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Broadly Reactive Polymerase Chain Reaction for Pathogen Detection in Canine Granulomatous Meningoencephalomyelitis and Necrotizing Meningoencephalitis

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

Hypothesis: Broadly reactive PCR might 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: A mixed prospective/retrospective case-control study was performed. Brain tissue prospectively collected at necropsy from GME, NME, and control cases was evaluated by broadly reactive polymerase chain reaction (PCR) for adenoviruses, bunyaviruses, coronaviruses, enteroviruses, flaviviruses, herpesviruses, paramyxoviruses, and parechoviruses. In addition, these tissues were retrospectively evaluated for the presence of mycoplasmas by PCR, culture, and immunohistochemistry (IHC).

Results: Brain tissue was collected from 11 GME and 27 NME cases and 30 controls. Viral nucleic acids were not identified in the 6 GME cases, 25 NME cases, and 2 controls evaluated by viral PCR. *Mycoplasma canis* was identified by *Mycoplasma* genus PCR in 1/5 GME and 4/25 NME cases and subsequently was cultured from 4/5 GME and 4/8 NME cases as well as 2/9 controls. The IHC did not detect *M. canis* in any of the 11 GME and 27 NME cases or 14 controls evaluated with strain PG14 polyclonal antiserum.

Conclusions and Clinical Importance: The negative results suggest that viral pathogens are not common in the brain tissue of dogs with GME and NME. Further investigation is warranted to determine the importance of *M. canis* in cases of GME and NME.

Key words: Central nervous system; Inflammation; *Mycoplasma*; Virus.

Abbreviations:

AAC	ambient atmospheric conditions
BKV	human polyomavirus type BK
bp	base pair
CAV	canine adenovirus
CNS	central nervous system
dNTPs	deoxynucleotide triphosphates
gDNA	genomic DNA
GME	granulomatous meningoencephalomyelitis
IHC	immunohistochemistry
LACV	La Crosse virus
NME	necrotizing meningoencephalitis
PCR	polymerase chain reaction
rRNA	ribosomal RNA
rRT-PCR	real-time RT-PCR
RT-PCR	reverse transcription PCR
SLE	St. Louis encephalitis
snPCR	semi-nested PCR

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 nonsuppurative 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

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CNS infection,[3–6] parainfectious immune dysregulation,[1] 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 NME[2,7] 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] However, a comprehensive search for viral and atypical bacterial pathogens by sensitive molecular techniques[9,10] has been lacking. Adeno-,[11] bunya-,[11] corona-,[12] entero-,[11,13] flavi-,[11,14] herpes-,[9,11,15,16] paramyxo-,[11,17] and parecho-[18] viruses all have been documented as causes of meningoencephalomyelitis in people. Moreover, *Mycoplasma edwardii* recently was reported as a cause of meningoencephalitis in a dog.[19]

The investigators evaluated the hypothesis that broadly reactive PCR may aid in finding occult viral and *Mycoplasma* infections associated with GME and NME. Brain tissue was prospectively collected from cases of histopathologically confirmed GME and NME to evaluate for the presence of known and novel pathogens by broadly reactive PCR for adeno-, bunya-, corona-, entero-, flavi-, herpes-, paramyxo-, and parecho- viruses. In addition, *Mycoplasma* genus-specific PCR was retrospectively performed on collected brain tissue.

Materials and Methods

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 necropsy examination. Age at disease onset, breed, sex, treatment administered, and survival time were recorded at time of sample collection.

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 cm^3 piece of tissue was collected from the frontal lobe and immediately transferred to −80°C until use. Time from dog 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).

Nucleic Acid Extraction and PCR Quality Control

Genomic DNA (gDNA) and total RNA were extracted from brain tissue[ab] 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.[20] The RNA integrity was confirmed in all samples by reverse transcription PCR (RT-PCR) amplification of superoxide dismutase (expected product size 440 bp).[21] To avoid contamination, nucleic acid extraction, PCR preparation (preamplification), 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. In addition, mock nucleic acid extraction of sterile water was performed in parallel with all clinical cases and utilized as a negative control for PCR reactions.

