Use of a multiplex polymerase chain reaction assay in the antemortem diagnosis of toxoplasmosis and neosporosis in the central nervous system of cats and dogs

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Objective—To develop a multiplex polymerase chain reaction (PCR) assay for the detection of *Toxoplasma gondii* and *Neospora caninum* DNA in canine and feline biological samples.

Sample Population—Biological samples from 7 cats with systemic (n = 4) or CNS (3) toxoplasmosis, 6 dogs with neospora- or toxoplasma-associated encephalitis, and 11 animals with nonprotozoal disease.

Procedure—Primers for *T gondii*, *N caninum*, and the canine ferritin gene (dogs) or feline histone 3.3 gene (cats) were combined in a single PCR assay. The DNA was extracted from paraffin-embedded brain tissue, CSF, or skeletal muscle. The PCR products with positive results were cloned, and sequence identity was confirmed.

Results—Of 7 cats and 4 dogs with immunohistochemical or serologic evidence of toxoplasmosis, PCR results were positive for all cats and 3 dogs for *T gondii*, and positive for *T gondii* and *N caninum* for 1 dog. Another dog had negative PCR results for both parasites. Of 2 dogs with immunohistochemical or serologic evidence of neosporosis, PCR results were positive for 1 for *N caninum* and positive for the other for *T gondii*. All negative-control samples yielded negative results for *T gondii* and *N caninum* on the PCR assay.

Conclusions and Clinical Relevance—Standard tests for toxoplasmosis or neosporosis associated with the CNS rely on serologic, histologic, or immunohistochemical analysis and can be difficult to interpret. The multiplex PCR assay with built-in control reactions could be a complementary clinical tool for the antemortem diagnosis of toxoplasmosis or neosporosis associated with the CNS. (Am J Vet Res 2003; 64:1507–1513)

*Toxoplasma gondii* is an apicomplexan protozoan that causes multisystemic disease in cats and dogs. *Neospora caninum* is a closely related organism that causes disease in dogs but not cats. Both organisms can cause abortion and neonatal morbidity in cattle, sheep, and horses. Prior to 1988, morphologic similarities between *T gondii* and *N caninum* led to frequent misdiagnoses. Diagnosis of toxoplasmosis or neosporosis associated with the CNS, in particular, is further complicated by a considerable overlap in clinical signs.

The most common neurologic syndromes described in dogs infected with these organisms include polyradiculoneuritis and meningoencephalitis. Toxoplasma-associated encephalitis has been reported in cats, but we are not aware of any reports of naturally occurring neosporosis in cats. Clinical signs of neospora- and toxoplasma-associated encephalitis reflect the location and severity of the lesion or lesions and include seizures, behavioral changes, cranial nerve deficits, and signs of cerebellar dysfunction. Because both organisms cause similar neurologic signs, serologic testing for *T gondii* and *N caninum* is generally conducted concurrently.

Presumptive diagnosis of neospora- or toxoplasma-associated CNS disease is generally based on clinical signs along with high antibody concentrations in serum or CSF. Because definitive diagnosis requires detection of the organisms in tissue sections of the brain, antemortem histologic diagnosis is usually impractical. Other findings that support the diagnosis include increased activities of serum enzymes associated with necrosis of myocytes and hepatocytes; spontaneous electromyographic activity associated with polyradiculoneuritis; and detection of parasites in CSF, muscle biopsy specimens, lung aspirates, and cutaneous lesions. However, antemortem diagnosis and discrimination between the 2 organisms continue to pose a diagnostic challenge.

Although *T gondii* and *N caninum* are immunologically distinct in many ways, they share cross-reactivity for several serologic assays and immunohistochemical analyses. Determination of serum antibody titers by use of an ELISA is the standard method used for...
antemortem diagnosis of toxoplasmosis, whereas indirect immunofluorescence antibody testing (IFAT) is the criterion-referenced standard for the diagnosis of neosporosis. Immunofluorescence antibody testing necessitates a continuous and reliable source of parasites or parasitic antigens, and false-negative results are not uncommon. Although serologic testing is generally accurate for the diagnosis of neosporosis, it can be difficult to interpret serologic results when testing for toxoplasmosis, especially in dogs.

