Canine adenovirus type 1 and Pasteurella pneumotropica *co-infection in a puppy*

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Keywords
Canine adenovirus type 1, Cholangiohepatitis, Dog, Encephalitis, Indirect fluorescent antibody, *Pasteurella pneumotropica*.

Summary
In 2008, a 2-month-old male German shepherd was presented with fever, depression, and evident organic wasting. The puppy died within 48 hours after the onset of clinical signs. A complete necropsy was performed. Bacteriological examination of samples from the brain, lung, liver, spleen, and bone marrow tested positive for *Pasteurella pneumotropica*. Histopathology demonstrated inflammatory and vascular lesions in the central nervous system and internal organs. Canine adenovirus type 1 (CAdV-1) nucleic acid was detected by polymerase chain reaction (PCR) in the frozen brain but not in the formalin-fixed, paraffin-embedded liver and lung samples. The positive PCR was subsequently confirmed by indirect fluorescent antibody testing of the paraffin-embedded brain and liver sections. Although the liver is the primary site of viral damage, these laboratory findings suggest that CAdV-1 infection should be included in the differential diagnosis of neuropathological diseases in dogs and that adenoviral infections could promote septicaemia caused by opportunistic pathogens.

Co-infezione di adenovirus canino tipo 1 e Pasteurella pneumotropica *in un cucciolo di pastore tedesco*

Parole chiave
Adenovirus canino tipo 1, Cane, Colangioepatite, Encefalite, Immunofluorescenza indiretta, *Pasteurella pneumotropica*.

Riassunto
Nel 2008, un pastore tedesco maschio di 2 mesi presentava alla visita clinica febbre, evidente depressione e deperimento organico. Il cucciolo è deceduto 48 ore dopo la comparsa dei primi segni clinici. È stata eseguita una necroscopia completa e successivamente si è proceduto ad esami batteriologici sui campioni di cervello, polmone, fegato, milza e midollo osseo che sono risultati positivi per *Pasteurella pneumotropica*. Istologicamente sono state riscontrate lesioni infiammatorie e vascolari rispettivamente nel sistema nervoso centrale (SNC) e negli organi interni. L'acido nucleico dell'adenovirus canino tipo 1 è stato rilevato mediante reazione a catena della polimerasi (PCR) nel cervello congelato ma non nei tessuti di fegato e polmone fissati in formalina e inclusi in paraffina. Il risultato positivo della PCR è stato successivamente confermato dal test di immunofluorescenza indiretta eseguito su sezioni di cervello e di fegato inclusi in paraffina. Anche se il fegato è il sito di elezione del virus, i risultati di laboratorio suggeriscono che l'infezione da adenovirus canino tipo 1 dovrebbe essere considerata nella diagnosi differenziale delle malattie neuropatologiche nel cane e che le infezioni da adenovirus potrebbero promuovere setticemia causata da agenti patogeni opportunistici quali *Pasteurella pneumotropica*.

Canine adenovirus type 1 (CAdV-1), a double-stranded DNA virus belonging to the genus *Mastadenovirus* of the family *Adenoviridae*, is responsible for infectious canine hepatitis (ICH) or Rubarth's disease, a systemic contagious disease of domestic and wild *Canidae* and other carnivores, including otters and bears (Park *et al.* 2007, Stephenson *et al.* 1982, Whetstone *et al.* 1988). First described in farmed foxes in 1930 (Green *et al.* 1930), CAdV-1 was recognised as a canine pathogen only some years later (Rubarth 1947). At the genetic and antigenic levels, the virus is closely related to canine adenovirus type 2 (CAdV-2), the agent of canine infectious tracheobronchitis. Canine adenovirus type 1 transmission occurs through direct contact with infected animals or through indirect exposure to contaminated saliva, urine, and faeces. After ingestion of contaminated material, CAdV-1 replicates in the tonsillar crypts, before spreading through a viraemic phase to all tissues including the central nervous system (CNS). Replication takes place mainly in the endothelial cells and hepatocytes. In animals less than 1 year old, clinical signs are more severe and the mortality rate is high (10-30%), whereas subclinical course may be observed in adults (Decaro *et al.* 2008). The main clinical findings associated with ICH are depression, anorexia, hyperventilation, vomiting, bloody diarrhoea, abdominal pain, epistaxis, lymphadenopathy, mucosal congestion, and jaundice (Gur and Acar 2009). Spontaneous recovery is common in dogs, although immune mediated complications due to the deposition of immune complexes may arise, *i.e.* glomerulonephritis, anterior uveitis, and corneal oedema leading to the characteristic clinical sign known as "blue eye" (Gur and Acar 2009, Pratelli *et al.* 2001). Encephalitis associated with CNS vascular lesions is uncommon in dogs (Caudell *et al.* 2005, Mandara *et al.* 2011). Common neurological signs associated with CAdV-1 are lethargy, ataxia, blindness, vomiting, seizures, and death. Recovered dogs may shed the virus for a long time.

