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Metagenomic Investigation of Idiopathic Meningoencephalomyelitis in Dogs

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Background: Meningoencephalomyelitis of unknown origin (MUO) is a common and life-threatening neuroinflammatory disease in dogs. Features of the disease are suggestive of an underlying immune-mediated process, but the association of this disease with a pathogen is still unknown.

Hypothesis/Objectives: To search for candidate etiologic agent associated with cases if MUO using next generation metagenomic sequencing.

Animals: Twenty-two dogs diagnosed with either MUO (11/22; 10 CSF and 3 brain), or noninflammatory CNS diseases inconsistent with MUO (11/22; 11 CSF and 2 brain) that served as negative controls.

Methods: A case control study was performed by identifying MUO and non-MUO cases. Samples were blindly processed and then unblinded for comparative analyses. Inclusion criteria for MUO cases included consistent MRI lesions and inflammatory CSF with a negative PCR panel for infectious agents or histopathologic diagnosis. Dogs with glucocorticoid therapy within 2 weeks of sample collection were excluded. Fresh-frozen cerebrospinal fluid (CSF; 21) and brain (5) samples were collected and RNA and DNA were extracted separately for shotgun metagenomic sequencing. Known positive samples were used as controls to validate our sequencing and analysis pipelines and to establish limits of detection. Sequencing results were analyzed at a nucleotide and protein level for broad comparison to known infectious organisms.

Results: No candidate etiologic agents were identified in dogs with MUO.

Conclusions and Clinical Importance: These results support but do not prove the hypothesis that MUO is not associated with infectious agents and might be an autoimmune disease.

Key words: Granulomatous; Leukoencephalitis; Necrotizing; Sequencing.

Meningoencephalomyelitis of unknown origin (MUO) is a common neuroinflammatory disease of dogs suspected to be caused by an underlying immune-mediated process. The classification of MUO includes several inflammatory diseases differentiated histopathologically, including necrotizing meningoencephalitis (NME), necrotizing leukoencephalitis (NLE), and granulomatous meningoencephalomyelitis (GME).[1] All of these diseases predominately affect small breed dogs, but disease occurs in other breeds.[1–8] These diseases are histologically distinct, but without histological confirmation of disease, NME, NLE, and GME tend to be collectively referred to as MUO.

Abbreviations:

AM	antemortem
CNS	central nervous system
Contigs	contiguous sequences
CSF	cerebrospinal fluid
dNTPs	deoxynucleoside triphosphates
E	euthanasia without a necropsy
EN	euthanasia and necropsy
EtOH	ethanol
F	female
FS	female-spayed
GME	granulomatous meningoencephalomyelitis
HP	histopathology
LTF	lost to follow-up
MC	male-castrated
ME	meningoencephalitis
M	male
MRI	magnetic resonance imaging
MUO	meningoencephalomyelitis of unknown origin
NIDP	negative infectious disease profile
NLE	necrotizing leukoencephalitis
NME	necrotizing meningoencephalitis
nt	nucleotide
PCR	polymerase chain reaction
PM	postmortem
qPCR	quantitative PCR
RT	room temperature
S	stable
TAXIDs	taxonomic identifications
WNV	West Nile virus

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MUO is thought to account for up to 25% of cases of inflammatory CNS disease in dogs.[9] The prognosis of untreated MUO is poor, but treatment with immunosuppressant drugs such as corticosteroids can alleviate clinical signs and delay progression of disease. This suggests that MUO is an immune-mediated disease. However, a study targeting the inflammatory components of GME found a predominance of MHC Class II and $CD3^+$ T cells, which might be the result of a delayed hypersensitivity reaction.[10] Therefore, whether the immune response is targeting an infection is a critical open question that this study sought to answer.

