Polymerase chain reaction analysis for viruses in paraffin-embedded myocardium from dogs with dilated cardiomyopathy or myocarditis

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Objective—To perform polymerase chain reaction (PCR) analysis on paraffin-embedded myocardium from dogs with dilated cardiomyopathy (DCM) and dogs with myocarditis to screen for canine parvovirus, adenovirus types 1 and 2, and herpesvirus.

Sample Population—Myocardial specimens from 18 dogs with an antemortem diagnosis of DCM and 9 dogs with a histopathologic diagnosis of myocarditis were evaluated.

Procedure—Paraffin-embedded myocardial specimens were screened for viral genome by PCR analysis. Positive-control specimens were developed from cell cultures as well as paraffin-embedded tissue specimens from dogs with clinical and histopathologic diagnoses of viral infection with canine parvovirus, adenovirus types 1 and 2, and herpesvirus. The histologic characteristics of all myocardial specimens were classified regarding extent, location, and type of inflammation and fibrosis.

Results—Canine adenovirus type 1 was amplified from 1 specimen from a dog with DCM. Canine parvovirus, adenovirus type 2, and herpesvirus were not amplified from any myocardial specimens. Histologic analysis of specimens from dogs with DCM revealed variable amounts of fibrosis; myocardial inflammation was observed in 1 affected dog. Histopathologic analysis of specimens from dogs with myocarditis disclosed variable degrees of inflammation and fibrosis.

Conclusions and Clinical Relevance—Viral agents canine parvovirus, adenovirus types 1 and 2, and herpesvirus are not commonly associated with DCM or active myocarditis in dogs. Additional studies evaluating for nucleic acid from viruses that less commonly affect dogs or different types of infectious agents may be warranted to gain insight into the cause of DCM and myocarditis in dogs. (Am J Vet Res 2001;62:130–135)

Dilated cardiomyopathy (DCM) is a primary disease of the heart muscle characterized by cardiac chamber enlargement and impaired systolic function of one or both ventricles. Cardiac arrhythmias and congestive heart failure are common sequelae. In 10 to 30% of humans with DCM, the disease is presumed to be secondary to myocarditis resulting from either an acute or persistent viral infection. Recent advances in molecular analysis of myocardial specimens have led to identification of viral nucleic acid within the myocardium of humans affected with DCM. Polymerase chain reaction (PCR) analysis of myocardial specimens has identified adenovirus, enterovirus, herpes simplex virus, parvovirus, and cytomegalovirus in myocardial specimens from adults, children, and infants with DCM.

Dilated cardiomyopathy is also observed in dogs and has a high prevalence in certain breeds, such as the Boxer, Doberman Pinscher, and Cocker Spaniel. Factors contributing to the development of DCM have been suggested in some breeds including myocardial carnitine deficiency in Boxers, serum or plasma taurine depletion in Cocker Spaniels, and deficiency of the creatine kinase isoenzyme MB in Doberman Pinschers; however, these factors do not explain the cause of DCM in all affected dogs. Less commonly, DCM develops in dogs with no known breed-predisposition (atypical dog breeds). The cause of DCM in these dogs is also unknown. An association between viral infection and DCM in dogs has not been explored.

The objective of our study was to evaluate archived formalin-fixed paraffin-embedded myocardial specimens from dogs with DCM for viral nucleic acid of several common viral pathogens. A second objective was to perform a similar evaluation on myocardial specimens from dogs with a histopathologic diagnosis of myocarditis. Additionally, histologic characterization was performed on myocardial specimens from dogs with DCM or myocarditis.
A second group of specimens was selected by screening the database for specimens from dogs with a histopathologic diagnosis of myocarditis by the Department of Veterinary Biosciences at The Ohio State University from January 1986 to July 1997. The histopathologic diagnosis of myocarditis included affected dogs in which myocyte degeneration and necrosis was predominantly accompanied by inflammatory cell infiltrate. None of these dogs had been evaluated ante-mortem with echocardiography; therefore, myocardial function status was unknown.

