# Fatal canine adenovirus type 1 acute infection in a Yorkshire Terrier puppy in Portugal: a case report

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**ABSTRACT**: This study reports the diagnostic algorithm followed for the identification of a fatal canine adenovirus type 1 (CAdV-1) infection in an unvaccinated 56 day-old puppy to overcome the limitations imposed by inconclusive histopathology hampered by body freezing. The animal was submitted to necropsy after a clinical history of lethargy, dehydration, vomiting and haemorrhagic diarrhoea. Pathological features, suggestive of infectious illness, included generalised gelatinous subcutaneous oedema, petechial, ecchymotic haemorrhages of the subcutaneous tissues and a friable uniformly yellow mottled liver. Differential diagnosis based on PCR ruled out the presence of most common gastrointestinal canine viruses and bacteriology and coprology confirmed that pathogenic bacteria and intestinal parasites did not account for the puppy's death. Strong amplification of CAdV-1 DNA was obtained from liver samples. Isolation of CAdV-1 in MDCK cells was subsequently demonstrated and sequencing analysis showed high similarity with CAdV-1 isolates from Europe. In the absence of serum, antibodies against CAdV-1 were investigated in lung tissue extracts. The presence of CAdV-1 infectious particles and absence of immune response was consistent with rapid progression of the infection and death of the animal two days after the onset of clinical signals, allowing a final diagnosis of the acute form of ICH. Antibodies against CAdV-1 were detected in sera collected from clinically healthy dogs from the same premises, 14-months after the index case, suggesting that the virus had circulated in the breeding kennel. We believe this to be the first report of CAdV-1 in Portugal where canine infectious hepatitis is considered a rare infection.

**Keywords**: canine adenovirus type 1; CAdV-1; infectious canine hepatitis; *Mastadenovirus*

**List of abbreviations**

BID = twice a day, CAdV-1 = canine adenovirus type 1; CAdV-2 = canine adenovírus type 2, CCoV = canine coronavirus; CDV = canine distemper virus, CHV = canine herpesvirus, CPIV = canine parainfluenza virus, CPV = canine parvovirus, CPE = cytopathic effect, ELISA = enzyme linked immunosorbent assay, FCS = foetal calf serum, FITC = fluoresceine isothiocyanate, ICH = infectious canine hepatitis, IgG = immunoglobulin G, IIF = indirect immunofluorescence, LTE = lung tissue extract, MDCK = Madin-Darby canine kidney, PBS = phosphate-buffered saline, PCR = polymerase chain reaction, RV = rotavirus, SID = once a day

Infectious canine hepatitis (ICH) is a highly contagious, often fatal, infection caused by CAdV-1, a double stranded DNA, non-enveloped icosahedral virus that belongs to the genus *Mastadenovirus* of the family *Adenoviridae* (Quinn et al. 2005).

ICH was first identified in North America in silver foxes in 1925 (Green 1925), but the disease was only described in domestic dogs in 1947 (Rubarth 1947).

Beside red and grey foxes, and other *Canidae,* such as coyotes, jackals and wolves, CAdV-1 can also infect members of the families *Ursidae* (black) (Pursell et al. 1983) and polar bears (Chaddock et al. 1950)), *Mustelidae* (skunks and otters) and *Procyonidae* (raccoons) (Spencer et al. 1999).

The course of the disease can evolve rapidly, varying from hyper-acute, where dogs may die before

or a few hours after the onset of clinical signs (fulminating fatal hepatitis), to acute, where death can occur one to two days after the observation of the first clinical signs, both usually occurring in puppies (Appel 1987; Greene 2006). Experimental studies demonstrated that partial immunity to the infectious agent (serum neutralisation titres between 1/16 and 1/500) favoured the establishment of prolonged forms of hepatitis following infection (sub-acute or chronic infections), while high levels of immunity confer full protection and no apparent illness follows inoculation (Gocke et al. 1967).

Common clinical signs of ICH include fever, loss of appetite, abdominal pain, lethargy, disorientation, hyperventilation, seizures, increased thirst, vomiting, and/or diarrhoea, which is sometimes haemorrhagic (Decaro et al. 2008; Piacesi et al. 2010). The classical "blue eye", due to immune complex formation, corneal endothelial damage and corneal oedema, may occur (Curtis and Barnett 1983). Animals that survive the infection will carry and shed the virus in body fluids, such as nasal discharge and urine, for at least six to nine months and 11 months, respectively (Piacesi et al. 2010).