Pasteurella pneumotropica, a Gram-negative coccobacillus with zoonotic potential, is a member of the family *Pasteurellaceae*. It was first isolated from laboratory mice in 1950 by Jawetz. It was so named because of the characteristic lung lesions it produces (Jawetz 1050). Rats and mice are the primary carriers, although the microorganism has been isolated also from hamsters, guinea pigs, rabbits, cats, and dogs. This opportunistic pathogen has been infrequently associated with clinical signs, but stressing factors may contribute to induce overt disease. *Pasteurella pneumotropica* is transmitted by direct contact with infected animals or their secretions and it does not survive for a long period in the environment. Transmission by means of contaminated material is not common (Scharmann Heller 2001). After entry through the oronasal route, the bacterium colonizes the respiratory and/or digestive system where it remains latent until concurrent immunosuppression predisposes to the onset of disease. The pathogen can be generally recovered from the conjunctiva and the respiratory and urogenital tracts of infected hosts.

To the best of our knowledge, no cases of co-infection with CAdV-1 and *P. pneumotropica* involving the CNS have been described so far. This report describes the pathological features of this unusual case.

In 2008, a 2-month-old male German shepherd, housed in a kennel, was presented with fever, depression and evident organic wasting. The puppy died within 48 hours after the onset of clinical signs and a complete necropsy was performed. Brain tissue was partly frozen for bacteriological and molecular investigations and partly fixed in 10% buffered formalin solution. Tissue samples from other organs were also subjected to bacteriological examination and fixed in formalin. Fixed tissue samples were routinely processed for histopathology; 4 ± 2 µ sections were cut and stained with haematoxylin and eosin (HE) for light microscopy evaluation. Indirect fluorescent antibody (IFA) testing was also performed on selected sections.

Primary bacterial cultures were obtained from the brain, lung, liver, spleen, and bone marrow. Briefly, after surface cauterization, a small piece from each organ was plated onto Columbia agar containing 5% sheep blood and onto MacConkey agar and incubated at 37°C in air. Analogously, all samples were plated onto 5% sheep blood agar plates, one incubated anaerobically and the other at 37°C in 5% CO_2-supplemented air. After incubation for 24 hours in aerobic atmosphere and for 48 hours in anaerobiosis and in 5% CO_2 atmospheres, suspected colonies were picked and streaked onto Columbia agar containing 5% sheep blood to obtain pure cultures for further biochemical identification. The oxidase-positive and Gram-negative colonies were tested using API 20NE which permitted their definitive identification after incubation for 48 hours at 30°C.

For the molecular analyses, samples from the frozen brain and the formalin-fixed, paraffin-embedded tissues (liver, spleen, kidney) were submitted to nucleic acid extraction using the DNeasy Tissue Kit (Qiagen, Milan, Italy). Tissue samples were also tested for molecular detection of the main viral pathogens of dogs, such as canine herpesvirus type 1 (Decaro *et al.* 2010), canine coronaviruses (Decaro *et al.* 2004, Decaro *et al.* 2005c), reoviruses (Decaro *et al.* 2005d, Leary *et al.* 2002), rotaviruses (Gouvea *et al.* 1994), canine parvovirus type 1 and type 2 (Decaro *et al.* 2005 a, b), CAdV-1 and 2 (Hu *et al.* 2001), and canine distemper virus (Elia *et al.* 2006). The PCR products