Currently, the etiology of MUO remains unknown. Studies searching for an infectious etiology have failed to reveal a consistent infectious agent.[9,11–13] Prior studies have utilized polymerase chain reaction (PCR), serology, culture, immunohistochemistry, or a combination of these tests to investigate viruses commonly implicated in CNS disease, including herpesviruses, adenoviruses, parvoviruses, canine parainfluenza virus, encephalomyocarditis virus, bunyaviruses, coronaviruses, enteroviruses, flaviviruses, paramyxoviruses, and parechoviruses.[12–14] Although the overwhelming majority of these studies have been negative or inconclusive, they have been limited by targeted testing for specific agents as opposed to utilizing less biased methodology to search for pathogens. This limitation has impacted our understanding of human neurologic disease as well: in large analyses of human encephalitis cases, targeted methods failed to detect an infectious agent in up to 70% of suspected cases.[15–17]

In this study, we utilized a pathogen discovery technique that bypasses many of the limitations of specific diagnostics: next-generation metagenomic sequencing. This technology has emerged in human and veterinary medicine as an invaluable tool for pathogen discovery in neurologic and other diseases of unknown etiology.[18–21] In metagenomic sequencing, total nucleic acids from a clinical or environmental sample are randomly sequenced and are taxonomically categorized by comparison to known sequences in public databases. In this study, the cerebrospinal fluid (CSF), brain, or both of 22 dogs with or without MUO were sampled by antemortem and postmortem techniques. In previous investigations of MUO in dogs, only brain samples were tested for infectious agents; however, CSF is a common sample utilized in the clinical evaluation of neurologic disease for the detection of infectious agent nucleic acids, especially by PCR.[12–17] CSF is more readily available as an antemortem sample and therefore more clinically relevant for the antemortem diagnosis of MUO. Additionally, CSF has been successfully utilized in previous metagenomics sequencing studies to detect infectious organisms in encephalitis.[18] Therefore, based on sample availability and clinical relevance, this study utilized CSF and brain to attempt to identify a candidate etiologic agent of MUO.

Materials and Methods

Inclusion Criteria

To be included in the diseased group for this study, dogs had to be greater than six months of age with a neurologic examination consistent with focal or multifocal neurological dysfunction. Additional inclusion criteria included negative PCR tests on cerebrospinal fluid, whole blood, or both for the infectious agents caused by members of the species or genera *Toxoplasma gondii*, *Neospora caninum*, *Ehrlichia canis, Ehrlichia ewingii, Anaplasma, Neorickettsia, Bartonella,* and *Rickettsia*; the presence of multifocal T2-weighted hyperintense lesions on MRI; and CSF pleocytosis with a nucleated cell count of greater than 5 cells/μL with greater than 50% mononuclear cells and a red blood cell count of less than 4,000 cells/μL.[1] For cases in which a necropsy was performed, histopathologic confirmation of disease was accepted in the absence of infectious disease testing, MRI, and CSF. Because of the inflammatory nature of MUO, any potential subject to whom glucocorticoids were administered within two weeks of CSF or antemortem brain collection were excluded from this study; however, this criteria was not used for postmortem brain samples. Three animals in the diseased group and one animal in the control group received antimicrobials within several days of sample collection, which could have altered the results of the infectious disease testing. Animals in the control (non-MUO) group were subject to the same age criteria as the diseased group (MUO). The control cases had a low index of suspicion of inflammatory disease based on a noninflammatory CSF analysis, inconsistent MRI findings, histologically confirmed non-MUO disease process, or a combination of these findings.

Sample Collection Methods

Samples of brain tissue were obtained using aseptic surgical technique and sharp dissection. In the diseased group, the samples were taken from the regions of the brain showing abnormalities on MRI or from areas of the brain associated with the clinical signs when MRI was not available (ie multiple areas of the cerebellum were sampled in dogs with cerebellar signs). In animals from the control group, samples were taken from the cerebral cortex. Portions of the tissue samples were then placed aseptically into sterile containers and stored at −80°C. The remainder of the sample was placed in 10% neutral buffered formalin and processed using standard paraffin-embedding and histologic techniques for microscopic evaluation of 5 μm hematoxylin and eosin-stained tissue sections. Brain samples collected at the time of necropsy were processed identically.

CSF was collected under general anesthesia via a cerebellomedullary cisternal or lumbosacral centesis using aseptic technique. For CSF collection, the area was surgically prepped using 4% chlorhexidine gluconate scrub. A 1.5 inch or 2.5 inch 20 or 22 gauge spinal needle was used to collect CSF in a sterile tube. The CSF was stored at −80°C.