Broadly Reactive Viral PCR

Consensus, degenerate, or consensus-degenerate hybrid primers were used for broadly reactive viral PCR (supporting information Table S1). Adenovirus PCR[c]; bunyavirus RT-PCR and coronavirus, flavivirus, and paramyxovirus seminested 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. The RT-PCR reactions contained 20 U RNase inhibitor[g] and PCR and snPCR reactions used 200 μM (each) of deoxynucleotide triphospates (dNPTs).[g] Initial reactions were performed with 5 μL template DNA or RNA, and seminested 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[22] and pan-paramyxovirus[23] primers were used as described previously.[4,23] 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.[24] 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 100–500 copies of target gene per reaction using Cache Valley virus and LACV.

Previously designed pan-coronavirus primers[25] 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 seminested 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 10–100 copies of target gene per reaction using representative viruses from each antigenic group.[25]

Previously designed flavivirus primers[14] cFD2 and MAMD were used for the initial reaction and cFD2 and FS778 for the seminested 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 100–500 copies of target gene per reaction using Japanese encephalitis, SLE, dengue, West Nile, and yellow fever viruses.

Previously designed pan-herpesvirus primers[26] DFASA and GDTD1B were used for the initial reaction and VYGA and GDTD1B were used for the seminested 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[h] (0.2 μM each) were used for rRT-PCR.[27,28] 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.[i] Threshold cycle values were determined using commercially available software.[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.

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[c] with a 50 μL final volume, 1 μM final primer concentration, and 200 μM (each) dNTPs. [g] The initial reaction was performed with primers MY-16S-447 (5′-GTCAGAAAGCGATGGCTAACTA-3′) and MY-16S-844 (5′-CGAGCATACTACTCAGGCGGAT-3′) using 2 μL gDNA, and the second amplification with primers MY-16S-483n (5′-CGGTAATACATAGGTCGC-3′) and MY-16S-748n (5′-TAT CTAATCCTGTTTGCTCC-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[j] containing 0.5% w/v glucose plus 0.21% w/v L-arginine (SP-4G/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% CO_2 (agar) until mycoplasmal growth was apparent. Individual colonies were passaged in SP-4G/A broth before isolate identification to segregate mixed infections. Isolates were presumptively identified by PCR-restriction fragment length polymorphism,[29] and the identification was confirmed by direct sequencing of the 16S rRNA gene as described previously.[30] The identity of the samples remained blinded until the end of the analysis.

Sequencing

Adenovirus, bunyavirus, coronavirus, flavivirus, herpesvirus, paramyxovirus, and *Mycoplasma* PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining under ultraviolet exposure, and amplicons were purified,[kl] or cloned[m] and purified[n] for sequencing. Purified amplicons and plasmids were sequenced[o] using the corresponding amplification primers. Species were defined by comparison of DNA sequences with GenBank database entries using the Basic Local Alignment Search Tool.

Immunohistochemistry

Unconjugated, lyophilized polyclonal antibodies generated in rabbits to *Mycoplasma canis* strain PG14[p] 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 *M. edwardii*[q] and *Mycoplasma spumans*[r] 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[s] of 1:150,000 with a staining time of 60 minutes. Nonspecific binding was blocked[t] for 5 minutes before incubation with the primary diluted antibody or universal negative control.[u] 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 before primary antibody incubation followed by addition of biotinylated goat antirabbit IgG,[v] horseradish peroxidase conjugated streptavidi.,[u] and 3, 3′-diaminobenzidine.[u] For the alkaline phosphatase detection system, incubation with primary antibody was followed by addition of biotinylated anti-Ig,[w] labeled with alkaline phosphatase conjugated streptavidin[x] and fast red.[y] Slides were counterstained with Gills II hematoxylin[z] and bluing.

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 Satterwaithe's approximation when necessitated by unequal variance, to assess a relationship between clinical parameters (age at disease onset, breed, sex status, treatment administered, and survival time) and *Mycoplasma* PCR or culture positivity. Commercially available software was used for all analyses,[aa] and significance was defined as $P < .05$.