Although there is no effective medication to treat ICH, vaccination with CAdV-2, which is antigenically similar to CAdV-1, protects and prevents the spread of CAdV-1 in the population (Emery et al. 1978). Modified live vaccines induce specific antibodies that persist for as long as 14 years conferring full protection against ICH infection. Following the introduction of widespread vaccination, only sporadic cases were reported, usually occurring in unvaccinated dogs (Carmichael 1999). Serological surveys conducted in wild carnivores revealed the previous

contact of these populations with CAdV (Table 1). The seroprevalence of CAdV-1 in foxes and wolves was highly variable, ranging from 4.7% to 100%. For wild carnivores from Portugal, data is limited to red foxes (n=4), with none testing positive for CAdV-1 (Duarte et al. 2013). Wild species are likely reservoirs of CAdV-1, from which sporadic spill over to susceptible unvaccinated domestic dogs may occur. Although the incidence of acute CAdV-1 infection in domestic dogs is currently low, the virus persists in dog populations worldwide, as evidenced by reports of the disease from North America (Caudell et al. 2005; Wong et al. 2012), South America (Oliveira et al. 2011; Headley et al. 2013), Asia (Kobayashi et al. 1993; Hai et al. 2009), Middle East (Cheema et al. 2012), and Europe (Pratelli et al. 2001; Decaro et al. 2007a; Gleich et al. 2009; Muller et al. 2010).

The disease is often not perceived by clinicians, particularly in urban areas where past vaccinations lead to CAdV-1 control. Although viral detection by classical or molecular methods demonstrates the presence of CAdV-1 in tissues, the observation of nuclear inclusion bodies in the liver through histopathological examination is the gold standard technique to confirm the involvement of virus in the disease. The present case constitutes an example of the difficulties faced by laboratories with regard to poor sample conditions since freezing of the animal in this report hampered histopathological analysis and posed a major challenge to laboratory diagnosis. Inconclusive histopathology findings necessitated the establishment of a comprehensive diagnosis strategy which took advantage of clinical, pathological, molecular, virological, serological and epidemiological data.

Table 1. Serological surveys that revealed the contact of wild carnivore populations with CAdV

| Common names | Scientific names | Geographic area | Serological investigation |
|---|---|---|---|
| Red fox | *Vulpes vupes* | North America | Amundson and Yuill 1981 |
| | | England and Scotland | Thompson et al. 2010 |
| | | Germany | Truyen et al. 1998 |
| | | Australia | Robinson et al. 2005 |
| | | Italy | Balboni et al. 2013 |
| | | Scandinavian mainland | Akerstedt et al. 2010 |
| Grey foxes | *Urocyon cinereoargenteus* | North America | Riley et al. 2004 |
| Wolves | *Canis lupus* | Scandinavian mainland | Akerstedt et al. 2010 |
| Island foxes | *Urocyon littoralis* | Channel Islands, California | Clifford et al. 2006 |
| Arctic foxes | *Vulpes lagopus* | Svalbard | Akerstedt et al. 2010 |
| Iberian lynx | *Lynx pardinus* | Spain | Millan et al. 2009 |

This is also the first report of canine adenovirus type 1 in domestic dogs in Portugal.

## Case description

The index case consisted of a 56-day old, Yorkshire Terrier female, from Vale de Cambra, District of Aveiro, in the Northwest of Portugal.

A retrospective inquiry showed that the puppy originated from a local multiple breeder that owned 41 adults, comprising a total of 15 breeds of dogs, mostly females (85.3%); Doberman (*n* = 1), Chihuahua (*n* = 4, two males), German Shepherd (*n* = 5), Pekingese (*n* = 4, one male), Pinscher (*n* = 3, one male), Pug Carlin (*n* = 1), Golden Retriever (*n* = 2 ) and Yorkshire Terrier (*n* = 3), Great Dane (*n* = 4), Pug (*n* = 4), Basset Hound (*n* = 2), Spitz (*n* = 2), Shih-Tzu (*n* = 2), Poodle (*n* = 2), Neapolitan Mastiff (*n* = 2, one male). Males from outside the family were also used for mating to avoid inbreeding. The mother of the index case is referred to as Yorkshire Terrier female 1. Vaccination of the adults was not practiced for at least six years.

The puppy, sold at the end of April 2012, had been vaccinated once against canine parvovirus (CPV) and canine distemper virus (CDV) (Nobivac Puppy DP Vaccine, MSD Animal Health) at six weeks of age, e.g., one week before being sold. Two days after purchase, the puppy developed anorexia, lethargy, abdominal pain, vomiting, and diarrhoea, which was observed by a local veterinarian one day after the disease onset. Pale mucosa, corneal oedema and hypothermia (33 °C) were also observed. Systemic antibiotic therapy included metronidazole (Metronidazol *i.v.* B. Braun, Portugal) 10 mg/kg BID *i.v.*, marbofloxacin (Marbocyl FD 1%, Vetoquinol, France) 2 mg/kg SID *s.c.*, associated with a combination of amoxicillin and clavulanate (Synulox, Pfizer) 15 mg/kg SID *s.c.* An antiemetic (metoclopramide, Primperan, Sanofi Aventis) 0.5 mg/kg BID *i.v.*, a histamine H2 receptor antagonist (ranitidine syrup, Zantac) 1 mg/kg BID *i.v.* and fluid therapy (Ringer Lactate, B Braun Medical Inc) were also administrated. The puppy's condition continued to deteriorate with haemorrhagic diarrhoea and drop in body temperature, and the animal fell into coma, dying during that day, i.e., two days after the onset of clinical signs. The animal's body was frozen and sent to the laboratory for diagnosis confirmation. No symptoms were observed in any of the adult dogs from the breeder.