Case Diagnostics

All animals in both the diseased (MUO) and control (non-MUO) groups received a physical and neurologic examination. In the MUO group, 11 of 11 animals had a complete blood count; 10 of 11 animals had a serum blood chemistry; 10 of 11 had CSF analysis; 9 of 11 had infectious disease testing of whole blood or CSF; 9 of 11 had an MRI; 4 of 11 animals were necropsied with histopathology and one of these animals also had an antemortem brain biopsy collected. Of the animals in the inflammatory group, 4 of 11 were euthanized, 2 of 11 died as a result of their disease, 1 of 11 is currently stable, and 4 of 11 have been lost to follow-up (Table 1).

In the control group, 11 of 11 animals had CSF analysis; 9 of 11 animals had a CBC and serum blood chemistry; 10 of 11 had an MRI; 7 of 11 were tested for infectious diseases by whole blood or CSF; 5 of 11 animals were necropsied with histopathology, and

Table 1. Case Summary.

	# of Cases	Avg age (years)	Breeds Included	Diagnostics Performed	Sample Used
Diseased (11/22)					
Antemortem diagnosis					
MUO	7	5.3	YT, Mix, Chi, MP, IG, Pug	CSF, NIDP, MRI	CSF
Postmortem diagnosis					
NME	1	2	Pug	CSF, HP (PM)	CSF
GME	1	4	Malt	CSF, NIDP, MRI, HP (AM, PM)	Brain (AM)
ME (unspecified)	2	2	Col, MS	CSF, NIDP, MRI (1/2); HP (PM) (2/2)	Brain (PM) and CSF
	11 Total				
Control (11/22)					
Diagnosis					
Neoplasia	5	8	Malt, MD, Mix, Box, WC	CSF, MRI (4/5); NIDP (3/5); HP (AM) (2/5); HP (PM) (4/5)	CSF (5/5) and Brain (PM) (2/5)
Degenerative	4	4	BM, Mix, DP, GSD	CSF, MRI (4/4); NIDP (3/4); HP (PM) (1/4)	CSF
Trauma	1	6	Wei	CSF, MRI	CSF
Epilepsy	1	10	SP	CSF, NIDP, MRI	CSF
	11 Total				

Diseased cases (11 of 22) represent animals diagnosed with MUO based on clinical presentation and antemortem diagnostics, with or without postmortem assessment. Antemortem diagnosis could not be further classified into the MUO subtypes. Postmortem diagnosis was made in 4 of 11 cases, two of which were diagnosed as either NME or GME and two of which had meningoencephalitis but lesions were not specific for any subset of MUO (see discussion). Control cases (11 of 22) are animals with noninflammatory CSF and either a definitive non-MUO diagnosis or additional clinical findings inconsistent with MUO. For "Diagnostics" and "Sample Used," if a fraction is not specified, then it applies to all in the group. MUO, meningoencephalomyelitis of unknown origin; ME, meningoencephalitis; NME, necrotizing meningoencephalitis; GME, granulomatous meningoencephalomyelitis; YT, Yorkshire Terrier; Mix, mixed breed; Chi, Chihuahua; MP, Miniature Pinscher; IG, Italian Greyhound; Malt, Maltese; Col, Collie; MS, Miniature Schnauzer; MD, Miniature Dachshund; BM, Belgian Malinois; Box, Boxer; WC, Welsh Corgi; DP, Doberman Pinscher; GSD, German Shepherd; Wei, Weimaraner; SP, Standard Poodle; CSF, cerebrospinal fluid; NIDP, negative infectious disease profile; MRI, magnetic resonance imaging; HP, histopathology; AM, antemortem; PM, postmortem.

2 of 5 of these animals also had an antemortem brain biopsy collected (one necropsied). All of the animals in the control group had diagnoses inconsistent with MUO (see Table 1 for listing). Of the animals in this group, 7 of 11 were euthanized, 1 of 11 is currently stable, and 3 of 11 were lost to follow-up (Table 1).