Methods for detection of nucleic acids for T. gondii and N. caninum that involve the use of body fluids or tissue biopsy specimens offer a more specific means of documenting active infection than is possible from the determination of serum antibody titers. Pathogen-specific polymerase chain reaction (PCR) assays, in particular, have become useful complementary diagnostic tools for the detection of T. gondii or N. caninum. In veterinary medicine, PCR assays have been used to amplify T. gondii from serum and aqueous humor obtained from clinical cases and from CSF to which T. gondii DNA has been added experimentally. The PCR assays have been used also to differentiate T. gondii from N. caninum in independent PCR assays. However, studies have not documented the use of PCR assays for the antemortem diagnosis of neospora- or toxoplasma-associated CNS disease in clinically affected animals. In the study reported here, we evaluated a multiplex PCR assay with built-in control reactions that could be applied to biological samples to differentiate between the 2 organisms.

Materials and Methods

Sample population—Biological samples were obtained from 24 animals for use in the study. Samples were obtained from 13 cats (7 cats with toxoplasmosis and 6 control cats that had nonprotozoal CNS diseases) and 11 dogs (6 dogs with neospora- or toxoplasma-associatedencephalitis and 5 control dogs that had nonprotozoal CNS diseases). All cats with toxoplasmosis were identified retrospectively. Four cats were identified with histologic (ie, encysted bradyzoites) or immunohistochemical evidence of systemic toxoplasmosis without CNS involvement. Three cats had histologic, immunohistochemical, or serologic evidence of toxoplasma-associated meningoencephalitis. Of these 3 cats, 1 had positive results when tested for FeLV, and another died as a result of lymphosarcoma in the gastrointestinal tract; both of these cats also had evidence of systemic toxoplasmosis. Six cats with nonprotozoal CNS diseases were chosen as negative-control animals. These cats had CNS disease associated with encephalitis (n = 1), granulomatous meningoencephalitis (1), canine distemper virus (1), meningioma (1), and oligodendroglioma (1).

DNA extraction—The DNA was extracted from CSF, frozen muscle, or paraffin-embedded tissue samples, as described elsewhere. Briefly, 2 to 4 sections (each section was 8 μm thick) were cut from each block of paraffin-embedded tissue by use of a microtome. Investigators ensured that the microtome blade was changed and the blade holder cleaned with acetone between blocks. As an extra precaution against contamination, blocks from control animals were sectioned before those from animals with toxoplasmosis or neosporosis.

The DNA was extracted from tissue sections by use of a commercially available kit for blood and tissues; the kit was used in accordance with the manufacturer's instructions but with omission of the dewaxing step. The DNA was extracted from samples of CSF and frozen muscle in accordance with the same protocol.

PCR assay—Primer pairs used to amplify T. gondii and N. caninum have been described elsewhere. For T. gondii, a 501 bp amplicon was amplified by use of primers derived from the B1 gene. A 327-bp N. caninum-specific amplicon was amplified by use of the Np6-Np21 primer pair. By use of the T. gondii-specific primers, the PCR assay was tested on N. caninum genomic DNA, and no amplicon was produced. Similarly, the N. caninum-specific primers were tested on T. gondii genomic DNA.
DNA, and no amplicon was produced. A 214-bp fragment of the canine histone 3.3 gene was amplified to assess the integrity of extracted canine DNA. The sense primer corresponded to nucleotide positions 547 to 566 (5′–CCA CTG AAC TTC TGA TTC GC–3′), and the antisense primer corresponded to positions 742 to 761 (5′–GGC TGC TAG CTG GAT GTC TT–3′). A 696-bp fragment of the canine ferritin gene was amplified to assess the integrity of extracted canine DNA. The sense primer corresponded to nucleotide positions 1 to 21 (5′–TTG TTC CTG CTT CAA CAG TGC–3′), and the antisense primer corresponded to positions 675 to 696 (5′–CAA AGA GAT ACT CTG CCA TGC–3′).