Results

Brain tissue was collected from 11 GME cases, 27 NME cases, and 30 control dogs. The GME cases included 4 Dachshunds, 2 Labrador Retrievers, and 1 of each of 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). The survival time from onset of clinical signs was 6.1 days (mean, 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 nonsteroidal 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), disk herniation (n = 1), and idiopathic meningitis (n = 1).

Six 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 (Table 1).

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 parvoviral enteritis) (Table 1 and supporting information Table S2). The 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 1 and supporting information Table S2). 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. In addition, 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 (Table 1).

The 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 14 controls evaluated (Table 1). In addition, 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.

Discussion

Using broadly reactive PCR for adeno-, bunya-, corona-, entero-, flavi-, herpes-, paramyxo-, and parecho-viruses, we found no evidence of viral nucleic acids in brain tissue of dogs with histopathologically confirmed GME and NME. In contrast, using PCR and culture, *M. canis* was identified 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,[1,3–6] 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 using broadly reactive PCR to identify unknown as well as known pathogens.[23,25,26] 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 noncanine viruses as PCR controls. In all cases, tissues were collected from the frontal lobe, which may or may not have resulted in direct sampling of inflammatory lesions. In addition, the PCR assays utilized ranged in sensitivity from 30 to 500 copies of target gene per reaction, 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.[31]

To fully utilize the GME and NME tissues collected for this investigation, *Mycoplasma* genus-specific PCR was performed in addition to viral screening. Interestingly, several *Mycoplasma* species regularly associated with the respiratory tract of dogs were identified, and detection of *M. canis* by PCR was significantly associated with a diagnosis of GME and NME. Although *M. canis* has been associated with respiratory and urogenital diseases,[32] this is the 1st report of *M. canis* associated with CNS disease or nervous tissue in any species.

Table 1. Summary of cases and controls evaluated by PCR, culture, and immunohistochemistry

	Broadly Reactive Viral PCR			*Mycoplasma* PCR			*Mycoplasma* Culture			*Mycoplasma canis* Immunohistochemistry		
	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total
GME	0	6	6	1	4	5	4	1	5	0	11	11
NME	0	25	25	5	20	25	4	4	8	0	27	27
Control	1	2	3	1	22	23	3	6	9	0	10	10
Total	1	33	34	7	46	53	11	11	22	0	48	48

The importance of finding *M. canis* associated with GME and NME is unclear. *M. canis* was not detected *in situ* and was detected in 2 controls, so contamination because of nonaseptic tissue collection cannot be ruled out. However, if contamination was the sole explanation for this finding, *M. canis* would be expected to occur at an equal frequency in GME and NME cases and controls. In addition, the polyclonal sera used for IHC were diluted significantly to avoid background, which may have contributed to the negative results obtained from this assay. Alternatively, *M. canis* may be associated with primary disease pathogenesis or may have been present secondary to previously existing disease or patient immunosuppression. Interestingly, *Mycoplasma pneumonia* has been implicated as a common yet elusive etiology in acute childhood encephalitis,[33] 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 before tissue collection, which may have altered the ability to detect or recover mycoplasmas in these cases. Of note, 2 *Mycoplasma* species were identified in several cases, which could support contamination with ubiquitous organisms or represent coinfection similar to *Mycoplasma synoviae* and *Mycoplasma meleagridis* in turkeys.[34] It also is noteworthy that CNS mycoplasmosis involving other species can result in suppurative inflammation,[19,35] 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 *M. canis* cannot be excluded.