**Virus selection, primer design, and synthesis**—Canine parvovirus, canine adenovirus type 1 (CAV-1), canine adenovirus type 2 (CAV-2), and canine herpesvirus (CHV) were chosen for PCR analysis to assay for viral nucleic acid (Appendix 1). Primers were synthesized on an automated oligonucleotide synthesizer. The parvovirus primer pair was designed to amplify a 301-bp product from the P1 domain of the hexon gene region. The third primer pair was created from the E1 gene region of CAV-2 and amplifies an approximately 1,500-bp segment. The fourth primer pair amplifies a 120-bp product of the glycoprotein B gene of CHV. As a positive-control specimen, a fifteenth primer set was designed to amplify a portion of host genome consisting of a highly conserved 106-bp product of the cardiac β-myosin heavy chain gene, exon 13.

**DNA Extraction**—Location and number of myocardial specimens varied for each affected dog. Specimens of 8 dogs with DCM and 5 dogs with myocarditis had been harvested only from the left ventricular free wall, the interventricular septum, or the right ventricular free wall, whereas the remainder of the dogs (10 with DCM and 4 with myocarditis) had specimens available from multiple sites. When specimens from multiple sites were available, specimens from each site were evaluated. Five-µm thick sections were obtained from each formalin-fixed myocardial tissue specimen. Sections were incubated with 2 ml of xylene for 5 minutes, homogenized by passing the xylene suspension repeatedly through a 1,000-µl volume pipet tip, followed by at least 8,000 rpm for 5 minutes. A second xylene extraction was performed with a 1-hour incubation at room temperature (approx 25°C). After centrifugation and decanting supernatant, 1 ml of 95% ethanol was added followed by a wash with 1 mh of 70% ethanol. The pellet was resuspended in 500-µl digestion buffer (0.02 M EDTA, 0.5 M Tris-HCl, 0.01 M NaCl, 0.5% SDS) and 50-µl proteinase K (10 mg/ml stock). Specimens were incubated at 42°C for 16 to 24 hours.

Five hundred microliters of phenol-chloroform-isooamyl alcohol (25:24:1, buffered pH 8) were added to each specimen and vortexed for 30 seconds. A final chloroform extraction followed by sodium acetate and isopropanol precipitation was performed, and specimens were incubated at −20°C overnight. The nucleic acid pellet was washed with 70% ethanol, allowed to dry completely, and resuspended in 50 µl of distilled water. Specimens were heated to 100°C for 5 minutes, allowed to cool slowly to room temperature (approx 25°C), and held at −20°C until PCR analysis. Efficacy of the DNA extraction technique was verified for each specimen by performing amplification of exon 13 of the cardiac β-myosin heavy chain. Spectrophotometric analysis was used to calculate the concentration of nucleic acid.

**PCR conditions**—For all viruses, 250 ng of extracted total DNA from each specimen was combined with 2.5 µl (50 pmol) of forward and reverse primers, 2.5 µl of 10X PCR buffer (260 mM Tris-HCl, pH 8.8, 260 mM MgCl2), 2.5 µl of 10-nmol dNTP mix, and distilled water to a total reaction volume of 25 µl. Taq polymerase (1 unit) was added after an initial incubation at 94°C for 5 minutes. Forty-five cycles of amplification were performed with the following conditions: 94°C for 1 minute, 53°C for 1 minute, and 72°C for 1 minute. Specimens were incubated at 72°C for 10 minutes.

Beta-myosin amplification was performed similarly with 250 ng of extracted total DNA from each specimen combined with 2.5 µl (50 pmol) of forward and reverse primers, 2.5 µl of 10X PCR buffer, 2.5 µl of 10-nmol dNTP mix, 0.2 µl (1 unit) of Taq polymerase, and distilled water to a total reaction volume of 25 µl. Thirty cycles of amplification were performed with the following conditions: 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds.

**Positive-control specimens**—Polymerase chain reaction conditions were optimized by amplification of viral genome for canine parvovirus, CAV-1 and CAV-2, and CHV, using nucleic acid extracts from wild-type strains of virus obtained from purified virus-infected tissue culture supernatant. Efficacy of amplification was confirmed, using nucleic acid extracts, processed as already indicated, from paraffin-embedded tissue specimens from dogs with clinical and histopathologic diagnoses of viral infection with parvovirus, CAV-1, CAV-2, or CHV.

**Product analysis**—Fifteen microliters of each reaction were analyzed by electrophoresis on a 1% agarose gel and viewed after ethidium-bromide staining. Amplified products were identified by sequence analysis performed manually, using a sequenase kit.