Optimized reactions for the multiplex PCR assay were used to amplify distinct bands for ferritin, *T. gondii*, and *N. caninum* from canine genomic DNA or histone, *T. gondii*, and *N. caninum* from feline genomic DNA. Optimized PCR assays were conducted in a final volume of 50 µL that contained 1× buffer, 1.5mM MgCl₂, 200mM deoxynucleoside triphosphate, 100 ng of each of the *T. gondii* B1 primers, 200 ng of each of the *N. caninum* Np6-Np21 primers, 25 ng of the canine ferritin or feline histone primers, and 0.25 units of Taq DNA polymerase. Multiple ratios of *T. gondii* genomic DNA to canine genomic DNA (1:500, 1:1,000, 1:2,000, 1:3,000, 1:4,000, and 1:5,000) were tested by use of PCR primers for the toxoplasma B1 gene. Positive-control samples for the PCR assays included *T. gondii* genomic DNA and *N. caninum* genomic DNA. Two types of negative-control samples were used for the PCR assays, including sterile water extracted in parallel with the clinical samples and a routine negative-control sample that used water as the PCR template.

Amplifications were performed for 40 cycles (denaturation, 10 minutes at 95°C; annealing, 1 minute at 55°C; extension, 1 minute at 72°C) followed by 39 cycles of 1 minute at 95°C, 1 minute at 55°C, and 1 minute at 72°C. Eight microliters of the 50-µL PCR product was analyzed by electrophoresis on a 1.5% agarose gel, and the bands were developed under ultraviolet light after incorporation of ethidium bromide.

Cloning and sequencing of PCR products—The PCR amplifications were ligated into a PCR cloning vector and transformed into *Escherichia coli*. Plasmid DNA was then extracted and purified by use of the commercially available kit. Bidirectional sequencing of the purified plasmid DNA was performed with plasmid-specific primers (ie, M13F and M13R) by use of a dye-terminator chemical reaction and a DNA sequencer, in accordance with the manufacturer's protocol. After 25 cycles of denaturation, annealing, and extension, the dye-terminated transcripts were precipitated with ethanol, washed with a 70% solution of ethanol, air-dried, and then analyzed. Homology analysis of the generated sequences was performed to confirm sequence identity.

**Results**

Reactions for the multiplex PCR assay were optimized by use of 200 ng of canine genomic DNA samples to which *T. gondii* genomic DNA was added (Fig 1). A ratio of 1:3,000 (200 ng of canine genomic DNA to which 0.07 ng of *T. gondii* genomic DNA was added) was selected as the optimal reaction because greater dilutions did not reliably yield an amplicon for the toxoplasma B1 gene.

Oligonucleotide primer concentrations were optimized by use of 200 ng of canine or feline genomic DNA to which 0.07 ng of both *T. gondii* and *N. caninum* were added. The amount of 0.07 ng was selected as a starting point for *N. caninum* genomic DNA because reproducible results were obtained when that amount of *T. gondii* genomic DNA was used in the aforementioned optimization experiment.

It was necessary to use 25 ng of ferritin or histone primers, compared with 100 ng of *T. gondii* B1 primers, to generate amplicons of similar intensity. Increasing the amount of the housekeeping primers (ferritin or histone) to ≥50 ng occasionally overwhelmed the mul-
multiplex reaction, producing intense ferritin or histone amplicons with only faint bands for either protozoal amplicon (data not shown).