Mycoplasmas have been infrequently associated with CNS disease[19,35–41] and the virulence factors that would allow *M. canis* to invade and colonize the CNS are unknown. Interestingly, neuraminidase activity recently was identified in *M. canis*.[42] 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[43] and recently was identified as critical for bacterial entry into the CNS in *Streptococcus pneumoniae* meningitis.[44]

Genetic susceptibility to *M. canis* infection also must be considered.[1,2,7] A missense mutation predisposing people to infection-triggered acute necrotizing encephalopathy recently was described[45] and breed-based susceptibility to certain pathogens also is likely in dogs.[46] It is possible that genetic immunosusceptibility to *M. canis* infection may contribute to a multifactorial etiopathogenesis in dogs that develop GME and NME. Genetic differences also may explain why *M. canis* infection could be associated with both GME and NME, despite their neuropathological distinctions.

In summary, broadly reactive PCR was used to evaluate histopathologically confirmed cases of GME and NME for viral pathogens and mycoplasmas. The results support that the viruses evaluated are not commonly associated with GME and NME but suggest a possible role for *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 GME and NME pathogenesis could favorably alter disease diagnosis, treatment, and outcome. Next steps include development of a monoclonal *M. canis* antibody for localization of *M. canis* via *in situ* hybridization and a prospective survey for *M. canis* from aseptically collected GME and NME brain tissue or cerebrospinal fluid.

Footnotes

[a] Qiagen DNeasy Blood and Tissue Kit, Qiagen, Valencia, CA
[b] TRIzol Reagent, Invitrogen, Carlsbad, CA
[c] Platinum *Taq* DNA Polymerase kit, Invitrogen
[d] SuperScript III One-Step RT-PCR System, Invitrogen
[e] HotStar*Taq* DNA Polymerase kit, Qiagen
[f] SuperScript III One-Step Quantitative RT-PCR System, Invitrogen
[g] Roche Diagnostics, Indianapolis, IN
[h] TaqMan, Applied Biosystems, Foster City, CA
[i] Roche LightCycler, Roche Diagnostics
[j] MinElute PCR Purification Kit, Qiagen
[k] QIAquick Gel Extraction Kit, Qiagen
[l] TOPO TA Cloning Kit, Invitrogen
[m] QIAprep Spin Miniprep Kit, Qiagen
[n] American Type Culture Collection (ATCC) 988, Rockville, MD
[o] BigDye Terminators v3.1 and ABI 3730xl, Applied Biosystems
[p] The Mollicutes Collection, World Federation of Culture Collections WDCM858, West Lafayette, IN
[q] ATCC 23462
[r] ATCC 19526
[s] Dako Antibody Diluent, Dako, Carpinteria, CA
[t] Power Block Universal Blocking Reagent, BioGenex, San Ramon, CA
[u] Dako
[v] Jackson ImmunoResearch Laboratories, West Grove, PA
[w] Super Sensitive MultiLink, Biogenex, San Ramon, CA
[x] Super Sensitive Label, Biogenex
[y] Vulcan Fast Red, Biocare Medical, Concord, CA
[z] Surgipath, Richmond, IL
[aa] SAS V 9.2, Cary, NC

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References

1. Talarico LR, Schatzberg SJ. Idiopathic granulomatous and necrotising inflammatory disorders of the canine central nervous system: A review and future perspectives. J Small Anim Pract 2010;51:138–149.

2. Greer KA, Wong AK, Liu H, et al. Necrotizing meningoencephalitis of Pug dogs associates with dog leukocyte antigen class II and resembles acute variant forms of multiple sclerosis. Tissue Antigens 2010;76:110–118.

3. Summers BA, Cummings JF, deLahunta A. Veterinary Neuropathology. St. Louis, MO: Mosby; 1995.

4. Schatzberg SJ, Haley NJ, Barr SC, et al. Polymerase chain reaction screening for dna viruses in paraffin-embedded brains from dogs with necrotizing meningoencephalitis, necrotizing leukoencephalitis, and granulomatous meningoencephalitis. J Vet Intern Med 2005;19:553–559.

5. Schwab S, Herden C, Seeliger F, et al. Non-suppurative meningoencephalitis of unknown origin in cats and dogs: An immunohistochemical study. J Comp Pathol 2007;136:96–110.

