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Virological and serological findings in dogs with naturally occurring distemper



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ABSTRACT

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Canine distemper virus (CDV) is the cause of a severe and highly contagious disease in dogs. The unpredictable and variable course of CDV-related disease may hamper correct diagnosis of infection and makes it crucial the collection of samples suitable for laboratory confirmation. In the present study we were able to follow the disease in two dogs infected naturally, collecting different biological matrices during the entire period of infection. By real time RT-PCR, viral RNA was detected and quantified, suggesting that urine and rectal swabs would be useful for ante-mortem diagnosis of distemper in dogs, regardless of the clinical stage and form of the illness.

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1. Introduction

Canine distemper (CD) is a contagious multisystem disease in dogs caused by canine distemper virus (CDV), classified in the Morbillivirus genus of the Paramyxoviridae (Greene and Appel, 1998).

Targets for CDV infection are mainly mucous membranes and lymphoid tissues. Following aerosol infection, the virus primarily replicates in lymphatic tissues of the respiratory tract and subsequently reaches various organs, e.g. the cells of the lower respiratory and gastrointestinal tracts, the lymphoid organs, the urinary bladder and the central nervous system (Appel, 1987). This can result in either subclinical infection or a combination of respiratory, ocular, gastrointestinal, neurologic and cutaneous signs or lesions, that appear simultaneously or sequentially (Greene and Appel, 1998). Nervous signs may be present in the chronic form of CD along with other manifestations, or may occur without any other signs (Appel, 1987).

A broad number of clinical parameters and laboratory assays have been suggested for definitive ante mortem diagnosis of CD. However the unpredictable and variable course of disease such as viremia duration, clinical signs, lack or delayed humoral or cellular immune response, hampers correct diagnosis of CD and makes

it crucial to collect samples suitable for laboratory confirmation. By the way, considering the great infectious potential of the disease, the detection of CDV from different biological specimens is essential for determining subsequent patient management.

Recent developments in molecular techniques revealed the suitability of these methods for diagnosis of CDV infection in dogs (Elia et al., 2006; Kim et al., 2006; Fischer et al., 2013; Li et al., 2013; Wilkes et al., 2014). However, the majority of those studies were carried out in dogs infected experimentally. In other studies, on dogs infected naturally with CDV, sampling was limited to a few biological matrices and was not covering the entire period of infection (Saito et al., 2006; Fischer et al., 2013; Wilkes et al., 2014).

In this note, the clinical findings, the virological and serological results of natural CDV infection in two dogs from an outbreak observed in a kennel are reported.

2. Materials and methods

2.1. Animals and clinical samples

On May 28 2013, two dogs, 359/13 and 360/13, referred to as A and B, respectively, were admitted to the Teaching Veterinary Hospital (isolation Unit) of University of Bari, Italy. The dogs came from a kennel with an ongoing outbreak of CD affecting mixed bred animals aged from 2.5 months to 12 years. At the introduction in the shelter, the dogs were not vaccinated. Both the animals displayed clinical signs suggestive of CD. When CDV infection was confirmed,

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the owners refused the option of euthanasia proposed for dog A. Also, medical care of the animals and inclusion in this observational study was approved by the owners. The study was approved by the Committee for Animal Ethics of the Department of Veterinary Medicine of Bari.

Dog A was a puppy of 3 months of age with respiratory signs including ocular and nasal discharge and coughing. The general health conditions were good and the appetite was conserved. After a few days, the pup developed fever (39.5–40.0 °C), anorexia, vomiting and hemorrhagic diarrhea which resolved within 20 days, while a slight prostration persisted after recovery. One month after hospitalization, fever and progressive neurological symptoms (ataxia, paresis and paralysis) were observed, and after 9 days the animal died.

Dog B was 8 months old and presented with systemic disease, characterized by fever (39.4 °C), lethargy, nasal discharge, conjunctivitis and hyperkeratosis of nose. However, the dog quickly recovered within one week.

The animals were examined daily and the clinical data relative were recorded. Whole blood, urine, rectal, nasal and conjunctival swabs were collected every four days from the animals. The samples were processed for RNA extraction immediately or stored for a maximum of 48 h at –20 °C before use.