Sequencing Library Preparation

Total RNA was extracted from 26 fresh-frozen CSF and brain samples from 22 dogs (*Canis familiaris*) that fit the inclusion or control criteria described above. These samples were blinded as to their case or control origin before processing. Additionally, RNA was extracted from postmortem brain samples from a mule deer (*Odocoileus hemionus)*, green tree python (*Morelia viridis*), American crow (*Corvus brachyrhynchos*), and American robin (*Turdus migratorius*), all of which had previously been tested by PCR, metagenomic sequencing, or both, and were found to be infected with specific known infectious agents. These were used as positive controls.[22,23] RNA was extracted using a combination of TRIzol (tissue; Ambion Life Technologies) or TRIzol LS (body fluid; Ambion Life Technologies) with RNA clean and concentrator columns (CC-5; Zymo Research). Approximately, 100 mg of brain tissue was added to 1 mL of TRIzol, and 250 μL of body fluid (CSF, serum, or blood) was added to 750 μL of TRIzol LS and incubated at room temperature (RT) for 5 minutes. Tissue samples were macerated using a single sterile metal BB shaken in a TissueLyzer (Qiagen) at 30 Hz for 3 minutes. Then, 200 μL of chloroform (Sigma-Aldrich) was added, shaken for 15 seconds by hand, and incubated at RT for 2 minutes. Samples were spun at 12,000 RPM for 10 minutes at RT. The aqueous phase was removed (approximately 450 μL) and was added to a mixture of 450 μL of RNA-binding buffer (CC-5; Zymo Research) and 450 μL of 100% ethanol (EtOH). This was added to an RNA clean and concentrator column (CC-5; Zymo Research). The interphase and organic phase were set aside for DNA extraction (see below). The RNA column was washed with 400 μL RNA wash buffer and then incubated with 6 U DNase enzyme (NEB), 1× DNase buffer (NEB), and RNA wash buffer for 15 minutes. The column was spun to remove DNase mixture and then washed with 400 μL RNA prep buffer. Additional washes with 800 and 400 μL RNA wash buffer were performed, the column was dried with a 1 minute high-speed spin, and then RNA samples were eluted in 30 μL of RNase-free water.

All CSF samples had undetectable concentrations of RNA by fluorometric quantification. These samples, along with a no template control, were reverse transcribed, the second DNA strand synthesized, and total DNA amplified using the Ovation RNA Amplification System V2 (NuGEN) according to the manufacturer's protocol.

For extracted RNA of brain samples, approximately 1000 nanograms of RNA was added to 200 pmol of a random hexamer oligonucleotide (5′-NNNNNN; MDS-286) and incubated for 5 minutes at 37°C; a separate no template control was also used for these samples. Reverse transcription reaction mixture containing 1× SuperScript III FS reaction buffer (Invitrogen), 5 mM dithiothreitol (Invitrogen), 1 mM each deoxynucleoside triphosphates (dNTPs), and 100 U SuperScript III reverse transcriptase enzyme (Invitrogen) was added to the RNA-oligomer mix (12 μL total reaction volume) and incubated for 30 minutes at 42°C, then 30 minutes at 50°C, then 15 minutes at 70°C. Then, 1 U RNase H (NEB) diluted in 5 μL 1× SuperScript III FS reaction buffer and 160 pmol MDS-286 was added to the reaction mixtures, which were incubated at 37°C for 20 minutes followed by 94°C for 2 minutes. Then, single-stranded cDNA was converted to double-stranded DNA by adding 2.5 U Klenow DNA polymerase (3′ to 5′ exo- NEB) in 5 μL 1× SuperScript III FS reaction buffer and

2 mM each dNTPs and incubated at 37°C for 15 minutes. DNA was purified using Sera-Mag Speed Beads at a 1.4:1 bead/DNA volume ratio according to the manufacturer's protocol. DNA was eluted in 20 μL molecular grade water (Sigma-Aldrich).