Figure 1. Gelatinous subcutaneous oedema of the head and neck, with petechial and ecchymotic haemorrhages (index case necropsy)

External examination of the carcass revealed mild emaciation, bilateral corneal oedema and pale mucous membranes. Internal examination showed generalised gelatinous subcutaneous oedema with spherical petechial and ecchymotic haemorrhages in subcutaneous tissues of the ventral abdomen (Figure 1) and a friable yellowish uniformly mottled liver with disseminated foci of haemorrhage. Petechial hemorrhagic foci in the visceral pleura, and white froth exuded from the cut surface of the lung were observed. Examination of the heart revealed a pale myocardium with haemorrhagic suffusion at the interventricular sulcus. Small haemorrhagic areas were observed at the pancreas. The renal parenchyma was pale. In the gastrointestinal tract, there were areas of congestion and haemorrhage in the intestinal mucosa and digested blood in the pylorus, terminal portion of the ileum, ileocaecal valve and caecum. Greenish faeces with traces of blood were detected. No macroscopic abnormalities were found in the bladder, gallbladder, spleen and brain. Histopathology was attempted on frozen liver and kidney tissues fixed in 10% buffered formalin and embedded in paraffin. Four-micrometer sections were stained with haematoxylin and eosin and examined using light microscopy but this analysis failed to provide reliable information to evaluate the pathogenic effect of CAdV-1 in these organs. Thus, the histopathological limitations posed a major challenge to laboratorial diagnosis, and this was overcome through the establishment of a comprehensive diagnosis strategy.

Samples of liver, spleen, lung, and kidney were taken during necropsy for bacteriological exami-

Table 2. Virological examinations: type of method, genomic regions targeted, size of the amplicon expected, tissues used for nucleic acid extraction, references of the methods and results obtained

| Virus[a] | Results | Type of PCR | Genomic region targeted | Amplicon size (bp) | Matrixes | Kits used for nucleic acid detection | Method reference |
|---|---|---|---|---|---|---|---|
| CPV | positive | real-time | *vp2* gene | 93 | small intestine, lymph node | FastStart TaqMan Probe Master Kit[a] | Decaro et al. 2005 |
| CCoV | negative | real-time | *7b* gene | 102 | small intestine, lymph node | OneStep RT-PCR[b] | Gut et al. 1999 |
| RV(group A) | negative | conventional | gene segment 6 | | small intestine | OneStep RT-PCR[b] | Elschner et al. 2002 |
| CDV | negative | real-time | *N* gene | 161 | lung | OneStep RT-PCR[b] | in house (unpublished) |
| CHV | negative | conventional | *gB* gene | 94 | lung | high fidelity PCR Master Mix[c] | Ma et al. 2008 |
| CAdV-1 | positive | conventional | E3 region/U-exon | 508 | liver | high fidelity PCR Master Mix[c] | Hu et al. 2001 |
| CAdV-1 | negative | conventional | E3 region/U-exon | 508 | kidney | high fidelity PCR Master Mix[c] | Hu et al. 2001 |
| CAdV-2 | negative | conventional | E3 region/U-exon | 1030 | lung | high fidelity PCR Master Mix[c] | Hu et al. 2001 |

CPV = canine parvovirus, CCoV = canine coronavirus, RV = rotavirus (group A), CDV = canine distemper virus, CHV = canine herpesvirus, CAdV-1 = canine adenovirus type 1, CAdV-2 = canine adenovirus type 2
[a]Roche, Roche Diagnostics GmbH, Mannheim, Germany, [b]Qiagen, Hilden, Germany, [c]Roche Diagnostics GmbH, Mannheim, Germany. Trivirovax (DHP) freeze-dried CPV, CDV, CAdV-2 vaccine (Tetradogâ, Merial), and preparations of RV RNA, CCoV RNA and CHV DNA from positive samples, were used as positive controls

nation, using standard and selective culture media according to the OIE (2008). Pathogenic bacteria, including *Salmonella* spp., were not detected in the tissue samples suggesting that bacterial infection was not the primary cause of death, although freezing might have hampered the isolation of some bacteria. Neither gastrointestinal helminthes, including *Toxocara canis*, *Toxascaris leonine*, *Ancylostoma caninum* and *Dypilidium caninum* were observed by macroscopic examination during necropsy nor were Coccidia oocysts detected in the direct examination of intestine and faeces of the female puppy.

