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Abstract

Many viruses have been identified in pericardial fluid and in tissue samples from humans with pericarditis by means of molecular diagnostics. In canine idiopathic pericardial effusion there is as yet no conclusive evidence to support the involvement of an infectious agent. This study was designed to investigate a possible relationship between idiopathic pericardial effusion in dogs and viruses most commonly encountered in humans affected with viral pericarditis. Coxsackievirus B3 RNA, influenza virus type A RNA, human adenovirus type 2 DNA, human cytomegalovirus DNA, and parvovirus B19 DNA were investigated using PCR on pericardial effusion samples and pericardial tissue specimens collected from 14 dogs with idiopathic pericardial effusion. PCR was also used to test for two bacteria, Borrelia burgdorferi and Chlamydia pneumoniae. The same microorganisms were also looked for in pericardial effusions or pericardial washes from 10 dogs with neoplastic pericardial effusion, and in samples collected from 10 dogs which died of a non-cardiac disease. One pericardial effusion sample from a dog with the idiopathic form of the disease tested positive for influenza virus type A and sequencing of the amplicon confirmed the PCR result. In another dog from the same group a cytomegalovirus was detected by PCR in the effusion, but sequencing showed this to be a false-positive result. The genomes of the microorganisms investigated were not detected in neoplastic effusions or pericardial washes. The results indicate that viral and bacterial DNA/RNA of relevance for human pericarditis is rare in pericardial samples from dogs with idiopathic pericardial effusion. The finding of influenza type A viral RNA in pericardial fluid from one dog with the idiopathic form of the disease warrants further investigation.

Evaluation of the presence of selected viral and bacterial nucleic acids in pericardial samples from dogs with or without idiopathic pericardial effusion

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Abstract

Many viruses have been identified in the pericardial fluid and in tissue samples from humans with pericarditis by means of molecular diagnostics. In canine idiopathic pericardial effusion there is yet no conclusive evidence to support the involvement of an infectious agent. The study was designed to investigate a possible relationship between idiopathic pericardial effusion in dogs and viruses most commonly encountered in humans affected by viral pericarditis. Coxsackievirus B3 RNA, influenza virus type A RNA, human adenovirus type 2 DNA, human cytomegalovirus DNA, and parvovirus B19 DNA were investigated by PCR in pericardial effusion samples and pericardial tissue specimens collected from 14 dogs with a diagnosis of idiopathic pericardial effusion. PCR was also employed to test the DNA for two bacteria: *Borrelia burgdorferi* and *Chlamydia pneumoniae*. The same microorganisms were also investigated in pericardial effusion or pericardial wash from 10 dogs with neoplastic pericardial effusion and 10 dogs which died of a non cardiac disease, respectively.

One pericardial effusion sample from a dog with the idiopathic form of the disease tested positive for influenza virus type A and sequencing of the amplicon confirmed the PCR result. In another dog of the same group a cytomegalovirus was detected by PCR in the effusion, but sequencing showed this to be a false positive result. The genomes of the microorganisms investigated were not detected in neoplastic effusions or pericardial washes. The results indicate that viral and bacterial DNA/RNA of relevance for human pericarditis is rare in pericardial samples of dogs with idiopathic pericardial effusion. The finding of influenza type A viral RNA in pericardial fluid from one dog with the idiopathic form of the disease warrants further investigation.

Keywords: Pericarditis; Canine; Aetiology; Virus; PCR

Introduction

Pericardial effusion is the third most common cardiac disease in dogs and accounts for around 10% of all cardiovascular diseases (Baumgartner and Glaus, 2004). Neoplasia and idiopathic pericardial effusion (IPE) are the most important aetiologies whereas congestive heart failure, atrial splitting, trauma, bacterial or fungal pericarditis, uraemia, peritoneopericardial hernias, intrapericardial cysts, and hypoalbuminemia are rare causes.

IPE is a diagnosis of exclusion, and is suspected when serosanguinous to hemorrhagic fluid accumulates in the pericardial space and routine diagnostic methods fail to demonstrate an underlying cause (Aronsohn and Carpenter, 1999). There is a wide variation in the course of the disease in IPE; some dogs may be cured after removal of all pericardial effusion by one single pericardiocentesis, whereas in others effusions may repeatedly recur (Aronsohn and Carpenter, 1999).