The interphase and organic phase from the TRIzol extraction described above were used for DNA extraction according to the manufacturer's protocol (Invitrogen) with minor alterations. Briefly, 300 μL of 100% EtOH per 1 mL TRIzol was added to the interphase and organic phase, gently mixed, and incubated for 2 minutes at RT. Samples were centrifuged for 5 minutes at RT, and the supernatant was removed and discarded. The DNA pellet was washed twice in 1 mL of 0.1 M sodium citrate in 10% EtOH pH 8.5 (per 1 mL TRIzol), with a 30 minute RT incubation, 5 minute centrifugation, and removal of the supernatant. The DNA pellet was then resuspended in 75% EtOH, gently mixed, and incubated for 20 minutes at RT. The samples were then centrifuged for 5 minutes, the supernatant discarded, and the pellet air-dried for 5 minutes. The DNA pellet was then resuspended in 100 μL molecular grade water (Sigma-Aldrich), heated to 55°C for 10 minutes, and then centrifuged for 10 minutes at 4°C. The supernatant containing DNA was then transferred to a 1.5 mL conical new tube and purified using Sera-Mag Speed Beads as previously described. All CSF samples were amplified to generate detectable levels of DNA for fluorometric quantification. This was performed using Phi29 isothermal strand displacement amplification. Five μL of template, including a no template control, was added to 50 μM of random hexamer primer and incubated at 95°C for 3 minutes and then placed directly on ice. Template and primers were then added to a mixture containing 1× Phi29 buffer (NEB), 1× bovine serum albumin (NEB), 2.5 mM each dNTPs, 4 mM dithiothreitol (Invitrogen), and 5 U Phi29 DNA polymerase (NEB). Samples were incubated at 30°C for 2 hours then 65°C for 10 minutes.

The DNA concentration from each sample (both RNA and DNA derived samples) was measured fluorometrically, and 10 ng was used as a template in 6.5 μL of 1× Tagment DNA buffer and 0.5 μL Tagment DNA enzyme (Illumina). The mixture was incubated at 55°C for 10 minutes and then placed directly on ice. Tagmented DNA was cleaned with Sera-Mag Speed Beads as previously described and used as a template (5.8 μL) in the addition of full-length adaptors with unique bar-code combinations by PCR. The 25 μL PCR reaction contained 1× Kapa real-time library amplification master mix (Kapa Biosystems), 0.33 μM (each) MDS-143 and MDS-445 primers (5′CAAGCAGAAGACGGCATACG3′ and 5′AATGATACGGCGACCACCGA3′, respectively), and 0.020 μM each of adapter 1 and 2 bar-coded primers.[24] Thermocycling conditions in consecutive order were 72°C for 3 minutes, 98°C for 30 seconds, and 8 cycles of 98°C for 10 seconds, 63°C for 30 seconds, and 72°C for 3 minutes. Relative concentrations of libraries were measured in quantitative PCR (qPCR) reactions containing home-made 1× qPCR master mix (10 mM Tris-HCl pH 8.6, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 5% glycerol, 0.08% NP-40, 0.05% Tween-20, 1× Sybr green (Life Technologies) and 0.5 U Taq polymerase) and 0.5 μM MDS-143 and MDS-445 primers. Equivalent amounts of DNA from each sample were pooled and then cleaned using Sera-Mag Speed Beads as previously described. The pooled libraries were run on a 2% agarose gel and size selected (400–500 nucleotides) by gel extraction with a gel DNA recovery kit (Zymo) according to the manufacturer's protocol. Size-selected pooled libraries were amplified once more in a PCR mixture containing 1× Kapa real-time library amplification mix, 500 pmol of MDS-143 and -445 each, and 5 μL of library template in a 50 μL total reaction volume. This PCR also included single reactions of 4 separate fluorometric standards (Kapa). Thermocycler conditions were 98°C for 45 seconds and 8 cycles of 98°C for 10 seconds, 63°C for 30 seconds, and 72°C for 2 minutes, which was when the sample curve passed standard 1. DNA was purified using Sera-Mag Speed Beads as previously described. Library quantification was performed with the Illumina library quantification kit (Kapa Biosystems) according to the manufacturer's protocol. Sequencing was performed on an Illumina NextSeq 500 instrument with a NextSeq 500/550 High Output Kit v2 (150 cycles).