Differential diagnosis was realised by investigating the presence of canine adenovirus type-1 (CAdV-1), canine herpesvirus (CHV), canine parvovirus (CPV), canine coronavirus (CCoV), rotavirus (RV) and canine adenovirus type-2 (CAdV-2) using the methods referenced in Table 2. Canine distemper virus (CDV) was investigated by means of a real time PCR designed in our laboratory and in use since 2003, which targets a 161 bp region within the N gene (Fevereiro et al. 2006). Different organs were homogenised with phosphate-buffered saline (PBS) for nucleic acid extraction using a BioSprint 96 nucleic acid extractor (Qiagen, Hilden, Germany), according to the manufacturer's protocol.

The amplification of a CAdV-1-specific fragment from a liver sample confirmed the suspicion of infectious hepatitis raised during necropsy.

After electrophoresis, the PCR fragment was excised and purified using a commercial kit (QIAquick gel extraction kit; Qiagen, Hilden, Germany). The resulting product was directly sequenced using a 3130 Genetic Analyser (Applied Biosystems, Foster City, CA, U.S.A.) and submitted to GenBank (KC577558).

Parvovirus DNA was detected by real-time PCR in the lymph nodes and small intestine of the female Yorkshire Terrier. High Ct values (mean 35.8) were obtained indicating a low amount of parvovirus DNA in the dog's tissues. The complete parvovirus *vp2* gene was amplified as previously described by Duarte et al. (2009). Polypeptide residues were deduced in order to determine the CPV subtype. Isolation of CPV in Madin-Darby canine kidney (MDCK) cells was not accomplished. The deduced amino acid residues showed the presence of $Met_{87}$, $Ile_{101}$, $Ser_{297}$, $Ala_{300}$, $Asp_{305}$, $Asn_{323}$, $Asn_{426}$, $Val_{555}$, $Ser_{564}$, and $Gly_{568}$, which are characteristic of the "old" CPV-2 parvovirus that does not circulate presently. To confirm that the CPV-2 strain detected in the animal tissues originated from the recent vac-

cination with CPV-2 Nobivac Puppy DP Vaccine (MSD Animal Health), the *vp2* sequences obtained directly from the vaccine suspension and from the puppy's organs were compared. The identity found between the two *vp2* complete sequences was 100%.

Isolation of CAdV-1 was achieved in MDCK cells infected with a liver homogenate. A liver sample was homogenised and suspended in PBS containing penicillin, streptomycin and amphotericin B (antibiotic-antimycotic), used according to the manufacturer (Gibco, Life Technologies Corporation, Carlsbad, USA). Following centrifugation, the supernatant was filtered through a 0.45-µm-pore-size filter (Milipore Express) and used to inoculate subconfluent MDCK epithelial cells, grown in Dulbecco's modified Eagle's medium supplemented with 8% foetal calf serum (FCS) (Gibco, Life Technologies Corporation, Carlsbad, USA) and 50 µg/ml gentamicin (Gibco, Life Technologies Corporation, Carlsbad, USA). Cells were maintained at 37 °C under a humidi-

fied atmosphere with 5% $CO_2$ and observed daily for cytopathic effects (CPE). Three passages were carried out and the supernatant of each passage was tested for the presence of CAdV-1 and CPV-2. Cytopathic effect (CPE) was observed at day 3 post-infection (Figure 2A). After the third passage, CPV DNA was no longer detected in the culture supernatant. Adenoviral typical basophilic intranuclear inclusion bodies were observed at 24 (Figure 2B), 48 and 72 h post-infection using haematoxylin and eosin staining, according to the protocol originally described by Bohmer (1865) and Fischer (1875)