**Histologic evaluation**—Formalin-fixed paraffin-embedded myocardial specimens varied in specimen location. Specimens of 8 dogs with DCM and 5 dogs with myocarditis had been harvested from only the left ventricular free wall, the interventricular septum, or the right ventricular free wall, whereas the remainder of the dogs (10 with DCM and 4 with myocarditis) had specimens available from multiple sites. When specimens from multiple sites were available, specimens from each site were evaluated. Masson’s Trichome and H&E stains were used to evaluate each site. Sections were examined under 10X and 40X magnification. For semi-quantitative analysis, 10 fields of each specimen were evaluated under 10X magnification, and changes were characterized according to the extent, location, and type of inflammatory cell infiltrate as well as the extent and location of fibrosis (Appendix 2). The classification scheme used in our study represents a modification of the Dallas criteria used in humans to characterize myocardial changes in myocarditis and is intended to provide descriptive information only.

**Results**

Specimens from dogs with DCM—A total of 216 dogs had DCM between January 1986 and July 1997. Myocardial specimens were available from 8 dogs that were from breeds typically underrepresented with DCM. The 8 included 2 mixed breeds, 2 German Shepherd Dogs, 1 Afghan, 1 Saint Bernard, 1 Border Collie, and 1 Labrador Retriever. Myocardial specimens were not recorded for 6 of the 8 dogs with DCM. For the remaining 2 dogs, specimens were available from the right ventricular free wall, interventricular septum, and left ventricular free wall.

A random selection was used to identify 10 dogs with DCM belonging to breeds with a presumed predisposition (ie, Doberman Pinschers, Boxers, Cocker Spaniels) that had myocardial tissue available for analysis. This included 1 Boxer, 3 Cocker Spaniels, and 6 Doberman Pinschers. Myocardial specimen sites were not recorded for 2 of the 10 dogs with DCM. For
the remaining 8 dogs, specimens were available from the right ventricular free wall, interventricular septum, and left ventricular free wall.

Specimens from dogs with myocarditis—Myocardial specimens from 9 dogs were identified for analysis on the basis of a histopathologic diagnosis of myocarditis. Myocardial specimen sites were not recorded for 5 of the 9 dogs with myocarditis. For the remaining 4 dogs, specimens were available from the right ventricular free wall, interventricular septum, and left ventricular free wall.

PCR analysis of positive-control specimens—Polymerase chain reaction amplification was successfully performed, using all viral primer sets with each positive-control specimen, including purified virus and virus-infected paraffin-embedded tissue (Fig 1). Exon 13 of the cardiac β-myosin heavy chain was amplified from all specimens successfully, confirming the presence of quality nucleic acid.

PCR analysis of specimens—Polymerase chain reaction amplification with the CAV-1 primers identified a 308-bp product from the myocardium in 1 of 18 dogs with DCM (Fig 2). The specimen was from a 5-month-old female German Shepherd Dog with clinical signs of congestive heart failure. The gel band was isolated and the identity of the amplimer confirmed by sequence analysis performed manually.

Polymerase chain reaction analysis for parvovirus, CAV-1, CAV-2, and CHV did not identify viral nucleic acid in myocardial specimens from any of the other dogs with DCM or myocarditis.

Histologic evaluation—Specimens from 5 of the 18 dogs with DCM had no evidence of fibrosis, and 5 had only mild grade-1 myocardial fibrosis. Specimens from the remaining 8 dogs had substantial grade-IV interstitial myocardial fibrosis, including specimens from the dog that had positive PCR results for CAV-1 viral genome. A specimen from 1 dog had moderate grade-III myocardial inflammation with lymphocytic-plasmacytic and neutrophilic cellular infiltrate in the regions of interstitial fibrosis. This was not the specimen in which CAV-1 was amplified. Specimens from the remaining 17 dogs with DCM had no histologic evidence of inflammation.

Specimens from 9 dogs with myocarditis were evaluated. Specimens from 3 of the 9 dogs had no evidence of fibrosis. Specimens from 2 dogs had grade-1 interstitial myocardial fibrosis, and 4 had grade-IV interstitial fibrosis. Grade-1 myocardial inflammation consisting of histiocytic cellular infiltrates was observed in specimens from 4 dogs. Specimens from 5 dogs had grade-IV myocardial inflammation. Specimens from all 5 of the dogs with grade-IV myocardial inflammation had neutrophilic inflammation, and 2 also had lymphocytic cellular infiltrates.