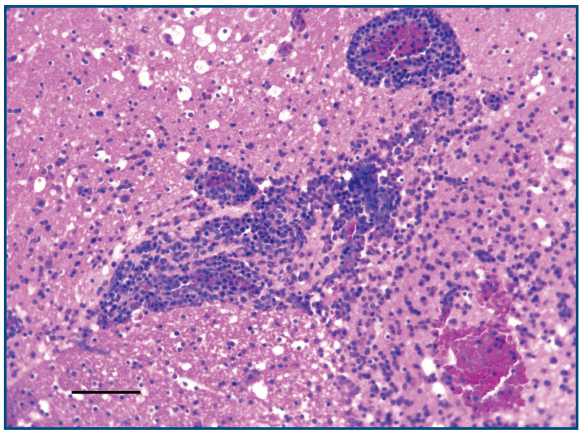

Figure 1. *Two-month-old male German shepherd's brain showing the presence of oedema, vasculitis and perivascular cuffs of mononuclear cells around the lateral ventricles, with infiltration of inflammatory cells in the surrounding tissue.* Hematoxylin and eosin. Bar = 50 µm.

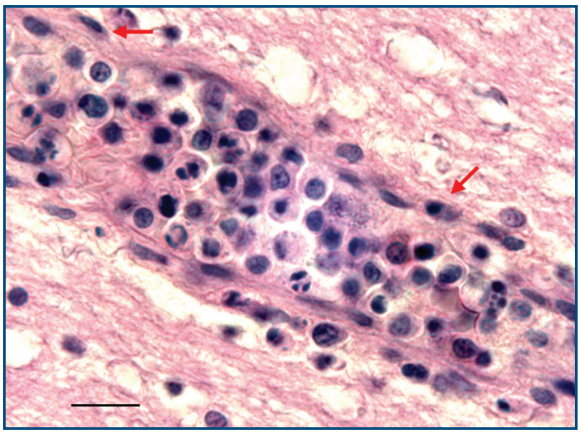

Figure 2. *Two-month-old male German shepherd's brain vessel.* Basophilic intranuclear inclusion bodies in the vascular endothelium (arrows). Hematoxylin and eosin. Bar = 10 µm.

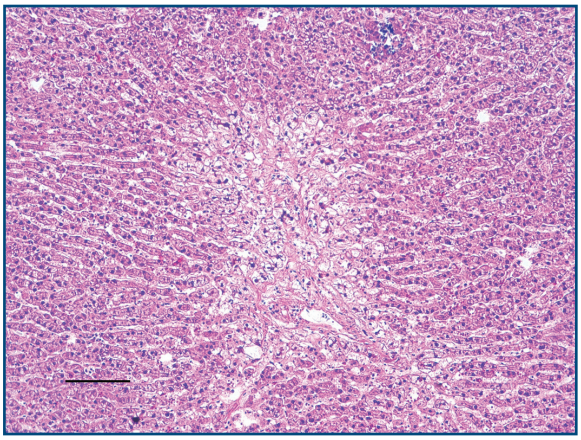

Figure 3. *Two-month-old male German shepherd's liver.* Fatty changes of hepatocytes with focal mononuclear infiltrate. Hematoxylin and eosin. Bar = 100 µm.

were detected by means of gel-electrophoresis and ethidium-bromide staining. Three-micrometer sections obtained from brain, liver, and lung were selected and compared against sections of the same organs from a healthy dog as control. The tissues were mounted on adhesive slides and dried for 24 hours at 37°C to maximize tissue adherence. The sections were then de-paraffinized and rehydrated in ethanol and distilled water for 6 minutes.

For IFA testing, the slides were incubated with a pH 7.8 solution containing 0.1% trypsin and 0.1% calcium chloride for 30 minutes at 37°C. Non-specific background staining was reduced by applying normal goat serum for 20 minutes at room temperature in a humidity chamber. The sections were incubated overnight at 4°C with a 1:100 dilution of the primary antibody, a CAdV-1 polyclonal rabbit hyperimmune serum. After rinsing in tris buffered saline (TBS), the slides were treated with a 1:200 dilution of fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin for 30 minutes. The slides were washed with TBS and mounted with cover slips using Vectashield® Mounting Medium fluid (Vector Laboratories). The edges of the slides were sealed with nail varnish and the samples were visualised with a fluorescent microscope and analysed using NIS-Elements software.