Identical nucleotide sequences were obtained from PCR fragments amplified from liver and MDCK cell culture supernatants. BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST) (Madden 2002) showed that the CAdV-1 genomic region analysed is highly similar (99% to 100%) to CAdV-1 isolates from other European countries – United Kingdom (M60937, Y07760), Netherlands (S38238), Canada (U55001), and India (EF057101,
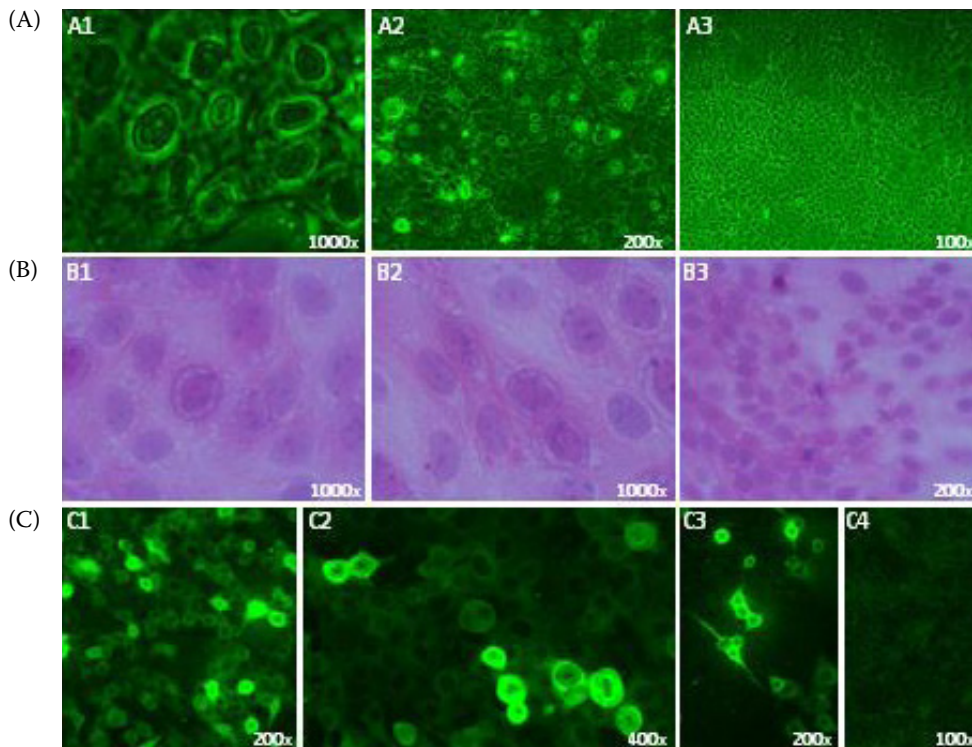


Figure 2. **A** = Light microscopy of MDCK cells infected with CAdV-1 PT/12 at 72 h post-infection (first passage) (A1 and A2) and MDCK cells mock-infected (A3). Intranuclear inclusion bodies are visible in A1 and A2. **B** = Haematoxylin- and eosin-stained MDCK cells infected with the isolate CAdV-1 PT/12. At third passage, eosinophilic nuclear inclusions with peripherally displaced and condensed chromatin were already visible 24 h post-infection (B1, B2). No evident CPE was observed at 6 h post-infection (B3). **C** = Indirect immunofluorescence performed on MDCK cells infected with the CAdV-1 PT/12 isolate fixed at 24 h post-infection with sera from female Pinscher 1 (C1, C2) and female Yorkshire Terrier 1 (C3), tested at dilution 1/10, and LTE from Yorkshire Terrier index case (C4). Magnification is indicated in the bottom left corner of each image

Table 3. Degree of genetic similarity between the CAdV-1 PT/12 isolate and other CAdV-1 and CAdV-2 strains, based on the 462 bp-long E3 region/U-exon region

| Strain | CAdV type | Geographic origin | Accession number | Nucleotide similarity with CAdV-1 PT/12 (KC577558) (%) |
|---|---|---|---|---|
| Glaxo | CAdV-1 | United Kongdom | M60937 | 100 |
| RI261 | CAdV-1 | United Kingdom | Y07760 | 100 |
| – | CAdV-1 | India | EF057101 | 99 |
| Utrecht | CAdV-1 | Netherlands | S38238 | 99 |
| CLL | CAdV-1 | Canada | U55001 | 99 |
| B 579 | CAdV-1 | India | GQ340423 | 98 |
| – | CAdV-1 | India | EF090910 | 94 |
| TorontoA26/61 | CAdV-2 | Canada | U77082 | 79[a] |
| – | CAdV-2 | – | S38212 | 79%[a] |

[a]homology based on the alignment of a sub-region (67%) of the CAdV-1PT/12 sequence

U77082, S38212) (Table 3). As expected, a lower similarity (79%) involving a partial region (67%) was observed with two CAdV-2 strains (Table 3). Kidney tested negative for CAdV-1 DNA.