When 100 ng of each of the toxoplasma B1 and Np6-Np21 primers were used (along with 25 ng of ferritin or histone), the 301-bp *T gondii* amplicon overwhelmed the reaction, and the 327-bp *N caninum* amplicon was barely discernible (data not shown). Use of twice the amount of Np6-Np21 primers (200 ng) produced *T gondii* and *N caninum* amplicons of similar intensity and a strong amplicon for canine ferritin or feline histone. These conditions (0.07 ng of intensity and a strong amplicon for canine ferritin or histone), the 501-bp Np6-Np21 primers were used (along with 25 ng of ferritin when tested by use of the multiplex PCR assay. The optimized multiplex PCR assay was applied to DNA extracted from selected tissues of the clinically affected and negative-control cats and dogs. The 4 cats with systemic toxoplasmosis all had positive results on the PCR assay for feline histone and the 301-bp fragment from the *T gondii* B1 gene (Fig 2). The 3 cats with toxoplasma-associated encephalitis all had positive results on the PCR assay for feline histone and the toxoplasma B1 gene.

Three of 4 dogs with only toxoplasma-associated encephalitis had positive results on the PCR assay for the toxoplasma B1 gene (Fig 3). Specimens yielding positive results included brain, muscle tissue, and CSF. One of these dogs also had been identified with neosporosis by use of immunohistochemical analysis of brain tissues; however, results of the PCR assay were negative for neospora but positive for toxoplasma in that dog. One dog with serologic evidence of toxoplasma-associated encephalitis had negative results when a sample of CSF was tested by use of the PCR assay; cryptococcal organisms were identified in this dog's CSF; antemortem biopsy specimens were not evaluated by histologic or immunohistochemical analyses.

A fifth dog had positive results for the toxoplasma B1 gene and the *N caninum*-specific amplicon when DNA extracted from paraffin-embedded brain, frozen muscle tissue, and CSF was tested (Fig 3). This dog was weakly seropositive for *N caninum* and had a strong positive result when tested for *T gondii* by use of immunohistochemical analysis.

The sixth dog had positive results when a sample of CSF was tested for the *N caninum*-specific amplicon by use of the PCR assay but a negative result for the toxoplasma B1 gene. Neosporosis was diagnosed in that dog on the basis of results of IFAT (titer, 1:1,600).

All of the dogs with neospora- or toxoplasma-associated encephalitis had positive results for canine ferritin when tested by use of the multiplex PCR assay.

Samples from 4 of 5 negative-control dogs had positive results on the PCR assay for canine ferritin, whereas samples from all 6 negative-control cats had positive results on the PCR assay for feline histone. None of the samples from the negative-control cats and dogs generated amplicons of the expected size for *T gondii* or *N caninum*.

Amplicons were successfully cloned into a PCR cloning vector. Sequence identity was confirmed for PCR products that had positive results (data not shown).

**Discussion**

The goal of the study reported here was to develop a multiplex PCR assay for the antemortem detection of *T gondii*, *N caninum*, or both in biological samples obtained from dogs and cats. The DNA targets for PCR assays of each parasite were selected to take advantage of the minor differences between *T gondii* and *N caninum*. The B1-gene, which exists in at least 35 copies within the toxoplasma genome, forms the basis for several PCR-based tests for *T gondii* and does not detect *N caninum*. Two *Neospora*-specific PCR products provide molecular discrimination from *T gondii*. Because of their specificities for *T gondii* and *N caninum*, respectively, we used the B1 gene and a *Neospora*-specific PCR ampiclon to help differentiate between the 2 parasites in a multiplex system.

Using this multiplex PCR assay, we amplified protozoal genomic DNA from formalin-fixed, paraffin-embedded tissues as well as from frozen CSF and skeletal muscle. Because formalin crosslinks DNA, PCR primers typically are designed to generate amplicons of < 200 bp when amplifying formalin-fixed tissues. However, to allow for sufficient separation of the PCR amplicons on agarose gels, we selected oligonucleotide primers that generate amplicons ranging from 200 to 700 bp. This should not be a substantial limitation for future antemortem applications in which we could rely mainly on muscle biopsy specimens or CSF samples for DNA extraction, as was performed in 4 dogs in the...
study reported here. Furthermore, only 1 of the reactions (for a negative-control dog) was negative for the ferritin amplicon (approx 700 bp), suggesting that both multiplex reactions will be sufficiently robust to amplify DNA targets > 200 bp, even from formalin-fixed tissues. Generation of longer DNA fragments depends largely on the amount of time that tissues are subject to formalin fixation prior to paraffin embedding; therefore, when protozoal disease is included as a differential diagnosis, a potential diagnostic strategy for PCR assay on tissues obtained during necropsy would be to limit the duration of formalin fixation to < 24 hours.