The carcass showed poor nutritional conditions and jaundice. The spleen and the liver were massively enlarged and displayed diffuse petechiae. Haemorrhages were also detected in the lungs, thymus, gastric mucosa, and bladder. The urine was red to brown-tinged. Neuropathological examination showed no macroscopic lesions in the brain. Histology demonstrated features of diffuse meningitis, vasculitis, and perivascular cuffs characterised mainly by macrophages, lymphocytes, and plasma cells, and few neutrophils. Multifocal lesions of congested blood vessels, swollen endothelium and vasculitis were surrounded by oedematous areas and scattered necrotic neurons. Visible around the lateral ventricles were a mononuclear inflammatory infiltrate and haemorrhages (Figure 1). Basophilic intranuclear inclusion bodies were detected in the vascular endothelium of the brain (Figure 2). Alveolar oedema and mild fibrinous pleuritis were observed in the lungs; the liver displayed fatty degeneration of hepatocytes with mild mononuclear cholangiohepatitis (Figure 3). Other histopathological changes included haemorrhagic gastritis, catarrhal enteritis, interstitial nephritis, and haemorrhagic cystitis. Diffuse granulomatous lymphadenitis and depletion of the white pulp in the spleen were also found.

Pure growth of light grey to yellow-coloured colonies about 1.5 mm in size was detected on blood agar from all cultured organs and all picked colonies were identified as *P. pneumotropica*.

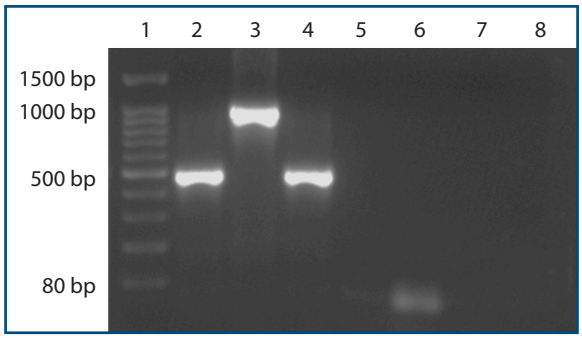

Figure 4. *PCR assay for detection of canine adenovirus type 1 (CAdV-1) and canine adenovirus type 2 (CAdV-2).* Lane 1 = marker GeneDirex 100 bp DNA Ladder RTUg; lane 2 = positive control for CAdV-1 (508 bp); lane 3 = positive control for CAdV-2 (1030 bp); lanes 4 to 7 = samples from the CAdV-1 infected dog (brain, liver, spleen, kidney); lane 8 = negative control (rectal swab from a dog negative for CAdV-1/CAdV-2).

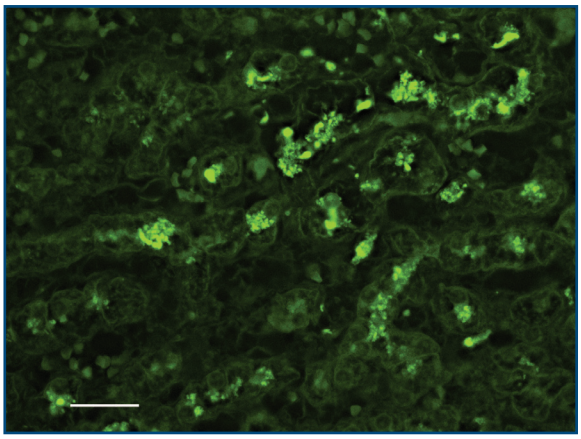

Figure 5. *Two-month-old male German shepherd's liver.* Cytoplasmatic and nuclear virus antigen in Kupffer cells and hepatocytes. Indirect immunofluorescence. Bar = 25 µm.

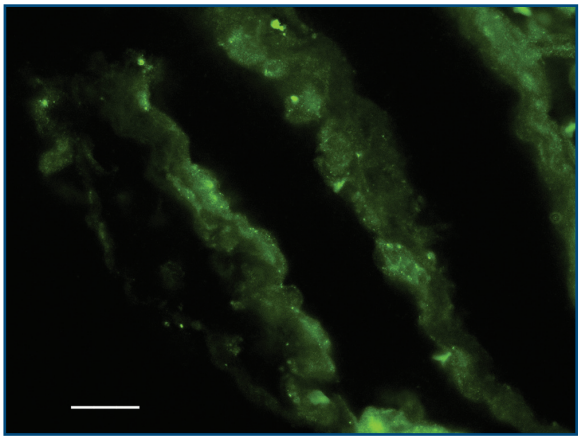

Figure 6. *Two-month-old male German shepherd's brain.* Viral antigen in the endothelial cells of the meningeal vessels. Indirect immunofluorescence. Bar = 17µm.