Discussion

Viral infection of the myocardium is associated with the development of DCM in humans.23 Recent technologic advances have allowed identification of...
viral nucleic acid in myocardial specimens, using PCR amplification to detect minute quantities of viral nucleic acid.14 Polymerase chain reaction analysis of myocardial specimens allows identification of adenovirus, enterovirus, herpes simplex virus, parvovirus, and cytomegalovirus in human specimens from adults, children, and infants with DCM.2,12 Many of these human pathogens are from the same viral families as prevalent viruses of dogs, including CAV-1 and CAV-2, parvovirus, and CHV. Dilated cardiomyopathy is documented in dogs, and although contributing factors have been identified, the cause remains unknown in most affected dogs. Because of the association of viral myocarditis with DCM in humans, we evaluated myocardial specimens for viral nucleic acid from dogs with DCM or myocarditis.

In our study, the CAV-1 genome was detected by PCR analysis of a formalin-fixed paraffin-embedded myocardial specimen from 1 dog with DCM. This German Shepherd Dog had clinical signs of congestive heart failure. No other systemic signs attributable to fulminant viral infection were identified. In human viral myocarditis, there are often no clinical signs, or flu-like symptoms may have been observed in the weeks to months preceding the diagnosis of DCM.3 In our study, the CAV-1 genome was detected by PCR analysis of a formalin-fixed paraffin-embedded myocardial specimen from 1 dog with DCM. This German Shepherd Dog had clinical signs of congestive heart failure. No other systemic signs attributable to fulminant viral infection were identified. In human viral myocarditis, there are often no clinical signs, or flu-like symptoms may have been observed in the weeks to months preceding the diagnosis of DCM.3

Identification of adenoviral DNA in a myocardial specimen in our study may represent true myocellular infection or may be the consequence of virus-infected lymphocytes coincidentally isolated in the paraffin-embedded tissue sections. The histologic finding of inflammatory cells in the myocardium would have supported a myocellular infection; however, this was not found in the specimens available for evaluation. Therefore, it is difficult to determine the importance of adenoviral isolation in this dog's clinical disease. It is of interest that adenovirus infection has been described in adult and pediatric human patients with DCM or myocarditis.5,8 Inflammation was observed in myocardial specimens from pediatric patients but appeared to be less than that associated with other viral infections of the myocardium.4 Inflammation was completely lacking from the specimens taken from adults with left ventricular dysfunction in which adenovirus was detected.12 This could suggest that adenovirus causes a milder inflammatory reaction in comparison to other viruses.12

The inability to detect virus in all but one of our myocardial specimens may represent the lack of infection with these viruses in dogs with DCM and myocarditis, or it could represent technical failure of the PCR or sampling technique. The efficacy of the techniques used in our study was evaluated in several ways. First, we were able to amplify β-myosin from all myocardial specimens, supporting the successful extraction of good quality DNA from each specimen. Second, we were able to amplify a product from the positive-control specimens with the designed PCR primers, supporting the accuracy of the primers as well as the PCR technique. Our sampling technique was limited because of the retrospective nature of specimen collection. We attempted to evaluate myocardial specimens from 3 areas of the heart (left ventricular free wall, interventricular septum, right ventricular free wall) to decrease the likelihood of missing focal disease. However, it is possible that a focal infection was missed, because many affected dogs only had specimens available from one area. Although it would have been desirable to evaluate a larger number of myocardial specimens, a total of 35 specimens and 27 affected dogs were evaluated with only 1 positive result on amplification. Nucleic acid degradation over time could have contributed to our inability to amplify a viral product, because 4 of the specimens had been fixed for >10 years (range, 1 to 11 years). However, successful PCR amplification of viral genome from long-term formalin-fixed biopsy tissue specimens has been reported.7,8