Serum samples were collected weekly for evaluation of the antibody response to CDV using virus neutralization and ELISA.

2.2. RNA–DNA extraction

Specimens were homogenized (10%, wt/vol) in Dulbecco's modified Eagle's medium (DMEM) and subsequently clarified by centrifuging at 2500 × g for 10 min. Each sample was prepared for RNA and DNA extraction by using QIAamp cadior pathogen mini kit (Qiagen S.p.A., Milan, Italy), according to the manufacturer's instructions.

2.3. CDV RNA detection and quantification

Real-time RT-PCR assay was performed for detection of CDV RNA. All RNA extracts were subjected to reverse transcription in a reaction volume of 20 µl containing PCR buffer 1× (KCl 50 mM, Tris–HCl 10 mM, pH 8.3), MgCl₂ 5 mM, 1 mM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), RNase Inhibitor 1 U, MuL V reverse transcriptase 2.5 U, random hexamers 2.5 U. Synthesis of c-DNA was carried out at 42 °C for 30 min, followed by a denaturation step at 99 °C for 5 min.

Real-time PCR and thermal protocols were carried out as previously described (Elia et al., 2006). Briefly, the real-time was carried out in a 50 µl-reaction containing 25 µl of IQTM Supermix (Bio-Rad Laboratories Srl, Milan, Italy), 600 nM of primer CDV-F (5'-AGCTAGTTTCATCTTAACATCAAAATT-3') and CDV-R (5'-TTAACTCTCCAGAAAACATCATGC-3') 400 nM of probe CDV-Pb (FAM-ACCCAAGAGCCGGATACATAGTTTCAATGC-TAMRA) and 20 µl of c-DNA.

Duplicates of log 10 dilutions of standard RNA were analyzed simultaneously in order to obtain a standard curve for absolute quantification. All standard dilutions and unknown samples were tested in duplicate. The following thermal protocol was used: activation of iTaq DNA polymerase at 95 °C for 10 min and 45 cycles consisting of denaturation at 95 °C for 15 s, primer annealing at 48 °C for 1 min and extension at 60 °C for 1 min.

2.4. CDV genotyping

Reverse transcription-PCR genotyping of the hemagglutinin (H) gene (Martella et al., 2007) was used to characterize CDV the strains. Briefly, the RNA was reversed transcribed and the cDNA used for the

first PCR amplification. The resulting amplicon was therefore used as template for the second-round PCR, using a panel of primers gives specific for different CDV genotypes.

2.5. Screening for other viral pathogens

The samples were screened molecularly for common canine viral pathogens such as canine parvovirus type 2 (CPV-2) (real-time PCR) (Decaro et al., 2005a,b,c), canine adenovirus type 1 and type 2 (CAV-1 and CAV-2) (PCR) (Hu et al., 2001), canine coronavirus (CCoV) (real-time PCR) (Decaro et al., 2004, 2005b), canine respiratory coronavirus (RT-PCR) (Erles et al., 2003) and reoviruses (Decaro et al., 2005a,b,c).

2.6. Serological assays

Serum samples were tested by virus neutralization and ELISA.

For virus neutralization, twofold serial dilutions starting from 1/2 of each serum were mixed with 100 tissue culture infectious doses (TCID₅₀) of the CDV strain Onderstepoort in 96-well microplates. After pre-incubation at room temperature for 60 min, 2 × 10³ VERO cells were added to each well. The plates were read after 3 days of incubation at 37 °C in a CO₂ incubator. The titer of each serum was expressed as the highest serum dilution neutralizing the virus.

