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CASE REPORT

Companion or pet animals

Different outcomes of *Canid alphaherpesvirus 1* infection in a litter of puppies: The silent viral spread

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Abstract

Canid alphaherpesvirus 1 causes worldwide infection with different outcomes in neonatal puppies and adults, followed by latency. Here, we report the varied outcomes of *Canid alphaherpesvirus 1* infection in a Dalmatian litter of 12 puppies in Italy. The diagnosis of *Canid alphaherpesvirus 1* was obtained by polymerase chain reaction on internal organs of one of the dead puppies. Another three puppies (one stillborn and two dead within the first 3 days of life) were not investigated (owner's request). Based on a positive result, an epidemiological investigation was performed. The outcome of the infection was particularly variable within the litter (one dead puppy, three healthy polymerase chain reaction-positive and another five virologically negative puppies). The present case report indicates the relevance of early detection of infection, how the features of herpetic infection can vary in the same litter and how viral spread can be underestimated in asymptomatic dogs. Effective control of *Canid alphaherpesvirus 1* infection should include hygienic measures and screening tests, especially in situations in which dogs, even asymptomatic, are mixed.

KEYWORDS
dogs, herpesviruses, infection outcomes, latent infection, PCR, puppies

BACKGROUND

Canid alphaherpesvirus 1 (CaHV-1, order *Herpesvirales*, genus *Varicellovirus*, species *Canid alphaherpesvirus 1*) is a temperature-sensitive virus with optimal replication at temperatures less than 37°C. It can infect puppies and adult dogs, inducing asymptomatic or clinically overt infections.[1] It has a worldwide distribution and has recently been defined as a transboundary disease because similar viral isolates have been found on different continents.[2]

The infection can occur transplacentally or through direct contact with contaminated secretions by oronasal, genital and orogenital routes. Clinical features vary based on the age when the contagion occurs and the physiological state of the infected dogs.[1,3] Ocular, respiratory and genital signs can be present in adult dogs, generally with self-limiting effects. Lesions on the genitalia of sexually active animals with typical bullous vesicles observed either in the vestibule or vagina and on the prepuce have been reported.[4] Most likely, the most clinically relevant signs are those linked to the reproductive area, depending on the stage of gestation at which the infection occurs, such as infertility (early unnoticed embryonic loss), embryonic resorption (nonexpulsed fetuses before ossification/calcification), abortion, stillbirth, delivery of poorly developed and compromised neonates, or neonatal mortality.[5]

Neonates are more susceptible than adults to CaHV-1, developing systemic infections due to the inefficiency of their thermoregulation system up to 2 weeks of life and their weaker immune response.[6,7] Clinical recovery is possible, especially in puppies older than 2 weeks, and is associated with lifelong latent infection localised in the nervous ganglia and lymphoid tissues, which represents the viral strategy of spread via its reactivation.[3] No estimates of the percentages of dogs becoming latent carriers after infection exist; however, serological investigations report positivity ranging from 30% to 100% in different kennels.[3]

CASE PRESENTATION

A 4-day-old male puppy underwent clinical examination for the presence of dyspnoea and neurological signs, consisting of opisthotonus and hyperextension of the limbs. It belonged to a litter of 12 puppies born in July 2018 in central Italy from a 5-year-old Dalmatian dog at her third pregnancy, which was completed without any complications. The bitch lived with

three other Dalmatian dogs, two males (17 and 25 months old), and an 8-month-old female (her daughter). They lived in the countryside but without contact with other dogs. The adult dogs never had previous health problems and were regularly dewormed and vaccinated with core vaccines. The owner explained that this pregnancy was not planned and that the bitch was not vaccinated against CaHV-1, whereas on the previous two occasions of pregnancy, she was vaccinated against CaHV-1 using a subunit vaccine available in Italy (Eurican Herpes 205, Boehringer Ingelheim Animal Health, Italy). The sire of the puppies was one of the two cohabiting males, but the owner was unable to identify which one.

At the time of the visit, the owner reported that a puppy was stillborn, and a second puppy died the previous night. However, these events were not considered worrisome but were attributed to the large number of newborn puppies. The owner subsequently reposted that 2 days later, a third puppy died. The owner did not consider it useful to submit the three puppies for postmortem examination. The remaining littermates were healthy, while the dam had a self-limiting diarrhoea, appeared after whelping and recovered within 2 days without medical treatment.