Antibodies against CDV, CPV and CCoV were investigated in six females and one male from the same premises, born between 2005 and 2011, by means of commercial ELISA kits from *Ingenasa*, Spain (Table 2). Serum samples were collected one year and two months after the fatal CAdV-1 infection reported in this paper. All dogs had been vaccinated at week 6 with Nobivac Puppy DP Vaccine, MSD Animal Health (attenuated canine parvovirus and canine distemper virus), and at week 9 with Nobivac DHPPi vaccine, MSD Animal Health, (attenuated vaccine for canine distemper, canine adenovirus type 2, parainfluenza (CPIV) and parvovirus) and Nobivac Lepto Mais vaccine, MSD Animal Health, (inactivated *Leptospira*). None of the animals, except the male (2010), which was revaccinated with Nobivac DHPPi vaccine and Nobivac Lepto Mais vaccine at week 13, followed the standard vaccination protocol. No further revaccinations were carried out in any of the animals. The animals tested included Yorkshire Terrier female 1, and the mothers of the puppies that developed symptoms compatible with CAdV-1 infection, designated as Yorkshire Terrier female 2 and Pinscher female 1. The results of the serology tests are shown in Table 4. Titres were calculated according to the manufacturer's instructions. Indirect immunofluorescence (IIF) was used to investigate the presence of CAdV-1 antibodies in the seven dogs (Table 4). Confluent cell cultures grown on cover slips in Leighton tubes were infected with the supernatant of the third passage of the isolate in MDCK

cells and, after 6, 14, 24, 48 and 72 h of incubation at 37 °C, fixed in acetone for 15 min and air-dried. Cells were washed with 1 × PBS prior to incubation at 37 °C for 1 h with serum samples diluted at 10% (v/v) in 1 × PBS. Adherent cells on cover slips were then washed with PBS three times and incubated for 1 h at 37 °C with FITC (fluorescein isothiocyanate) conjugated horse anti-dog IgG (SIGMA) diluted 1 : 100 in PBS – Evans Blue solution. Following an additional washing with PBS, stained sections were examined under a Nikon Eclipse Ti-5 microscope equipped for incident light fluorescence.

As serum from the index case was not available, LTE prepared from a lung sample collected during necropsy, as previously described (Ferroglio et al. 2000), was tested for the presence of CAdV-1 and CPV antibodies. Briefly, lung tissue was homogenised in PBS (10 g/10 ml), incubated at room temperature for 20 min and centrifuged at 10 000 × g for 10 min and the supernatant was stored at −20 °C. Antibodies against CAdV-1 were not detected but LTE was positive to CPV antibodies, up to 1/10 dilution (S/P > 0.150; titre of 410), indicating the puppy had developed some immunological protection either through CPV vaccination administrated one week before or by maternal colostrum (Table 4). Serum from Yorkshire Terrier female 1, collected 14 months after the death of the puppy, tested positive for CPV (1/1800) (Table 4) and CAdV-1 antibodies (Figure 2).

To determine the shedding of CAdV-1 in the breeding kennel, we analysed the faeces and nasal swabs of 21 dogs (18 adults and three three-month-old puppies) for the presence of CAdV-1 DNA. Faecal samples and nasal swabs collected 6 months after the Yorkshire's death were suspended

Table 4. Results of the serological survey for canine parvovirus (CPV), canine distemper virus (CDV), canine corona-virus (CCoV) and canine adenovirus type 1 (CAdV-1) in adult dogs

| Method for antibodies detection | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Animal | Gender | Year of birth | Age of vaccination (weeks) | Vaccine administrated | CPV (ELISA[d]) | CDV (ELISA[e]) | CCoV (ELISA[f]) | CAdV-1 Ab (IIF[g]) |
| Pinscher1 | F | 2005 | 6 | Nobivac Puppy DP[a] | 3153 | low | > 1/400 | strong positive |
| | | | 9 | Nobivac DHPPi+[b] | | | | |
| | | | 9 | Nobivac lepto Mais[c] | | | | |
| Chihuahua | F | 2006 | 6 | Nobivac Puppy DP[a] | 599 | low | > 1/400 | positive |
| | | | 9 | Nobivac DHPPi+[b] | | | | |
| | | | 9 | Nobivac lepto Mais[c] | | | | |
| Yorkshire Terrier 1 | F | 2009 | 6 | Nobivac Puppy DP[a] | 1800 | high | > 1/400 | positive |
| | | | 9 | Nobivac DHPPi+[b] | | | | |
| | | | 9 | Nobivac lepto Mais[c] | | | | |
| Yorkshire Terrier 2 | F | 2009 | 6 | Nobivac Puppy DP[a] | 1692 | med | > 1/400 | positive |
| | | | 9 | Nobivac DHPPi+[b] | | | | |
| | | | 9 | Nobivac lepto Mais[c] | | | | |
| Pinscher | F | 2008 | 6 | Nobivac Puppy DP[a] | 5409 | low | 1/200 | positive |
| | | | 9 | Nobivac DHPPi+[b] | | | | |
| | | | 9 | Nobivac lepto Mais[c] | | | | |
| Chihuahua | M | 2010 | 6 | Nobivac Puppy DP[a] | 179 | low | > 1/400 | positive |
| | | | 9 | Nobivac DHPPi+[b] | | | | |
| | | | 12 | Nobivac lepto Mais[c] | | | | |
| Chihuahua | F | 2011 | 6 | Nobivac Puppy DP[a] | 908 | low | 1/100 | positive |
| | | | 9 | Nobivac DHPPi+[b] | | | | |
| | | | 9 | Nobivac lepto Mais[c] | | | | |