A more likely explanation for our inability to detect viral DNA is that DCM and myocarditis in dogs is rarely viral-induced or that viruses other than those evaluated in our study are involved. Other viruses that may be involved include coronavirus, canine distemper virus, or possibly other less commonly recognized canine pathogens (eg, coxsackie virus). Histologic evaluation was performed on all available specimens. It should be emphasized that the histologic information obtained in our study was for descriptive purposes only, because we were limited in sampling area by a retrospective study design and the ability to take multiple sections from multiple areas. Histologic evaluation of myocardial specimens from dogs with DCM revealed changes identical to those described for idiopathic DCM.5 The most common abnormalities were nonspecific and included varying degrees of interstitial fibrosis with multifocal myocardial degeneration and necrosis with no inflammation. One myocardial specimen from a dog with no breed predisposition for DCM, and that did not amplify viral genome, had moderate lymphocytic inflammation. A similar finding of rare lymphocytic myocardial inflammation was previously reported in a study of idiopathic DCM in large breed dogs.21 In humans, histologic evidence of myocarditis may differ in the acute phase of viral infection in contrast to the chronic or persistent infection. The acute phase may have marked inflammatory cell infiltrate accompanying myocellular degeneration and necrosis with minimal or no fibrosis. The chronic or persistent phase may have poorly defined changes such as reparative fibrosis and nonspecific myocellular morphologic changes similar to those described in tissue from people with DCM.3

The most common histologic finding in dogs with myocarditis was a neutrophilic inflammatory infiltrate. In humans, neutrophilic infiltrates are most often associated with bacterial myocarditis, although they can be associated with viral myocarditis.32 Viral myocarditis is more typically characterized by lymphocytic inflammation. The evaluation of myocardial specimens from dogs with myocarditis for bacterial nucleic acid may be of interest.

In conclusion, results of our study suggest that viral myocarditis associated with canine parvovirus, CAV-1 and CAV-2, or CHV is an uncommon cause of DCM in dogs. Similarly, it also suggests that infection with these viruses is not a common cause of active histologically apparent myocarditis in dogs.
### Appendix 1

Oligonucleotide sequences of primers used to amplify viral nucleic acid and the cardiac β-myosin heavy chain gene in myocardial specimens from dogs with dilated cardiomyopathy (DCM) and dogs with a histopathologic diagnosis of myocarditis

<table>
<thead>
<tr>
<th>Virus</th>
<th>Primer sequence (upstream/downstream)</th>
<th>PCR (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canine parvovirus</td>
<td>5'-TGCATGTTTGGAGTTACAGT-3'</td>
<td>1500</td>
</tr>
<tr>
<td></td>
<td>5'-TCCAGTGGTCTGCAAATTG-3'</td>
<td>1500</td>
</tr>
<tr>
<td></td>
<td>5'-CGCAGTGTGCTGCAAGATTG-3'</td>
<td>1500</td>
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<tr>
<td></td>
<td>5'-AGGACTATCTGGATGCGG-3'</td>
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<tr>
<td></td>
<td>5'-CTTCCTGACGTCTCTCT-3'</td>
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<tr>
<td></td>
<td>5'-CAGGACTATGCTGATAGT-3'</td>
<td>1500</td>
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<tr>
<td></td>
<td>5'-TGCAATGGCCCTATAAT-3'</td>
<td>1500</td>
</tr>
<tr>
<td></td>
<td>5'-TACAGGAGCTGCTGTACTCTTGCACT-3'</td>
<td>1500</td>
</tr>
</tbody>
</table>

**PCR** = Polymerase chain reaction product size. **CAV-1** = Canine adenovirus type 1. **CAV-2** = Canine adenovirus type 2. **CHV** = Canine herpesvirus.

### Appendix 2

Description of histologic scoring system for inflammation and fibrosis applied to the characterization of the changes in myocardial specimens of dogs with DCM and dogs with a histopathologic diagnosis of myocarditis. Degree of myocardial fibrosis and inflammation was scored by examining fields of each specimen with 10× and 40× objective lenses, respectively.

<table>
<thead>
<tr>
<th>Histologic finding</th>
<th>Grade No.</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation</td>
<td>I</td>
<td>Infrequent multifocal perivascular inflammation with a low cell number (≤ 5 cells/field).</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>Frequent multifocal perivascular or interstitial inflammation with a low cell number.</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>Frequent multifocal perivascular or interstitial inflammation with a high cell number (≥ 5 cells/field).</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>I</td>
<td>Infrequent multifocal perivascular or interstitial inflammation with a low cell number.</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>Frequent multifocal perivascular or interstitial inflammation with a high cell number (≥ 5 cells/field).</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>≥ 15% of fields affected with any degree of fibrosis.</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>≥ 20% of fields affected with any degree of fibrosis.</td>
</tr>
</tbody>
</table>

References