6. Cordy DR, Holliday TA. A necrotizing meningoencephalitis of pug dogs. Vet Pathol 1989;26:191–194.

7. Barber RM, Schatzberg SJ, Corneveaux JJ, et al. Identification of risk loci for necrotizing meningoencephalitis in pug dogs. J Hered 2011;102:S40–S46.

8. Vite C. Inflammatory disorders of the central nervous system. In: Braund K, Vite C, eds. Clinical Neurology in Small Animals: Localization, Diagnosis and Treatment. Ithaca, NY: International Veterinary Information Service; 2005.Available at http://www.ivis.org. Accessed August 25, 2011.

9. Redington JJ, Tyler KL. Viral infections of the nervous system, 2002: Update on diagnosis and treatment. Arch Neurol 2002;59:712–718.

10. Calvario A, Bozzi A, Scarasciulli M, et al. Herpes consensus PCR test: A useful diagnostic approach to the screening of viral diseases of the central nervous system. J Clin Virol 2002;25 (Suppl 1):S71–78.

11. Whitley RJ, Gnann JW. Viral encephalitis: Familiar infections and emerging pathogens. Lancet 2002;359:507–513.

12. Ann Yeh E, Collins A, Cohen ME, et al. Detection of coronavirus in the central nervous system of a child with acute disseminated encephalomyelitis. Pediatrics 2004;113:e73–e76.

13. Kammerer U, Kunkel B, Korn K. Nested PCR for specific detection and rapid identification of human picornaviruses. J Clin Microbiol 1994;32:285–291.

14. Scaramozzino N, Crance J-M, Jouan A, et al. Comparison of flavivirus universal primer pairs and development of a rapid, highly sensitive heminested reverse transcription-PCR assay for detection of flaviviruses targeted to a conserved region of the NS5 gene sequences. J Clin Microbiol 2001;39: 1922–1927.

15. Shoji H, Wakasugi K, Miura Y, et al. Herpesvirus infections of the central nervous system. Jpn J Infect Dis 2002;55: 6–13.

16. Koskiniemi M, Rantalaiho T, Piiparinen H, et al. Infections of the central nervous system of suspected viral origin: A collaborative study from Finland. J Neurovirol 2001;7: 400–408.

17. Debiasi RL, Tyler KL. Molecular methods for diagnosis of viral encephalitis. Clin Microbiol Rev 2004;17:903–925.

18. Wolthers KC, Benschop KSM, Schinkel J, et al. Human parechoviruses as an important viral cause of sepsis like illness and meningitis in young children. Clin Infect Dis 2008;47: 358–363.

19. Ilha MRS, Rajeev S, Watson C, et al. Meningoencephalitis caused by *Mycoplasma edwardii* in a dog. J Vet Diagn Inves 2010;22:805–808.

20. Schatzberg SJ, Haley NJ, Barr SC, et al. Polymerase chain reaction (PCR) amplification of parvoviral DNA from the brains of dogs and cats with cerebellar hypoplasia. J Vet Intern Med 2003;17:538–544.

21. Schatzberg S, Olby NJ, Steingold S, et al. A polymerase chain reaction screening strategy for the promoter of the canine dystrophin gene. Am J Vet Res 1999;60:1040–1046.

22. Thomson D, Meers J, Harrach B. Molecular confirmation of an adenovirus in brushtail possums (*Trichosurus vulpecula*). Virus Res 2002;83:189–195.

23. Tong S, Chern S-WW, Li Y, et al. Sensitive and broadly reactive reverse transcription-PCR assays to detect novel paramyxoviruses. J Clin Microbiol 2008;46:2652–2658.

24. Kuno G, Mitchell C, Chang G, et al. Detecting bunyaviruses of the bunyamwera and California serogroups by a PCR technique. J Clin Microbiol 1996;34:1184–1188.

25. Tong S, Conrardy C, Ruone S, et al. Detection of novel SARS-like and other coronaviruses in bats from Kenya. Emerg Infect Dis 2009;15:482–485.

26. Rose TM, Strand KB, Schultz ER, et al. Identification of two homologs of the Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in retroperitoneal fibromatosis of different macaque species. J Virol 1997;71:4138–4144.