CPV = canine parvovirus, CCoV = canine coronavirus, CDV = canine distemper virus, CAdV-1 = canine adenovirus type 1, CAdV-2 = canine adenovirus type 2, CPIV = canine parainfluenza virus
[a]live attenuated CDV and CPV, [b]live attenuated CDV, CAdV-2, CPV, CPIV, [c]inactivated leptospira, [d]15.CPV.K.1, Ingenasa, Spain, [e]15.CDG.K.1 (IgG), Ingenasa, Spain, [f]15.CCV.K.1, Ingenasa, Spain, [g]in house IIF method developed during this work

in PBS buffer and submitted to CAdV-1 PCR, all testing negative.

## DISCUSSION AND CONCLUSIONS

The puppy had been vaccinated against CPV and CDV eight days before the appearance of the first clinical signs. In addition to the strong amplification of CAdV-1 DNA, traces of CPV DNA were also detected in the puppy's tissues but molecular characterisation of the complete *vp2* gene showed it to be an old CPV-2 strain. Furthermore, the 100% identity between the CPV-2 detected in the intestine (CPV-2/12250/PT12) and the C-154 vaccine strain indicated that the former originated from the vaccine that had been administered very recently. Due to the absence of a serum sample from the puppy, the presence of CPV

and CAdV-1 antibodies was investigated in LTE by means of a commercial ELISA and IIF, respectively. Although less sensitive with LTE when compared to serum samples, ELISA was able to detect antibodies against CPV in the puppy, demonstrating seroconversion induced by the vaccine or passively transferred immunity. CAdV-1 antibodies were not detected in the index case (Figure 2), which is consistent with rapid progression of the infection and death of the animal before an effective immune defense could be raised. Experimental CAdV-1 infections in fully susceptible animals showed that none of the dogs with acute disease had detectable antibodies to the virus at the time of infection nor did they develop antibodies during the brief course of their illness (Gocke et al. 1967). Although the clinical signs and gross pathological findings are not exclusive to classical ICH, these results, in view of the strong amplification of CAdV-1

DNA from the liver and absence of field CPV, CCoV, RV, CDV and CHV, suggest that CAdV-1 infection caused the death of the puppy. The acute course of the disease may explain the lack of CAdV-1 DNA detection in the kidneys. While viraemia probably spread the virus throughout the puppy's body, virus titres in some internal organs may have remained low due to the sudden death of the puppy, which prevented the development of ICH-typical lesions such as the oedema of the gall bladder and the congestion and oedema of the kidneys.

Vomiting and diarrhoea are described in the literature as common signs of ICH. Haemorrhagic diarrhoea was described as a clinical sign of CAdV-1 infection (Decaro et al. 2008; Piacesi et al. 2010). The extension of the haemorrhages to several tissues and organs of the puppy, indicating ample damage of endothelial cells and/or impairment of the coagulation system, may have accounted for the observations in the gastrointestinal tract. Interestingly, haemorrhagic diarrhoea was described in four CAdV-2-vaccinated puppies that tested CAdV-1 and CPV-2c PCR-positive (Decaro et al. 2007a). In our case, however, no field CPV was detected. The possibility that CPV-2 vaccine virus may have also contributed to the clinical disease of the Yorkshire puppy is remote, since vaccine parvovirus was not isolated in cell culture after three passages indicating that, if present, the viral charge was very low, in agreement with the high Ct value (38.5) obtained in the real time PCR. Furthermore, we obtained lower Ct values with fresh samples from cats and dogs with active disease (Duarte et al. 2013), in contrast with the high Ct value obtained for the index case. Moreover, previous studies confirmed that most cases of parvovirus-like disease occurring shortly after vaccination were related to infection with either field strains of CPV type 2 (CPV-2) or other viruses, bacteria or parasites, rather than to reversion to virulence of the modified live virus contained in the vaccine. In fact, these authors observed that only three of the 29 samples analysed were found to contain the vaccine strain without evidence of any of the canine pathogen investigated (Decaro et al. 2007b). In the present study, parasites were excluded as gastrointestinal bleeding causes, but we cannot guarantee that other parasites or viruses, such as giardia or norovirus, were not present. Although the possibility of co-infection with other pathogens remains unclear in this particular case, the presence of CAdV-1 and the absence of field parvovirus were demonstrated.