An important feature of this multiplex reaction is the incorporation of a housekeeping gene to control for the integrity of DNA in the clinical samples that were tested. A true-negative result for protozoal DNA can be made only when the reaction generates a product of the expected size from the housekeeping gene. When no amplicon is generated for the housekeeping gene, it is probable that an insufficient amount of DNA was extracted from a sample or an inhibitory substance affected the reaction. This positive-control housekeeping gene as part of the multiplex is distinct from the standard positive-control samples included simply to test that the PCR reagents are working. The only way to duplicate this control housekeeping gene to ensure sample integrity is to conduct a separate reaction on each sample to be tested, which adds to expense and may not be done routinely by all laboratories. As well as a positive-control housekeeping gene for sample integrity, we also chose to run a negative-control sample (ie, water) that had been subjected to the same extraction protocol as the clinical samples tested. This ensured that the DNA extraction reagents and the sample itself had not been inadvertently contaminated with PCR amplicons, which would then generate false-positive results. Finally, we confirmed the identity of the PCR products generated by use of sequence analysis. Future use in a clinical laboratory setting could employ techniques such as restriction fragment-length polymorphism analysis, use of reporter fluorophores for end-point detection, or real-time PCR assays.

In the dogs with neospora- or toxoplasma-associated encephalitis, we were able to eliminate DNA degradation as a cause for false-negative data by amplifying a 700-bp fragment of the canine ferritin gene. This amplicon is not well conserved in cats, and a 200-bp fragment of the feline histone gene was substituted. Results for 1 of the negative-control dogs (a dog with a meningeoma) documents the use of the ferritin positive-control sample to confirm sample integrity. Despite the fact that it is unlikely that this dog harbored protozoal parasites, the negative result for the PCR assay cannot be interpreted because the negative PCR result for canine ferritin suggests that the DNA was degraded.

Results of PCR assays were consistent with immunohistochemical or serologic diagnoses in all clinical cases except for 3 dogs. One of these dogs was particularly interesting in that amplicons were generated for both *T gondii* and *N caninum*. Previous testing of samples from that dog revealed seemingly discordant immunohistochemical and serologic results, with strong positive results for immunohistochemical analysis of *T gondii* (immunohistochemical analysis was not conducted to test for *N caninum*) but negative serologic results for *T gondii* and weak seropositive results for *N caninum*. Results of the PCR assay confirmed DNA from both organisms in samples of CSF, brain tissue, and muscle tissue, which, to our knowledge, has not been reported in a naturally occurring infection. The second of those 3 dogs had positive results for immunohistochemical analysis of *N caninum*; however, the PCR assay reproducibly generated an amplicon for only *T gondii*. It is possible that this immunohistochemical result was attributable to cross-immunoreactivity of the *Neospora* antibody for *T gondii*. Alternatively, it is possible that this patient harbored a combined infection; the sections from which DNA was extracted for PCR analysis may have contained a different population of organisms from that for the sections on which we conducted immunohistochemical analysis. We believe the former is more likely because several studies in which immunohistochemical analyses were conducted have documented cross-reactivity of antibodies for *T gondii* and *N caninum*. Finally, the last of those dogs had a strong positive result on multiple serologic tests for *T gondii* in CSF, but had negative results for both *T gondii* and *N caninum* when tested by use of the PCR assay. One potential explanation is that protozoal organisms were not in the CSF and were restricted to neural tissue, which was not available for antemortem study. Alternatively, the toxoplasma antibodies may have been induced by a nonspecific antibody response secondary to prolonged antigenic stimulation by the cryptococcal infection.