In human patients, even with exhaustive examinations a specific cause may not be identified in many cases of IPE although in 20-30% of pericardial effusions viruses have been identified and considered causative (Zayas et al., 1995; Maisch and Ristic, 2002). In dogs, the viral hypothesis has been proposed because many cytological reports of the effusions and histopathological findings of the pericardial sac of dogs affected by IPE present inflammatory features not indicative of a bacterial, protozoal or fungal infection, and attempts to culture such microorganisms have been unrewarding (Kienle, 1998). Immunological studies have been also performed in these dogs and have shown a predominance of IgA positive plasma cells within inflammatory aggregates in the pericardial tissue (Day and Martin, 2002) and, in the peripheral blood, a depletion of T lymphocytes with helper cells being mostly affected (Guglielmino et al., 2004). In a more recent investigation, however, differences were not

78 detected between immunoglobulin concentrations in peripheral blood and pericardial effusion
79 in dogs with idiopathic or neoplastic pericardial effusion (Martin et al., 2006). The immune
80 system appears thus involved in the pathogenesis of the disease, although modestly, but the
81 aetiology remains unknown.
82
83 There is scarce veterinary literature on viral agents associated with cardiovascular
84 disease in adult dogs. Molecular genomics have been employed on one occasion to detect
85 viruses in the myocardium of dogs with active myocarditis and dilated cardiomyopathy.
86 Among 27 dogs only one dog with dilated cardiomyopathy had amplified canine adenovirus
87 type 1 (CAV-1) (Maxson et al., 2001). In a second report, West Nile virus was amplified in
88 the myocardium of a dog showing neurological signs (Buckweitz et al., 2003). To our
89 knowledge, there are no published data on infectious pericarditis other than rare pyogenic
90 bacterial infections (Aronson and Gregory, 1995; Stafford Johnson et al., 2003).
91
92 The aim of this retrospective study was to evaluate the potential role of human viruses
93 in the pathogenesis of canine IPE, by means of polymerase chain reaction (PCR) performed
94 on samples of pericardial fluid and biopsies of pericardial tissue. Samples were collected from
95 dogs with a diagnosis of IPE and from dogs with neoplastic pericardial effusion. In addition, a
96 pericardial wash was collected from dogs which died of a non-cardiac disease. In the light of
97 the available cooperation between the authors' affiliations, primers were directed toward the
98 genome of some of the most important viruses involved in human pericarditis and
99 myocarditis, including coxsackievirus B3, influenza virus type A, human adenovirus type 2,
100 human cytomegalovirus and parvovirus B19. Some of them, such as influenza virus type A
101 and coxsackieviruses have been identified in dogs, but their role as cardiovascular pathogens
102 has not been investigated in this species (Grew et al., 1970; Kilbourne and Kehoe, 1975). In

addition, common bacterial pathogens of the human cardiovascular system were also included in the investigation, such as *Borrelia burgdorferi* and *Chlamydia pneumoniae*. Recently, *C. pneumoniae* has been demonstrated in canine atherosclerotic lesions and *B. burgdorferi* myocarditis has been suspected in some dogs (Levy and Duray, 1998; Sako et al., 2002).

Materials and methods

Patients

Between January 2000 and April 2007, samples of pericardial effusion from dogs with suspected IPE were collected at our institutions. A diagnosis of IPE was made according to the following criteria: (1) absence of detectable masses within the pericardial space using echocardiography; (2) absence of pulmonary lesions by thoracic radiography; (3) negative pericardial fluid cytology for neoplastic disorders; (4) absence of suppurative inflammation of pericardial fluid cytology or pericardial sac histopathology, or sterile anaerobic/aerobic bacterial and fungal culture from pericardial effusion; (5) exclusion of underlying metabolic diseases such as hypoproteinaemia and uraemia by means of a biochemical profile; (6) exclusion of cardiac diseases causing congestive heart failure and defects of the pericardial sac (i.e., pericardial cysts) by echocardiography and radiography.