The puppy stopped sucking milk and showed progressive wasting, with incessant vocalisation. It was hospitalised at the veterinary clinic, and a clinical assessment was performed. It was weighed and monitored for physiologic and behavioural parameters. Heart rate (240 heart rate), respiration (tachypnoea, with 30 respiratory rate, and dyspnoea), irritability reflex (vigorous), motility (active motion) and mucus membrane colour (pale pink) were observed. Body temperature was 35.5°C–36°C.

The puppy died at 5 days of age, despite supportive treatment.

LEARNING POINTS/TAKE-HOME MESSAGES

- Multiple outcomes of *Canid alphaherpesvirus 1* infection can appear in the same litter.
- Early detection of *Canid alphaherpesvirus 1* infection is critical to prompt appropriate treatment in a litter with infected puppies.
- Multiple tests, detecting both the virus directly and indirectly (serology), are needed to characterise timing and spread of *Canid alphaherpesvirus 1* infection.
- *Canid alphaherpesvirus 1* systematic screening in canine infertility, stillbirth or neonatal mortality is necessary.

INVESTIGATIONS

Postmortem examination was conducted looking for lesions attributable to congenital or infectious diseases. At the same time, blood tests were carried out on the mother (complete blood count, including white blood count differential measuring neutrophils, lymphocytes, monocytes, eosinophils and basophils, and a basic biochemical profile), but only slight and nonspecific alterations were found (not reported here). At postmortem examination, general congestion was observed, and histopathological examination was not performed; samples of kidney, liver, lung and gut were collected and subjected to routine microbiological investigations. For the bacteriological examination, kidney, liver and lung samples were inoculated onto blood agar, mannitol-salt agar and MacConkey's agar plates and incubated for a minimum of 24 hours at 37°C under aerobic and anaerobic conditions. No significant bacteria were isolated. For virological screening, CaHV-1 infection was investigated. DNA extraction from a pool of kidney, liver, lung and gut tissues was carried out using the GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA). The concentration and purity of the extracted DNA were assessed using a NanoDrop spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Milan, Italy). A nested polymerase chain reaction (PCR) protocol previously described,[8] amplifying a fragment of 493 bp at the first step and 168 bp at the nested run of the CaHV-1 thymidine kinase gene, was used to identify CaHV-1 DNA, using a positive control (CaHV-1 reference strain) and negative controls (water instead of DNA and DNA negative for CaHV-1). Nested PCR revealed the presence of CaHV-1 DNA from all tested organs of the puppy. Consequently, an epidemiological investigation was carried out to identify the source and state of infection of all the dogs on the property.

The day following the death of the puppy, a vaginal swab was collected from the bitch. Nasal swabs were individually collected from the puppies when they were 16 and 31 days old. On the same dates, blood samples were obtained from the adults to assess the presence of CaHV-1 in buffy coats. When the puppies were 31 days old, nasal swabs were also collected from the other adult dogs, whereas blood samples without anticoagulants were taken to evaluate the antibody response in serum. Blood was not collected from puppies to avoid invasive sampling. Nasal and vaginal swabs were individually collected by rotating a sterile polyester swab on the nasal mucosa and vagina, respectively, and dipped in 500 μl of phosphate-buffered saline (PBS, pH 7.2). DNA was extracted from swabs and blood samples using a QIAmp DNA mini-kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and submitted to the nested PCR protocol described above to detect CaHV-1 DNA.[8] Real-time PCR was also performed to quantify the viral DNA from the specimens according to a protocol described elsewhere.[9] A plasmid containing a CaHV-1 DNA fragment was used as a positive control; a negative control (canine DNA negative for CaHV-1) and a no-template control were included in each assay.[9]

The serological response was assessed by indirect immunofluorescence as previously described.[10] Briefly, CaHV-1 strain 257/01 was propagated in confluent canine fibroma A-72 cells seeded onto multispot glass slides. When the cytopathic effect was evident in 30% of the infected monolayer, the cells were fixed with an 80% acetone solution and incubated with twofold dilutions of each serum sample (in duplicate). After washing, the virus-bound CaHV-1 antibodies from the sera were detected with anti-canine IgG conjugated with fluorescein isothiocyanate. The CaHV-1 antibody titres were calculated as the highest serum dilutions producing nuclear fluorescence in the infected cells. The results of the analyses are reported in Tables 1 and 2.