Sequence Analysis

Sequences were trimmed using Cutadapt (version 1.9.1) to trim adaptor sequences and low-quality bases, and remove trimmed sequences that were less than 80 nt long.[25] Quality base was set to 33 (default) and quality cutoff was set to 30 for the 5′ and 3′ ends. The first base of each sequence was also trimmed. The CD-HIT-DUP sequence clustering tool was then used to collapse reads with 99% global pairwise identity, leaving only unique reads remaining.[26] Host-derived sequences were then filtered using the Bowtie2 alignment tool (version 2.2.5).[27] First, a bowtie index was generated from the host genomic sequence (assembly CanFam3.1 for dogs, assembly Python_molurus_bivittatus-5.0.2 for the green tree python, Bos_taurus_UMD_3.1.1 for mule deer, assembly ASM69197v1 for American crow, and all available assemblies in the NCBI Assembly database in the order *Passeriformes* for the robin [ASM128173v1, GWvir1.0, GWplu1.0, Passer_domesticus-1.0, Taeniopygia_guttata-3.2.4, FicAlb1.5, GeoFor_1.0, PseHum1.0, Zonotrichia_albicollis-1.0.1, SCA1, ASM69197v1, ASM69201v2, ASM69581v1, Hooded_Crow_genome, Sturnus_vulgaris-1.0, Parus_major1.0.3, Lepidothrix_coronata-1.0]) and then sequences aligning with a local mode alignment score greater than 60 were removed. SPAdes genome assembler (version 3.5.0) was used to generate contiguous sequences (contigs).[28] Then, to taxonomically categorize sequences, the NCBI nt database was queried with all contigs greater than 150 nt using the BLASTn alignment tool (version 2.2.30+).[29,30] Any hit with an expect value less than 10^{-8} was assigned taxonomically according to the sequence with the highest alignment score.[29,31] Additionally, to attempt to categorize contigs that were too divergent to produce a high scoring nt–nt alignment, the NCBI nr database was queried in a RAPSearch2 (version 2.23) with a minimum length of 20 amino acids and an expect value of 0.01.[32] The same process was performed using all the reads that did not form contiguous sequences from SPAdes genomic assembly, except GSNAP alignment tool (version 2016-11-07) was used instead of BLASTn.[33] Raw sequence data was deposited in the NCBI Short Read Archive database (accession SRP118690).

We then looked for taxa that were specifically associated with cases and not controls. Samples were unblinded, and datasets were identified as either MUO or non-MUO (NM). All taxonomic identifications (TAXIDs) present within MUO samples that were also present in NM samples were removed from further analysis. Next, remaining TAXIDs were compared between MUO samples. A fraction was generated for each TAXID to determine the number of MUO samples that had alignments to the specific TAXID over the total number of samples evaluated. If a TAXID occurred in two MUO samples or more, the sequences associated with the TAXID were manually inspected by again querying using NCBI BLASTn and BLASTx to corroborate initial taxonomic assessment.[34,35] This was performed four times for each sample using the different sequencing outputs: SPAdes generated contiguous sequences queried to (1) BLASTn and (2) RAPSearch2 and individual reads queried to (3) GSNAP and (4) RAPSearch2.

Results

Case Collection Results

Eleven cases of MUO were collected for this study (Table 1). Seven cases were diagnosed based on an

inflammatory CSF that was collected prior to immunosuppressive therapy, a negative infectious disease panel, and MRI findings. Four of these cases were diagnosed with meningoencephalitis by postmortem histopathology, and two of these cases were definitively diagnosed with subtypes of MUO: NME and GME. The GME case included full diagnostics with an MRI, image-guided brain biopsy, and postmortem histopathology showing classical histologic features of GME. Diagnosis in this case was ultimately made by postmortem microscopy of necropsy brain tissue as histopathology of the brain biopsy obtained antemortem was considered "nondiagnostic." Neither of these two cases received immunosuppressive therapy prior to postmortem evaluation of the brain.