To increase diagnostic reliability and exclude other potential causes of pericardial effusion (e.g. neoplasia) the animals needed a follow-up of at least 12 months during which they did not develop any other disease. Pericardial effusions due to primary or secondary neoplastic infiltration of the pericardium or heart were also stored. In addition, pericardial fluid obtained through pericardial wash was collected from dogs which died of a non cardiac disease. The two last groups served as control.

Sample collection

Pericardial effusions were collected in all dogs by echocardiography guided pericardiocentesis. To avoid any contamination, routine aseptic techniques were used for preparation of the thoracic area. The pericardial effusion was collected by the operator wearing sterile gloves and rapidly placed into a sterile vacuum tube. Pericardial tissue fragments were collected under thoracoscopy in order to minimize pericardial sac handling and contamination. In dogs which died due to a non cardiac disease a pericardial wash was collected within 3 h from death. After thoracotomy the pericardial sac was identified and injected with 15-20 mL of sterile saline using a 21-G needle. After 5-10 s the fluid was removed through the same needle. Care was provided in order to prevent contamination as above. Effusion samples and pericardial tissue fragments were stored at -70 °C until examination.

Polymerase chain reaction (PCR)

Conventional PCR was used to investigate pericardial effusion or wash samples and pericardial tissue fragments of dogs. Pericardial fluids and pericardial biopsies were investigated for the presence of Coxsackievirus B3 RNA, influenza virus type A RNA, human cytomegalovirus DNA, parvovirus B19 DNA and human adenovirus type 2 DNA (Table 1). Primer pairs were also used to test for the presence of *B. burgdorferi* and *C. pneumoniae* DNA. At the laboratory of two of the authors (SP, BM), primers for these two bacteria are normally part of the investigative panel of human patients with pericarditis or myocarditis.

To extract RNA/DNA from pericardial fluids and pericardial biopsies, the QIAamp Viral Mini Kit and the QIAamp Tissue Kit (Qiagen) were used. To avoid false-positive results, extraction amplification and electrophoresis were performed in separate areas and in duplicates. Also, a water sample was extracted as well, as a negative control. Ten microlitres

of extracted RNA/DNA were incubated with 25 pmol of the appropriate primer, 5 µL 10x PCR buffer (1.5 mmol $MgCl_2$), 10 mmol dNTPs, and 2.5 U Taq polymerase gold (Applied Biosystems) and deionised H_2O in a 50 µL final reaction volume. After an initial incubation at 94 °C for 12 min, 40 rounds of amplification were performed under the following conditions: 94 °C (denaturation) for 45 s, appropriate temperature for each primer pair for 45 s (Table 1), 72 °C (extension) for 1 min. A final cycle of 72 °C for 5 min for complete polymerisation followed. For detection of the RNA-viruses (influenza virus A and coxsackievirus B3) an initial reverse transcription using the RT-One-step-PCR-KIT (Qiagen) was performed. Two negative controls (sterile distilled water) and three serial dilutions of positive control were included in every PCR assay. Beta-actin was used as internal control for PCR (data not shown). Ten microlitres of each reaction were analysed on a 1.5% agarose gel (Sigma) containing 0.5 µg/mL ethidium bromide. For the control of size, basepair marker No. VIII ranging from 37-1114 basepairs (Boehringer Mannheim) was used. Primer sensitivity was determined for each pair by our laboratory (Table 2). Specificity was assessed in silico for all primer pairs through a GenBank database search and, apart the target genes, other sequences could not be identified.

Sequencing

To obtain enough material to be sequenced, influenza A and cytomegalovirus positive samples were reamplified using the same PCR conditions as described above using 2 µL of the previous PCR reaction. Products were analyzed on 3% agarose gels; amplicons were purified using the MinElute Gel Extraction Kit (Qiagen) and sequenced from both sides (Microsynth).

Results

Thirty-four dogs were enrolled in the study. Fourteen dogs had a diagnosis of IPE, 10 a diagnosis of pericardial or heart neoplasia and 10 had a non cardiac disease. Among dogs with neoplastic effusion, five had pericardial mesothelioma, three heart haemangiosarcoma, and one each a pericardial carcinoma or an unclassified metastatic neoplasia. Six dogs which died of a non cardiac disease had metastatic tumours and two each end-stage renal or liver failure.