TABLE 1 Results of nested PCR and real-time PCR for CaHV-1 from tissues of the dead puppy, vaginal swab of the mother and nasal swabs of the other puppies

ID dog	Type of sampling	Date of sample collection and testing (days from birth)	CaHV-1 nested PCR	CaHV-1 real-time PCR on nasal swab (Ct)	Date of re-testing (days from birth)	CaHV-1 nested PCR on nasal swab (re-testing)	CaHV-1 real-time PCR on nasal swab (re-testing)
Puppy no. 1	Pool of organs	8 July 2018 (5)	+	+ (18.51)	−	Not available	Not available
Mother	Vaginal swab	9 July 2018 (6)	+	−	−	Not done	Not done
Puppy no. 2	Nasal swab	18 July 2018 (16)	−	−	2 August 2018 (31)	−	−
Puppy no. 3	Nasal swab	18 July 2018 (16)	+	+ (33.74)	2 August 2018 (31)	−	−
Puppy no. 4	Nasal swab	18 July 2018 (16)	−	−	2 August 2018 (31)	−	−
Puppy no. 5	Nasal swab	18 July 2018 (16)	+	+ (31.14)	2 August 2018 (31)	−	−
Puppy no. 6	Nasal swab	18 July 2018 (16)	+	+ (29.21)	2 August 2018 (31)	−	−
Puppy no. 7	Nasal swab	18 July 2018 (16)	−	−	2 August 2018 (31)	−	−
Puppy no. 8	Nasal swab	18 July 2018 (16)	−	−	2 August 2018 (31)	−	−
Puppy no. 9	Nasal swab	18 July 2018 (16)	−	−	2 August 2018 (31)	−	−

Abbreviations: CaHV-1, *Canid alphaherpesvirus 1*; Ct, cycle threshold; PCR, polymerase chain reaction; +, positive result; −, negative result.

An interview with the owner was conducted to identify the source of infection. Considering that the four dogs lived together and were isolated from other dogs, the only event identified as at risk was participation in a dog show abroad, which occurred when the bitch was at mid-gestation, but the owner did not know that the bitch was pregnant. All four dogs were enrolled in the show. It was not possible to exclude contact with other dogs, which may have occurred in the previous months or years (after reactivation of latent infection); however, the dogs have never shown clinical signs that suggested any disease before this event. The owner reported no contact with other dogs that could have acted as fomites.

DIFFERENTIAL DIAGNOSIS

Differential diagnosis included infectious causes (mainly bacterial infections, canine brucellosis, canine distemper, Rubarth disease, neosporosis, 'toxic milk syndrome' for ingestion of toxins from milk of the dam due to uterine or mammary gland infections) and non-infectious causes, such as placental deficiency and poor maternal care.

Bacterial infections were excluded based on the results of the bacteriological examination. Canine distemper and Rubarth disease were excluded because the bitch was regularly vaccinated with core vaccines, including viruses causing these infectious diseases. Canine brucellosis was excluded because no mating with external dogs had been performed, although a serological investigation on adult dogs might be appropriate. Neosporosis was excluded based on the absence of clinical neurological signs (ascending paralysis) in the puppies of the littermate.

Other unidentified factors may have contributed to the severity of CaHV-1 infection in some of the puppies.

TREATMENT

Considering the low body temperature (35.5°C–36°C), the puppy was kept at room temperature, above 32°C, with humidity at 45%–55% and oxygen therapy using a human incubator for premature babies. The specific gravity of the urine was evaluated to estimate the state of hydration, which was higher than 1017; accordingly, fluid therapy was administered using Lactated Ringer's solution (60 ml/kg per day, for instance, 10 ml during each feeding, to reduce stress). No blood test was performed due to the invasive procedure and to the limited volume of blood that could be collected; the puppy was fed with milk replacer (Professional Babydog Milk - Milk Replacer for Puppies, Royal Canin, Italy) by orogastric tube. Antibiotic therapy was administered (cefazolin 20 mg/kg day, intramuscular, until the day of death). However, the puppy died at day 5 postpartum, 1.5 days after its hospitalisation. The other puppies were reared at home and were monitored by means of video clips provided by the owner; no abnormal behaviours were noticed. The use of an infrared lamp to warm the remaining puppies was suggested to the owner, but was not adopted.