The other two histologically examined cases were diagnosed as meningoencephalitis (ME). These cases had small numbers of macrophages, lymphocytes, and plasma cells within the meninges and around cerebral blood vessels in the gray and white matter, as well as variable regions of vacuolization within the neuropil, axonal degeneration, and encephalomalacia, consistent with ME. However, these cases did not exhibit classical histologic patterns associated with any specific subtype of MUO. In contrast to the histologically diagnosed GME and NME cases, both of these animals had received doses of glucocorticoids (either prednisone or dexamethasone) and chemotherapy (cytarabine) prior to postmortem evaluation; one for six months and the other for three days prior to euthanasia or death.

The remaining 11 cases (control group) were diagnosed with diseases inconsistent with MUO, including neoplastic, degenerative, traumatic, and idiopathic epilepsy (Table 1). These served as negative control cases.

Sequencing Results

RNA and DNA were extracted from CSF and brain samples from 11 MUO dogs and 11 non-MUO dogs as well as multiple positive controls samples. Nucleic acids were then converted into sequencing libraries and sequenced on an Illumina NextSeq 500 instrument. The datasets contained on average 1.16×10^7, 150-nucleotide sequences per sample. A stepwise data analysis pipeline was used to remove adaptor sequences and low-quality reads, collapse sequences to unique reads, and filter out dog-derived sequences. Approximately, 2% of sequences remained in each sample after filtering (Table S1). Remaining sequences were assembled into longer contiguous sequences (contigs), which were queried against databases of nucleotide and protein sequences to identify possible pathogen-derived sequences. Sequences from no single organism were found in more than 3 MUO samples (of 11), and organisms were inconsistent between DNA and RNA from the same tissue as well as brain and CSF collected from the same animal. A majority of sequences lacked specificity to any single organism based on nucleotide and protein sequence analysis. This was because of either poor quality of the read, or sequences that were low complexity or highly conserved, and thus taxonomically ambiguous. This was the case for all eukaryotic organisms detected. A number of bacterial-aligning reads were also detected; however, because of the range of bacterial species and the inconsistency of any given organism among samples, these were deemed environmental contaminants. The most common bacteria detected were *Pseudomonas*, *Streptococcus*, and *Staphylococcus* species. A low number of viral species were detected, but all that were present solely within MUO samples were bacteriophages, and therefore unlikely to be associated with disease. Overall, a consistent and specific candidate etiological candidate was not detected.

Positive Control Cases

We sequenced and analyzed in parallel a number of known positive samples to validate our approach and to establish limits of detection. These included (1) brain from a captive green tree python positive for python nidovirus[22]; (2) brain from a wild mule deer positive for caprine herpesvirus 2; (3) brain from a wild-caught American robin experimentally infected with West Nile virus (WNV)[23]; and (4) brain from a wild-caught American crow experimentally infected with WNV.[23] These samples had previously tested positive by metagenomic (green tree python and mule deer) or targeted next-generation sequencing (crow and robin). We used an identical analysis pipeline for positive control samples, except we used different, appropriate genome assemblies for filtering host sequences. As expected, we detected python nidovirus, caprine herpesvirus 2, and WNV in the green tree python, mule deer, and crow, respectively, using our metagenomic sequencing approach (Table S2). We did not detect WNV in the experimentally infected robin brain,[23] but confirmed that the sample was positive for WNV RNA by qRT-PCR.[36] We quantified the WNV copy number in the bird brain samples at 168 genome copies/μL RNA in the robin and 8.82×10^4 genome copies/μL RNA in the crow.[36]

Discussion

MUO is an idiopathic inflammatory neurologic disease, including GME and the necrotizing encephalitides (NME and NLE). The pathogenic mechanisms underlying MUO remain unknown. Similar to previous targeted diagnostic studies,[9,11–14] our study using a less biased approach failed to detect any infectious agents that were consistently associated with canine MUO cases.

There are several possible biological and technical explanations for our study's inability to identify a candidate etiologic agent for MUO, including the underlying pathogenesis of the disease, sample type and collection methods, case inclusion criteria, sensitivity of diagnostics, and database limitations.

First, it is possible that the inflammation observed in MUO does not have an infectious etiology.