For ELISA, in-house test based on whole-virus antigen was developed, as described previously for CCoV (Pratelli et al., 2002). Supernatants of VERO cell cultures infected with CDV strain Onderstepoort were used for antigen preparation. Briefly, polysorb microtiter plates (Nunc, Milan, Italy) were coated with antigen (250 ng/well). After treatment with blocking solution (0.2% gelatin in carbonate buffer) and repeated washing, 100 µl of each dog serum diluted 1:50 in PBS-Tween 20 was added in duplicate and the plates incubated at 37 °C for 90 min. After a wash cycle, goat anti-dog IgG antibody labeled with peroxidase (Sigma–Aldrich, Milan, Italy) was added and the plates incubated for 60 min at 37 °C. After an additional three washes, ABTS [2,2'-azino-di-(3-ethylbenzothiazoline sulfonate)] substrate solution (Sigma–Aldrich, Milan, Italy) was added to each well and the plate was incubated at room temperature for 25 min. The optical density (OD) was read at 405 nm using an automatic ELISA reader.

Ten control negative sera were used to adjust the Elisa cut-off value (three standard deviations higher than the arithmetic mean of the absorbance of concordantly negative samples). Samples with value exceeding than 0.040 were considered to be positive.

3. Results

By real-time RT-PCR, CDV infection was confirmed in both animals at the day of recovery. In animal A, all the collected samples tested positive for CDV, while in dog B CDV was detected only in urine and rectal swab. Sample from both animals tested negative for other viral pathogens. Based on the RT-PCR genotyping, the CDV strains were characterized as Arctic genotype.

Real-time RT-PCR was used to screen all the collected samples. Dog A was found to shed virus in all the samples with consistently higher viral RNA load in urine, rectal, nasal and conjunctival swabs (Table 1). Moreover, all the samples tested positive for CDV at all the sampling times, with a virus load higher than 1.06 × 10⁴ RNA copies/µL of template 37 days after hospitalization.

Dog B was consistently positive for CDV in urine samples until 50 days after hospitalization (Table 1). Peaks in viral loads were recorded from 10 to 22 days after hospitalization with a mean titer of 1.47 × 10⁴ RNA copies/µl of template. In the remaining observation period, the mean viral load detected in urine was 7.22 × 10²

Table 1
Detection of CDV RNA by real time RT-PCR in dogs with natural infection.

Day of observation	N		C		R		WB		U	
	A	B	A	B	A	B	A	B	A	B
1	5.60×10^5	neg	5.60×10^5	neg	3.72×10^5	3.10×10^2	5.95×10^5	neg	9.29×10^4	3.37×10^2
5	2.01×10^6	neg	1.31×10^5	1.52×10^3	7.99×10^5	7.24×10^2	3.59×10^5	neg	4.87×10^6	2.88×10^3
10	3.86×10^5	1.06×10^3	1.63×10^5	neg	1.62×10^6	5.71×10^4	1.32×10^5	1.17×10^3	6.02×10^5	2.02×10^4
14	1.25×10^6	1.96×10^2	1.46×10^6	neg	9.45×10^5	3.53×10^2	9.57×10^4	neg	8.29×10^4	5.04×10^3
18	3.12×10^6	8.56×10^2	3.22×10^5	8.66×10^2	7.75×10^5	8.06×10^2	1.14×10^5	neg	1.91×10^5	8.70×10^3
22	3.07×10^6	3.49×10^2	2.26×10^6	1.09×10^3	7.94×10^5	8.71×10^2	4.71×10^4	1.06×10^2	6.20×10^5	2.49×10^4
27	9.01×10^5	neg	6.67×10^5	9.77×10^2	3.32×10^5	1.49×10^3	4.71×10^4	4.58×10^2	9.24×10^4	5.26×10^2
32	4.46×10^5	neg	2.21×10^5	neg	5.25×10^5	7.50×10^2	3.20×10^4	neg	7.85×10^4	2.83×10^2
37	4.65×10^4	neg	5.03×10^5	neg	8.04×10^4	neg	1.06×10^4	neg	9.81×10^4	7.36×10^2
44	ND	neg	ND	neg	ND	neg	ND	neg	ND	1.19×10^2

A, dog A; B, dog B; N, nasal swab; C, conjunctival swab; R, rectal swab; WB, whole blood; U, urine; ND, not determined.

RNA copies/ μ l of template. Interestingly, rectal swabs resulted consistently positive from 1 to 36 days of hospitalization with the highest viral load (5.71×10^4 RNA copies/ μ l of template) detected at day 10.