OUTCOME AND FOLLOW-UP

The puppy died at 5 days of age, despite supportive treatment. The three healthy PCR-positive puppies described herein did not show any systemic disease, neurological signs or ocular

TABLE 2 Results of nested PCR, real-time PCR and serology for CaHV-1 from the mother of the litter and adult dogs cohabitant with the mother

ID dog	CaHV-1 nested PCR from buffy coat, 16 days after puppy birth	CaHV-1 real-time PCR from buffy coat, 16 days after puppy birth	CaHV-1 nested PCR from nasal swab, 31 days after puppy birth	CaHV-1 real-time PCR from nasal swab, 31 days after puppy birth	Serology (indirect immunofluorescence antibody titre), 31 days after puppy birth
Mother	–	–	–	–	1:1920
Adult male 17 months old	–	–	–	–	1:240
Adult male 25 months old	–	–	–	–	1:480
Adult female 8 months old	–	–	–	–	1:60

Abbreviations: CaHV-1, *Canid alphaherpesvirus 1*; PCR, polymerase chain reaction; +, positive result; –, negative result.

lesions. Three years later, the owner reported that these dogs remained healthy. The other PCR-negative puppies also remained healthy.

DISCUSSION

CaHV-1 infection is generally suspected when stillbirth and neonatal mortality are present in the whole litter. This report describes a less frequent clinical picture of CaHV-1 infection in a litter where very different outcomes of infection were present. If an early diagnosis of CaHV-1 infection had been made at the time of death of the first stillborn puppy, supportive treatment could have been undertaken promptly, increasing the survival rate. Awareness of the impact of the infection and the possibility of latent infection by CaHV-1 should be a reason for the owner to investigate further and implement preventive measures.

The index case was likely the dam or one of the cohabiting dogs, which may have been directly infected at the dog show or by contacts with other dogs. Interestingly, participation in competitions or shows has previously been associated with CaHV-1 seropositive bitches.[11]

Therefore, the onset of infection may be dated either a month or 10–15 days before whelping if it was secondary to the infection of a cohabiting dog, but in any case, it could have occurred transplacentally, at least in some fetuses. It is legitimate to suspect that the virus crossed through the placenta when systemic herpetic infection causing the death of puppies within the first 7–9 days of life occurs,[3] as observed in the present litter. However, the mortality rate in transplacental or neonatal infections can reach almost 100%.[12,13] Cases of puppies infected in utero that overcome the systemic infection have rarely been described, but almost all generally had residual herpesvirus-induced lesions affecting the central nervous system, eyes, lungs and kidneys.[9,14,15] These puppies showed neurological deficits within the first year of life due to direct damage to the brain by the virus, cardiac defects and renal failure.[16,17] As mentioned above, the three healthy PCR-positive puppies did not show any systemic disease during the 3 years of observation.

We ruled out the possibility of transmission by the venereal route because if CaHV-1 had crossed the uterus in the early days of pregnancy, it would have resulted in implantation failure or embryonic resorption. It was also probably a primary infection for the bitch, considering the fatal outcome in four puppies.

Apart from three puppies in which it was not possible to investigate the diagnosis, very different outcomes were observed in the nine puppies of the present report: CaHV-1 was associated with the death of one puppy, which occurred 5 days after birth; eight were completely asymptomatic puppies, three of which were PCR-positive; and five were already negative by PCR for the detection of CaHV-1 DNA from nasal swabs collected at 16 days from birth. This heterogeneity of outcome is generally reported in litters with a high number of puppies.[9,18] Although the use of two different molecular tests gives robustness to the results, the puppies testing negative may have had a viral load lower than the detection limit of the test at the time of the sampling, resulting in a negative PCR result. Moreover, it is possible that the dam, during infection, had time to develop partial immunity consequent to natural infection, which was transferred to the offspring, resulting in different grades of protection. Puppies suckling milk from seropositive bitches, which become asymptomatic, have rarely been reported.[3] Partial protection due to a longer persistence of low-titre antibodies from vaccinations against CaHV-1 performed during the two previous pregnancies cannot be ruled out.