Although we documented that the multiplex PCR assay reported here shows promise as an antemortem diagnostic tool, PCR assays are unlikely to be a panacea for diagnosing CNS-associated protozoal disease in veterinary medicine. In animals with neospora- or toxoplasma-associated CNS disease in which encysted bradyzoites are embedded deep in the brain parenchyma or spinal cord, PCR assays may yield false-negative data, which may have occurred in 1 of the dogs of this study. In human medicine, PCR assays performed on CSF samples obtained from human immunodeficiency virus-positive patients with confirmed toxoplasma-associated encephalitis yields false-negative results in many instances.36-38 False-negative data are an inherent risk to any procedure that necessitates finding a pathogen's DNA in biological samples. Although PCR techniques have emerged as extremely sensitive and specific diagnostic tools, it is crucial to interpret results in light of the type of sample examined, biological behavior of the organism, and limitations of the test when attempting to rule in or rule out protozoal-induced encephalitis.

In human medicine, the diagnosis of toxoplasmosis may be established on the basis of results of PCR assays or serologic tests, histologic detection of the parasite or its antigens, or isolation of the organism.39 Other less commonly used methods include analysis of antigene-mia and antigen in serum and body fluids, results of a toxoplasmin skin test, and evaluation of antigen-specific...
ic lymphocyte transformation.\textsuperscript{40} Detection of antibody specific to \textit{T gondii} is the initial and primary method of diagnosis. A number of serologic tests for antibodies with unique patterns are then used routinely to differentiate recent infection from prior exposure.\textsuperscript{16,17} A combination of serologic tests (ie, the \textit{Toxoplasma} serologic panel, which consists of the Sabin-Feldman dye test,\textsuperscript{40,41} double-sandwich IgM ELISA,\textsuperscript{42} IgA ELISA,\textsuperscript{43} IgE ELISA,\textsuperscript{44} and differential agglutination test\textsuperscript{45}) is usually required to establish whether an individual has been infected in the distant past or has only recently been infected. The necessity to use such an elaborate serologic panel in humans suggests that similar strategies should be used in veterinary medicine.

Discriminating between exposure and active infection is particularly problematic in cats with toxoplasmosis. In 1 study\textsuperscript{46} of cats in Georgia, 54.3\% of healthy cats were seropositive for toxoplasma, whereas 65.4\% of sick cats were seropositive. The use of convalescent titers and antibody coefficients for the diagnosis of toxoplasmosis has substantially increased the specificity of serologic testing; however, serologic results do not always correlate with clinical disease.\textsuperscript{15,16,17} In addition to serologic testing and PCR assay, positive results for immunohistochemical analysis are suggestive of active infection, but acquiring tissue samples is often impractical in protocol diseases that involve the CNS. Because of such limitations and the inherent challenge of serologic interpretation, we propose a combination of PCR data, serologic results, and, when practical, evaluation of biopsy specimens by use of immunohistochemical analysis for the antemortem diagnosis of toxoplasmosis and neosporosis that involves the CNS.

\textsuperscript{40}QiAmp, Qiagen Inc, Chatsworth, Calif.
\textsuperscript{41}Taq DNA polymerase, Promega Corp, Madison, Wis.
\textsuperscript{42}Neospora caninum genomic DNA was provided by Dr. J. P. Dubey, Parasite Biology, Epidemiology & Systematics Lab, BARC-East, 10300 Baltimore Ave, Beltsville, Md.
\textsuperscript{43}QiPOPO TA Cloning Kit Dual Promoter with One Shot MAX Efficiency DH5\textsuperscript{a} T1 E coli, Invitrogen Corp, Carlsbad, Calif.
\textsuperscript{44}BigDye terminator cycle sequencing ready reaction kit, Applied Biosystems Inc, Foster City, Calif.
\textsuperscript{45}3700 DNA analyzer, Applied Biosystems Inc, Foster City, Calif.

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