In the group of dogs with IPE, 11 were males and 3 were females. Ten dogs belonged to large or giant breeds and four belonged to medium-size breeds. The age ranged from 3-13 years, with a median age of 8 years. In nine dogs pericardial effusion was collected during the first episode of IPE, in five others IPE was already diagnosed and they were referred when many relapses had occurred (three relapses in four dogs and four relapses in one dog) and thoracoscopic fenestration of the pericardial sac was required.

During the 12-month follow-up period, one dog had a single recurrence of pericardial effusion requiring removal of the pericardial fluid and another had two relapses. In the group of dogs with neoplastic effusion, 6 were males and 4 females. Six dogs belonged to large or giant breeds and the remaining to medium or small-size breeds. The age ranged from 5-14 years, with a median age of 10 years. In the group of dogs with non cardiac disease, 5 were males and 5 females. Five dogs belonged to large or giant breed and five to small-size breeds. The age ranged from 7-14 years, with a median age of 11 years.

In dogs with neoplastic effusion or non-cardiac disease a single effusion sample or pericardial wash was submitted for the viral and bacterial screening, respectively. With PCR none of the samples tested positive for the investigated microorganisms. In dogs with IPE a

total of 11 effusion samples and of five pericardial biopsies were analysed. Nine dogs had only the pericardial effusion investigated, two had investigated both the pericardial effusion and the pericardial tissue, and three the pericardial tissue only. In the pericardial fluid samples positive results were obtained for influenza virus type A (Fig. 1) and for human cytomegalovirus in one dog each. In each of these dogs both duplicates examined were positive. Samples of both dogs were collected during the first episode of pericardial effusion.

Definitive confirmation by genomic sequencing was successfully obtained only in the case of influenza virus type A (GenBank accession No: AY920454). The sequenced 229 bp-long segment of the matrix protein (M1) showed 96-100% homology with the first 100 sequences gathered from GenBank; the homology was always with an influenza virus type A (several subtypes). In the case of cytomegalovirus the sequence obtained was not consistent with the target product and did not show any homologies with published sequences; thus the amplicon was not cytomegalovirus. None of the pericardial tissue fragments or pericardial effusions was positive for coxsackievirus B3, parvovirus B19 and human adenovirus type 2. *B. burgdorferi* or *C. pneumoniae* were not detected in any dog with IPE.

Discussion

To the best of our knowledge, so far no study has yet been performed to investigate viruses as possible agents involved in the development of IPE in dogs. This is the first report to show that one of the viruses involved in human infectious pericarditis could also be recognised in a dog affected by IPE. Specifically, we were able to detect and sequence part of the genome of influenza type A in 1/14 dogs with IPE and in none of the dogs with neoplastic effusions or in pericardial washes from dogs without cardiovascular disease.

Influenza viruses type A, as well as types B and C, can cause infections in dogs both naturally and experimentally (Todd and Cohen, 1968; Nikitin et al., 1972; Kilbourne and Kehoe, 1975; Manuguerra and Hannoun, 1992), however current data suggest influenza viruses in dogs to cause only mild disease, whereas cardiovascular disorders have not yet been described or investigated.

During the last decade an increasing number of viruses have been identified in the pericardial fluid and tissue from affected humans, and viral pericarditis is now an established cause of pericardial effusion in this species (Zayas et al., 1995). Although in humans influenza viruses type A are implicated as cause of pericarditis, these viruses are more commonly associated with myocarditis, and when pericardial effusion occurs, it is mainly observed in conjunction with myocarditis (Engblom et al., 1983; Proby et al., 1986; Pankuweit et al., 2000). In our dog where the viral genome was sequenced, isolation was coincident with the diagnosis of IPE and, in contrast to human patients, the pericardial participation was apparently isolated, without concomitant involvement of the myocardium. It must be noted, that without histopathology the possibility of a focal transitory and subclinical myocarditis cannot be definitely excluded. However, the blood biochemical panel did not include markers of myocardial damage such as creatine-kinase or troponins, but echocardiographic evaluation after pericardiocentesis did not identify any systolic dysfunction, diskynesia or focal myocardial lesion, suggestive of a significant myocardial damage.