Second, it might be that MUO has an infectious cause, but that we are sampling at a point in the

natural history of the disease when the initiating pathogen is no longer present in detectable amounts. This possibility could be investigated by the development of a comprehensive serological panel of known canine pathogens that would enable retrospective sampling of dogs with and without MUO.[37]

Third, CNS lesions could be secondary to a primary infection elsewhere in the body, resulting in a systemic response that manifests as meningoencephalitis. Or the lesions could be a disproportional response to a very low-level CNS infection. The evaluation of multiple tissue types in dogs diagnosed with MUO, beyond CNS samples, could help assess this possibility.

Fourth, it might be that we sampled the wrong regions of the CNS. MUO, like many other neurologic diseases, can be focally or multifocally distributed. This limitation is likely to apply more to biopsy/postmortem samples than to pathogen detection in CSF. However, low or inconsistent shedding of organisms into the CSF could reduce the likelihood of detection. Future studies could benefit from more consistent use of antemortem image-guided biopsies (only 1 of 11 of our MUO cases) and sampling of multiple sections of the CNS postmortem (only 4 of 11 MUO cases), as well as multiple time-separated CSF sample collections.

Furthermore, although four of the diseased cases were histologically confirmed as having inflammatory brain disease, seven cases were presumptively diagnosed with MUO. Strict inclusion criteria were used for antemortem diagnosis in this study. However, the lack of histopathology does not definitively rule out other disease processes, such as lymphoma. Therefore, it is possible that not all of the presumptively diagnosed MUO cases were GME, NME or NLE. Additionally, only two of the four cases evaluated by histopathology yielded a definitive diagnosis of GME or NME, whereas the other two were diagnosed as meningoencephalitis of undetermined subtype. The use of a greater number of cases with histologic confirmation could have strengthened the diagnostic certainty of each case and allowed for a more specific investigation of MUO based on histologic type.

There are also several possible technical reasons that could have prevented us from identifying an infectious agent underlying MUO. First, it might be that we lacked the necessary sensitivity. Although metagenomic sequencing can detect any pathogen, it is generally less sensitive than targeted methods such as PCR. The sensitivity of PCR is typically defined in absolute units (eg 100 genome copies in a quantitative PCR reaction), but the sensitivity of metagenomic sequencing is limited by read depth and the relative pathogen concentration. For example, if a metagenomic dataset contains 1 million unique sequences and if a pathogen's nucleic acid is present at a concentration lower than 1 part per million host nucleic acid molecules, then it is unlikely to be detected. The development and use of methods to deplete mammalian nucleic acids could have improved the sensitivity of our study by eliminating dog sequences and enriching for microorganismal nucleic acids. Our analysis of bird brain samples with high and low WNV copy numbers illustrates this sensitivity threshold. We detected WNV by sequencing in the crow brain, which had 8.82×10^4 viral RNA copies per microliter of RNA but did not detect WNV in the robin brain, which had 1.68×10^2 genome copies per microliter of RNA. It can, therefore, be deduced that our limit of detection lies somewhere between these values. This range is large, and the use of WNV-positive samples with intermediate copy numbers could have allowed us to narrow this empirically determined limit of detection. Additionally, CSF has inherently low nucleic acid content because of the low number of nucleated cells present when compared to tissue. Therefore, DNA and RNA extraction generally have a low yield and further amplification is required for library preparation in these samples. Amplification can introduce base-composition bias and increases the number of nonunique reads, contributing to reduced sequencing quality and read depth. Finally, it is also possible that the cause of MUO is an infectious agent so divergent from known pathogens that its sequence was unrecognizable. This is not likely, however. Eukaryotic and bacterial pathogens typically have characteristic conserved sequences that are easily recognizable (eg ribosomal RNA sequences), and viruses can typically be recognized by viral polymerase sequences, especially when compared at the protein level, as we did.

In summary, we applied the best available molecular methods to continue the search for an MUO etiology, and did not find a candidate agent. There are several technical and biological reasons that could have prevented us from doing so. However, the thoroughness of our approach, our inclusion of internal positive controls, similar negative results from previous studies, and the clinical responsiveness to immunosuppressant therapy all provide support for the hypothesis that MUO is a primary autoimmune disease.

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Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Average reads per sample and sequencing analysis summary in dog datasets.

Table S2. Positive control sequencing summary.