An epidemiological inquiry revealed that by the time the Yorkshire litter was born (second week of March) two other females (Pinscher female 1 and Yorkshire Terrier female 2) also gave birth. Shortly after the clinical case reported in this study, and two days after being sold, one of the Pinscher puppies born in the middle of May 2012, developed lethargy, vomiting, enlargement of the liver and diarrhoea. The puppy then died two or three days following the onset of clinical signs. A Yorkshire Terrier puppy born in August 2012 developed similar clinical signs but recovered following clinical treatment.

Laboratory confirmation of CAdV-1 was not carried out in either of these puppies. Hepatomegaly, which is compatible with a CAdV-1 infection, was observed in the Pinscher. Apart from the clinical signs described by the veterinarian, no other clinical data could be gathered regarding the second puppy. Also, no clinical signs were observed in any of the adult dogs from the breeder suggesting immune protection, either from previous subclinical infections, or from earlier vaccinations. Although the epidemiological source could not be clearly identified, the access to males from other kennels for breeding and the owner's regular contact with a second local breeder may have been connected with the origin of the disease. No animals had recently been introduced into the kennel.

About six months after the death of the Yorkshire puppy, CAdV-1 DNA could not be detected in the faeces or nasal fluids from 18 adult dogs, which represented 44% of the adult kennel population, nor in three puppies, two Pugs (Carlin) and one Yorkshire Terrier, born in July 2012. None of these animals had contact with either the confirmed CAdV-1-infected Yorkshire or the Pinscher and Yorkshire that were suspected of having undergone CAdV-1 infections. Therefore, negative PCR results in faeces from these three puppies indicate that they were not infected, or that colostrum antibodies may have prevented clinical signs and viral shedding. To investigate the CAdV-1 immune status of the adults, serum samples from seven animals were tested at 1/10 by IIF 14 months after the index case and all were positive (7/7). It is uncertain if the immunity found for CPV, CDV and CAdV-1 is the result of vaccination as it was demonstrated that a single dose of modified live virus against CPV-2, CDV and CAdV-2, when administered at 16 weeks or older, could provide long-term immunity against these viruses in a very high percentage of animals, while also increasing herd immunity (Schultz et

al. 2010). None of the animals were, however, re-vaccinated, and therefore did not complete the full recommended vaccination program. Heterogeneity of CDV and CPV antibody responsiveness or continuous natural boosting through contact with field strains of CDV and CPV may explain the variability found in antibody titres. Positive serology results for CCoV may indicate ongoing or previous infections (Table 4). The 100% CAdV-1 seroprevalence seems to reflect virus circulation in the kennel. We observed that the oldest of the seven dogs tested, female Pinscher 1, showed the highest CAdV-1 immunofluorescence staining intensity compared to the other six dogs. Although the number of animals tested is very limited (*n* = 7), the correlation between immune protection for CAdV-1 and age observed is in accordance with expectations for continuous contact with the virus.

Curiously, the breeder reported similar previous clinical cases. At the time, they were considered to be parvovirus infections although laboratory confirmation was not carried out. If those episodes were in fact caused by CAdV-1, acute infections in the young appear to have alternated with silent periods of disease. This cyclicity could be the result of a dynamic equilibrium between infection dose exposure and level of maternal immunity. In addition, the CAdV-1 immunity of the adult breeders may have been boosted by periodic contact with the virus.

In this work, we report the clinical, pathological, serological, virological and molecular findings of a CAdV-1 acute infection in a puppy, which occurred at the beginning of May 2012, in Portugal. To our knowledge, this is the first report of a CAdV-1 infection in the country.

In Portugal, both veterinarians who use our services in the North and Centre of the country, as well as our team of pathologists who reported having seen very few cases of suspected ICH, all confirmed by histopathological examination, in the last two decades agree on the rarity of this disease in our country.

The incidence of CAdV-1 infection in dogs appears to have decreased worldwide since the 1950's due to vaccination of dogs with CAdV-2, which is antigenically similar to CAdV-1 (Philippa 2010). Occasional reports in domestic dogs have been published suggesting a recrudescence of the disease (Decaro et al. 2007a). However, surveys conducted in domestic dogs between 1964–2006 in Brazil (Inkelmann et al. 2007) detected 2.1% of ICH cases, while in UK 80 cases of ICH were reported

in dogs submitted to one centre in the UK from 1986 to 2007 (Thompson et al. 2010), providing evidence of ongoing exposure of susceptible dogs to CAdV-1 and suggesting that the occurrence of ICH has probably been underestimated due to underreporting in the last decades. The importance of considering CAdV-1 as a putative aetiological agent when clinical signs are consistent with ICH (Muller et al. 2010), as well as persisting with vaccination against CAdV-1/ICH at a worldwide level is necessary in order to increase the immunity of dog populations and to reduce the risk of CAdV-1 transfer from wildlife reservoirs to susceptible dogs.

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