Although the influenza virus type A genome was not identified in dogs with neoplastic effusions or in pericardial washes of dogs without cardiovascular disease, it is important to emphasize that the identification of the viral RNA in a dog with IPE may not represent the

explanation for the pericardial disorder. Even if influenza viruses are commonly associated with pericarditis in humans, and even if dogs may be infected with these viruses, finding the viral genome does not prove a causative role of these pathogens. It may actually be possible that the dog organism behaved like a passive bystander without replication in cardiac tissue. The virus could have been acquired by the close proximity with the owner; in fact, exposure of dogs to influenza virus as assessed by seroconversion has been demonstrated after human pandemics (Todd and Cohen, 1968; Nikitin et al., 1972). Even though further characterization of the viral subtype (i.e., haemagglutinins, neuraminidase) has not been attempted, according to currently published gene sequences, the human and very rarely the pig and camel are the only species where the influenza virus type A expressing the sequenced matrix protein (M1) is found. Based on the dog's clinical history, contact with pigs and camels is excluded, and the hypothesised viral transmission from human to dog seems most probable.

Finally, it is important to note that the sensitivity of the PCR protocol used in the present investigation may not have been high enough to detect very low concentrations of viral or bacterial gene copies. Also, the sensitivity could have been negatively influenced by the detection of pathogen variants, common in particular for some viruses, such as influenza viruses. Both factors may have underemphasised the strength of our results. It is also possible that the identification of the viral genome could have been merely due to contamination of the pericardial effusion during sample handling. In the authors' opinion, however, this possibility seems less likely as collection of the pericardial fluid was achieved in aseptic conditions and then, through the laboratory procedures, the sample was examined in duplicate.

Conclusions

The finding of human influenza virus in a dog supports the concept that viral pericarditis may be a cause of IPE also in dogs. However, a cause-and-effect relationship is not proven by the identification of one human virus in one dog with the disease, and further studies including canine pathogens and different strains are thus needed to elucidate the role of infectious organisms in canine IPE.

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Table 1. Primer pairs used for sequence detection in this study.

Pathogen	Target gene	Primer	Sequence (5’-3’)	Annealing temperature (°C)	Length of the PCR product (bp)	NCBI accession number
Coxsackievirus B3	5' UTR	Forward Reverse	TCCGGCCCCTGAATG CACCGGATGGCCAATCCA	60	195	M74567
Influenza virus type A	Matrix protein M1	Forward Reverse	CAGATTGCTGACTCCCAGCA GACCAGCACTGGAGCTAGGATGA	67	229	J02145
Human adenovirus type 2	Hexon	Forward Reverse	GCCGCAGTGGTCTTACATGCACATC CAGCACGCCGCGGATGTCAAAGT	68	301	J01917
Human cytomegalovirus	US10/11	Forward Reverse	GTTCTCTCGTCTCCTCCGTG CCTGTGGAGCTCGTTAGAGG	57	362	AY446894
parvovirus B19	ORF2 (VP1)	Forward Reverse	GATACTCAACCCCATGGAGA GCCCTAACACATATGGGTACTT	59	249	AF264149
B. burgdorferi	16S rRNA	Forward Reverse	ACACTGGAACTGAGATACGGT ATTCCACCCTTACACCAGA	61	386	X85195
C. pneumoniae	Major outer membrane protein	Forward Reverse	CTTGCCTGTAGGGAACCCTT CTTTAAGATACGGTCGAAAACATAG	54	160	L04982

355 **Table 2.** Sensitivity of PCR primers.

356

Pathogen	Primer sensitivity (genome copies)	Isolates used for determination of sensitivity	NCBI accession number.
Coxsackievirus B 3	50	coxsackievirus B3 (strain Woodruff)	M74567
Influenza virus type A	50	influenza A (strain PR8)	J02145
Human adenovirus type 2	50	Human adenovirus (strain ADV type 6)	DQ149613
Human cytomegalovirus	100	Human cytomegalovirus (strain AD169)	C17403
Parvovirus B19	50	Human serum (human PVB19 pos)	M50517
B. burgdorferi	100	*B. burgdorferi* (strain B 29)	X85195
C. pneumoniae	100	*C. pneumoniae* (strain TWAR)	L04982

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