27. Nix WA, Maher K, Johansson ES, et al. Detection of all known parechoviruses by real-time PCR. J Clin Microbiol 2008;46:2519–2524.

28. Oberste MS, Peñaranda S, Rogers SL, et al. Comparative evaluation of Taqman real-time PCR and semi-nested VP1 PCR for detection of enteroviruses in clinical specimens. J Clin Virol 2010;49:73–74.

29. Spergser J, Rosengarten R. Identification and differentiation of canine *Mycoplasma* isolates by 16S–23S rDNA PCR-RFLP. Vet Microbiol 2007;125:170–174.

30. Brown DR, Farley JM, Zacher LA, et al. *Mycoplasma alligatoris* sp. nov., from American alligators. Int J Syst Evol Microbiol 2001;51:419–424.

31. Schatzberg SJ, Li Q, Porter BF, et al. Broadly reactive pan-paramyxovirus reverse transcription polymerase chain reaction and sequence analysis for the detection of canine distemper virus in a case of canine meningoencephalitis of unknown etiology. J Vet Diagn Inves 2009;21:844–849.

32. Chalker VJ. Canine mycoplasmas. Res Vet Sci 2005;79:1–8.

33. Bitnun A, Ford-Jones EL, Petric M, et al. Acute childhood encephalitis and *Mycoplasma pneumoniae*. Clin Infect Dis 2001;32:1674–1684.

34. Rhoades K. Turkey sinusitis: Synergism between *Mycoplasma synoviae* and *Mycoplasma meleagridis*. Avian Dis 1977;21: 670–674.

35. Brown D, Nogueira M, Schoeb T, et al. Pathology of experimental mycoplasmosis in American alligators. J Wildl Dis 2001;37:671–679.

36. Tsiodras S, Kelesidis I, Kelesidis T, et al. Central nervous system manifestations of *Mycoplasma pneumoniae* infections. J Infect 2005;51:343–354.

37. Chin RP, Daft BM, Meteyer CU, et al. Meningoencephalitis in commercial meat turkeys associated with *Mycoplasma gallisepticum*. Avian Dis 1991;35:986–993.

38. Hill A. *Mycoplasma pulmonis* infection in the brains of rodents. J Infect Dis 1983;147:540–545.

39. Thomas L, Aleu F, Bitensky MW, et al. Studies of PPLO infection II. The neurotoxin of *Mycoplasma neurolyticum*. J Exp Med 1966;124:1067–1082.

40. Schumacher VL, Hinckley L, Liao X, et al. Meningitis caused by *Mycoplasma mycoides* subspecies *capri* in a goat. J Vet Diagn Inves 2011;23:565–569.

41. Beauchamp DJ, da Costa RC, Premanandan C, et al. *Mycoplasma felis*-associated meningoencephalomyelitis in a cat. J Feline Med Surg 2011;13:139–143.

42. May M, Brown DR. Secreted sialidase activity of canine mycoplasmas. Vet Microbiol 2009;137:380–383.

43. Corfield T. Bacterial sialidases—Roles in pathogenicity and nutrition. Glycobiology 1992;2:509–521.

44. Uchiyama S, Carlin A, Khosravi A, et al. The Surface-anchored NanA protein promotes pneumococcal brain endothelial cell invasion. J Exp Med 2009;206:1845–1852.

45. Neilson DE, Adams MD, Orr CMD, et al. Infection-triggered familial or recurrent cases of acute necrotizing encephalopathy caused by mutations in a component of the nuclear pore, RANBP2. Am J Hum Genet 2009;84:44–51.

46. Eggers J, Parker G, Braaf H, et al. Disseminated *Mycobacterium avium* infection in three Miniature Schnauzer litter mates. J Vet Diagn Inves 1997;9:424–427.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Broadly reactive viral polymerase chain reaction primers and probes.

Table S2. Cases and controls positive for mycoplasmas by polymerase chain reaction (PCR) or culture.