It is possible that the surviving positive puppies may have acquired the infection not in utero but later in the birth canal (since DNA from a vaginal swab of the mother was also positive for the presence of CaHV-1, albeit only at nested PCR) or following licking by the mother or by direct contact with infectious secretions, limiting viral presence to a primary replication phase localised in the oronasal mucosa. However, it remains to be understood why, albeit in close contact, some puppies became infected before 16 days of age but without showing any clinical signs or were not infected at all. Unfortunately, to date, few studies have been carried out on the mechanisms and timing of transmission of CaHV-1 infection in the uterus.[19,20] We hypothesise that transplacental transmission does not immediately involve all fetuses in the same way but spreads progressively, perhaps by contiguity, and the fetuses may come into contact with the virus at different times of gestation; this hypothesis is supported by the results of a previous study.[20]

In any case, within 30 days of life, the contagious process had ended as all puppies were CaHV-1 negative by PCR carried out on nasal swabs. Prolonged viral shedding has been reported for 35–36 days both in neonates and adults.[9,21] It was hypothesised that 14 days could be the time of viral spread in natural infection, and a longer time (32 days) was observed after immunosuppressive treatment.[22]

Initially, the clinical and infectious problem was considered limited to the puppies, not considering the possible epidemiological role of adult dogs, and no samples from adult dogs were obtained; we collected predominantly nasal swabs, considered to be the best specimen to evaluate viral spread among newborns to identify possible animals developing infectious disease[9]; however, investigation of antibodies against CaHV-1 in the dam, adult dogs and puppies could have better indicated the time of infection and determined if the infection of the mother occurred during the pregnancy and that of the puppies in utero or after the birth. Unfortunately, the serum was not collected from the bitch initially because it was not valued as being helpful or from the puppies due to the invasive nature of the sampling. In general, CaHV-1 serology may be helpful to monitor CaHV-1 infections, even retrospectively. The serological test, on the other hand, documented the infection in all four adult dogs. In one study, the CaHV-1 antibody titres in dogs under 1.5 years of age were lower than those in older dogs,[23] which is consistent with what we observed in the current investigation, with the 8-month-old dogs showing lower antibody titres (1:60) than older dogs (Table 2).

At least the puppies that are CaHV-1 PCR-positive should be monitored over time, both for reproductive purposes (to detect asymptomatic carriers) and in the case of any immunosuppressive treatments for a possible reactivation of latent virus. In fact, from an epidemiological point of view, asymptomatic individuals are the most dangerous for the spread of the virus.

In conclusion, this report describes a further clinical picture of CaHV-1 infection; usually clinical cases refer to infections involving the whole litter and with a fatal outcome. This outcome depends on several factors, such as the age at which the animals become infected, the availability of antibodies in the colostrum and its intake, the amount of virus spread and the body temperature of the puppies. As little is still known about CaHV-1 infection, and different outcomes can be present in the same litter, veterinary practitioners should not use preestablished clinical models for CaHV-1 infection to make a diagnosis. They should suspect this infection in any case of infertility, stillbirth or neonatal mortality and then increase screening for it to prevent spread of the infection.

Moreover, the ability of the virus to spread via asymptomatic dogs is probably underestimated, and health screening for situations at risk (promiscuity of animals of different origins, such as exhibitions or entry of new animals into breeding, mating, purchase of animals) and preventive measures against the virus, including vaccination during pregnancy and biosecurity procedures, should be implemented. Early detection and more intensive supportive therapy could help infected puppies overcome the critical period of infection (the first 15 days) and increase the survival rate.

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CONFLICT OF INTEREST

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The authors confirm that they adhered to ethical policies of the journal, as noted on the journal's author guidelines page and of the institution. Authorisation from the ethics commission was not required because the analyses were performed for diagnostic reasons. Informed client consent was obtained.

AUTHOR CONTRIBUTIONS

MLM conceived and designed the project. MLM, AT, IG, LS and ND acquired, analysed and interpreted the data. MLM, AT, IG, LS and ND wrote the paper, and MLM, AT, LS and ND reviewed the paper.

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