Blood, nasal and conjunctival swabs of dog B tested negative at hospitalization. The virus was detected only occasionally and with low viral titer in the first month, while it was no longer detected after the 32nd day of hospitalization.

3.1. Serology

The animals were screened for the presence of antibodies against CDV. Blood samples were collected at arrival (day 1), and then at day 15 and at day 35 of hospitalization.

Antibodies against CDV were not detected in the sera collected from dog A by virus neutralization. However, in ELISA a slightly immune response against CDV was detected with an OD value of 0.067 at day 1. This reactivity did not increase with time (0.071 and 0.060, at day 15 and day 35, respectively). By converse, in dog B the virus neutralization antibodies against CDV ranged from 1:320 at day 1 to 1:160 at day 15 and day 35. These results were confirmed by ELISA, with an high OD mean value of 0.245.

4. Discussion

CD is a major disease of dog, characterized by high morbidity and mortality rates. In affected dogs, CDV may cause a large variety of clinical signs. Depending on the age, the immune status of the host and the virus strain, the infection can lead to abortive, clinical or subclinical disease. Many dogs do not display the classical clinical picture, thus making CD diagnosis difficult (Amude et al., 2007).

The cases of CD described in this study confirm the clinical variability of CDV infection and the importance of choosing appropriate biological samples for diagnosis. Previous studies on CDV shedding were carried out in dogs infected experimentally. In this study we were able to follow the disease in dogs infected naturally, collecting different biological matrices during the entire period of infection.

Dog A experienced systemic clinical signs of CD followed by a 10-days period of apparent health, during which it was difficult to suspect CD. However, in this phase the animal shed the virus at high titers in all the body fluids, thus posing a serious threat for other susceptible dogs in close contact with the animal. In such epidemiological circumstances, a wrong or delayed diagnosis of CDV infection may have dramatic consequences. As expected, in the serum samples collected from dog A, CDV-specific antibodies were not detected in virus neutralization, and were rather low in ELISA. These results may be accounted for by the fact that ELISA technique can detect antibodies to all the viral proteins, including the major internal N protein that elicits the highest antibody response in morbillivirus infections (von Messling et al., 1999). This would suggest

that it is better to use assays based either on the whole virion or on the N protein when evaluating CDV serological status.

Dog B showed only few mild symptoms of infection and recovered completely within a week. This is the typical pattern of CDV infection with dogs mounting an immune response able to clear the virus from the epithelial cells. Dog B displayed a good humoral reactivity against CDV, as observed by ELISA and virus neutralization, confirming this hypothesis. At hospitalization, dog B tested positive for CDV only in the urine and rectal swab. In these samples, CDV RNA was detected continuously during the entire observation period.

Whole blood, conjunctival swab and urine are usually recommended for CDV laboratory diagnosis (Saito et al., 2006; Kim et al., 2006; Shin et al., 2004; An et al., 2008). However, it is important to consider also the stage of the infection and the clinical form of the disease. In this study blood and urine were found to be the samples more suitable for CDV diagnosis. In dog A, CDV was detected by real-time PCR in blood and urine much more consistently than in the other samples (nasal and conjunctival swabs). However only the urine tested positive in both dogs. Regardless of the clinical stage and form of the illness, urine seems to be the most appropriate sample for diagnosis. Interestingly, as previously reported (Fischer et al., 2013), both the animals were also positive in the rectal swabs, although the duration of shedding of CDV was shorter in the feces than in the urines. Fecal samples are of easier collection in non-clinical settings and can also be used for parallel screening of other gastrointestinal pathogens.

Several recent reports suggest both the re-emergence and increased activity of CDV worldwide (Griot et al., 2003). Respiratory and enteric signs in CDV infected dogs may be confused with the signs caused by other respiratory and enteric pathogens, hindering early CDV diagnosis (Appel, 1970; Griot et al., 2003). Therefore, rapid and accurate diagnosis of CDV infection would enable veterinarians to implement appropriate and timely strategies necessary to improve disease management and to prevent virus diffusion, particularly within a shelter environment. The local and global epidemiology of CDV reinforces the importance of vaccination in dogs and the need for implementing an accurate and reliable diagnostic.

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