Canine adenovirus type 1 nucleic acid was detected by polymerase chain reaction (PCR) in the frozen brain (Figure 4) but not in any of the formalin-fixed tissues. Molecular analyses for all other searched pathogens were negative; IFA testing detected CAdV antigens within the endothelium of the nervous system, hepatocytes and Kupffer cells (Figures 5 and 6), whereas the lung samples tested negative.

This report describes a case of co-infection with CAdV-1 and *P. pneumotropica* in a dog. Generally, the liver is the organ most severely affected by CAdV-1 infection due to the virus affinity for hepatocytes, Kupffer cells, bile duct epithelial, and endothelial cells. In the present case, the only finding that matched previous observations was the mild to moderate liver damage (Caudell 2005).

Signs of encephalitis caused by damage to the vascular endothelium are rare in dogs but, when present, they may include rapid and progressive tetra paresis, convulsions, coma, and death. Morphologically, multiple haemorrhages can be found at the level of the brainstem, thalamus, and caudate nuclei, which are rarely visible under macroscopic examination. The cerebral and cerebellar cortex are not usually involved (Elia *et al.* 2006). The neuropathological changes observed in the present case were similar to those previously described (Andreani *et al.* 1998, Caudell 2005, Chouinard *et al.* 1998, Summers *et al.* 1995), except for the presence of neutrophils in the perivascular cuffs, which could be explained by the simultaneous infection with *P. pneumotropica*. In contrast to previous reports (Caudell 2005), intra-nuclear inclusion bodies were present only within the brain vascular endothelial cells, probably because the extensive haemorrhage in the liver prevented their detection in the hepatic tissue.

So far, few cases of neurological disorders caused by CAdV-1 infection have been described in dogs (Caudell 2005), whereas CAdV-1 encephalitis is more common in foxes (Green *et al.* 1930, Green and Shillinger 1933). Considering that CNS manifestations are rare during ICH, it has been suggested that a certain variation in CAdV-1 strains exists with respect to their CNS endothelial tropism (Caudell 2005). However, this was not ascertained in the present case, as no sequence analysis was conducted on the detected CAdV-1 strain.

In this study, histopathological examination suggested a CAdV-1 diagnosis, which was subsequently confirmed by IFA and PCR testing on the brain and internal organs. Although CAdV-1 DNA was not detected in the liver as well as in the other formalin-fixed tissues, this could be expected as a consequence of the DNA degradation due to the formalin fixation. Vaccination is the only effective way to control CAdV-1 infection. Currently, the disease is only sporadically observed in developed countries thanks to extensive vaccination programs,

but its re-emergence may be expected as puppies continue to be imported from endemic areas (Decaro *et al.* 2007). The puppy described in the present report had not received vaccination against adenovirus and there are no data about its dam.

Although uncomplicated, CAdV-1 infection usually lasts 5-7 days and may be followed by rapid recovery; still there may be serious consequences in dogs with concurrent infections, such as canine distemper virus (Kobayashi *et al.* 1993), canine coronavirus (Decaro *et al.* 2007, Pratelli *et al.* 2001), canine parvovirus (CPV) (Decaro *et al.* 2007), and parasites (Sanchez-Cordon *et al.* 2002). To date, no cases of co-infection with CAdV-1 and *P. pneumotropica* have been reported. The role of *P. pneumotropica* as a primary pathogen is uncertain and infection in immunocompetent animals is usually asymptomatic. However, in immune suppressed animals this microorganism can act as an opportunistic pathogen (Rogers *et al.* 1973). Occasional infections in humans have been reported, some of which may have occurred as a result of bites by infected dogs or cats (Minton 1990, Rogers *et al.* 1973, Serre *et al.* 2004).

In the present case, histopathological and bacteriological examinations suggested that the bacterium had spread systemically subsequent to CAdV-1 infection, which may have predisposed the animal to brain colonization by *P. pneumotropica*.

This case report confirms that CAdV-1 is still circulating in the canine population in Italy and that its clinical course is more severe when other (concurrent) pathogens are endemic, management is poor, and systematic vaccination is not carried out. The increasing re-emergence of CAdV-1 in Italy underscores the need to continue to vaccinate dogs, also in areas where the disease has been satisfactorily controlled (Decaro *et al.* 2007, Gur and Acar 2009). Although the liver is the primary site of viral damage, this case report suggests that CAdV-1 infection should be included in the differential diagnosis of neuropathological diseases in the dog, given that it could predispose to superinfection by zoonotic agents in immunocompromised animals.

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