tr>
<tr>
<td>4</td>
<td>AGAA</td>
<td>0.50</td>
<td>0.59</td>
<td>0.44</td>
<td>2.90</td>
<td>0.0888</td>
<td>0.3772</td>
<td>32183184</td>
<td>32225068</td>
</tr>
</tbody>
</table>
6.7 REFERENCES


CHAPTER 7
SUMMARY AND CONCLUSIONS

The canine idiopathic meningoencephalomyelitides, particularly GME, NME and MUE, are clinically important inflammatory disorders of the CNS that are associated with a poor prognosis.\textsuperscript{1} Although the cause of these disorders is unknown, it has been speculated that they are multifactorial in origin and require contribution of both environmental and genetic factors for development.\textsuperscript{1} To address this problem, the work presented here focused on two goals: 1) to rigorously evaluate the CNS of dogs diagnosed with GME, NME and MUE for a wide variety of microorganisms to identify infectious agents potentially associated with these disorders and 2) to identify genetic risk factors associated with the development of NME.

The hypothesis of goal 1 is that viral pathogens affecting the canine CNS are associated with the development of GME, NME and MUE. The specific aims that addressed this hypothesis were:

\textit{Specific Aim 1:} To determine if vector-borne bacterial pathogens can be detected in the CNS of dogs with GME, NME and / or MUE. The \textit{working} hypothesis is that \textit{Ehrlichia, Anaplasma, Rickettsia, Bartonella} and \textit{Borrelia} species are uncommon causes of meningoencephalomyelitis in dogs. The data presented in Chapter 3 confirm that species in the genera \textit{Ehrlichia, Anaplasma, Rickettsia, Bartonella} and \textit{Borrelia} are uncommonly associated with GME, NME and MUE. Interestingly, \textit{Bartonella vinsonii} subsp. \textit{berkoffii} was identified in 1 GME case. Based on the fact that numerous pathogens, including West Nile virus, canine parainfluenza and encephalomyocarditis virus\textsuperscript{2} have been suggested to play a role in individual
cases of GME, this finding may reflect the possibility that GME is a non-specific inflammatory response to various antigens, of which pathogens comprise an important subset.¹

**Specific Aim 2:** To determine if viruses can be detected in the CNS of dogs with GME and NME. The working hypothesis is that viral antigens in the CNS are associated with the development of GME and NME. The data shown in Chapter 4 suggest that viral pathogens are not commonly associated with GME and NME. However, as a part of this viral screening *M. canis* was identified in association with several cases of GME and NME. Although the pathogenic significance of finding *M. canis* is unclear at this time, further evaluation is warranted to assess the potential role of *M. canis* as an etiological agent for GME and NME.

**Specific Aim 3:** To determine if viruses can be detected in the CSF of dogs with MUE. The working hypothesis is that viral antigens in the CNS are associated with the development of MUE. The data presented in Chapter 5 suggest that LACV CNS infection in dogs is more common than previously thought. Additional work is necessary to confirm the significance of LACV infection in cases of MUE.

The hypothesis of goal 2 is that NME demonstrates polygenic inheritance within the Pug breed with specific genetic variants leading to an altered risk for disease development. The specific aim that addressed this hypothesis was:

**Specific Aim 1:** To identify genetic susceptibility loci in Pug dogs with NME through genome-wide SNP association. The working hypothesis is that multiple genes contribute to NME inheritance in Pug dogs. As presented in Chapter 6, at least two genetic loci are associated with NME development, a region on chromosome 8 containing the *STYX* and *GNPNAT1* genes and a 4.1 Mb region on chromosome 12 that contains the DLA class II genes.
Together, these findings support the idea that GME, NME and MUE likely have a multifactorial etiopathogenesis. Although a definitive infectious etiology was not identified in cases of GME, NME and MUE, two important candidate etiological agents were detected: *M. canis* in cases of GME and NME and LACV in cases of MUE. Additionally, the genome-wide association study of NME supports that this disease has a complex genetic basis, suggesting that multiple genes and/or environmental factors play a role in disease development. It can be speculated that functional mutations in the DLA II genes on chromosome 12 contribute to this multifactorial etiopathogenesis by altering the presentation of peptide antigens, resulting in an increased susceptibility to infection and/or autoimmunity.

Ultimately, the research presented in this dissertation raises several key questions: Does *M. canis* contribute to disease pathogenesis in cases of GME and NME? How common is LACV infection in dogs and how frequently does LACV infection cause clinical disease of the nervous system in dogs? Does antigenic stimulation from exposure to various pathogens, such as *B. vinsonii* subsp. *berkhoffii*, *M. canis* and LACV, contribute to disease development in GME, NME and MUE? What are the specific functional genetic mutations that contribute to NME development? Can specific genetic risk loci also be identified in other canine idiopathic meningoencephalomyelitides, such as GME and necrotizing leukoencephalitis? Does disease pathogenesis in these disorders result from genetic immunosusceptibility to infection, autoimmunity or both?

To begin answering these questions, several key steps need to be taken. First, determining if *M. canis* truly is associated with GME and NME will require prospective evaluation of aseptically collected GME and NME brain tissue or CSF for *M. canis* organisms as well as localization of *M. canis* in GME and NME inflammatory lesions via *in situ* hybridization. If *M.
*canis* truly is associated with GME and NME, a determination needs to be made whether it acts as a direct CNS pathogen, initiates disease by a parainfectious mechanism or is present secondary to disease-induced or iatrogenic immunosuppression. Sequencing of the full *M. canis* genome to evaluate for virulence factors also may provide key information. To further elucidate the role of LACV in cases of MUE, additional screening of healthy and diseased dogs needs to be performed. To do this, it will be important to develop a reliable serological assay to test for anti-LACV IgG and IgM antibodies in canine serum and CSF. Finally, to further explore the genetic basis of NME, fine mapping needs to be performed across multiple disease-affected breeds to narrow the disease-associated genetic region, followed by DNA sequence analysis to identify functional variants. There also is a critical need to collect enough cases from individual breeds affected by GME to perform genome-wide association studies. The information that could be gained from these further analyses would not only improve our knowledge of disease pathogenesis, but aid in efforts to develop accurate antemortem diagnostic tests and improve prevention and treatment of canine idiopathic meningoencephalomyelitis.

REFERENCES
