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## Long-term shedding of *Canine alphaherpesvirus 1* in naturally infected newborn pups

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### ABSTRACT

The long-term shedding of *Canine alphaherpesvirus 1* (CaHV-1) by neonatal pups with natural infection is reported. The pups belonged to a litter of 11 pointers of a breeding kennel in southern Italy, 9 of which developed a fatal form of systemic infection, as resulted by the detection of CaHV-1 in internal organs (kidney, liver, lung and brain) of one of this dogs and in the vaginal swab of their mother. The two remaining animals displayed a milder form of disease, with one pup showing ocular involvement, and underwent a progressive recovery. These pups were monitored from 11 to 36 days of age, showing a long-term shedding of the virus through the nasal and ocular secretions and the faeces. CaHV-1 shedding, as assessed by means of a specific and sensitive real-time PCR assay, occurred mainly through the nasal secretions, although the pup displaying ocular disease shed the virus at high titres and for a long period even in the ocular secretions.

*Canine alphaherpesvirus 1* (CaHV-1) is an enveloped, double-strand DNA virus of the genus *Varicellovirus*, which is strictly related to other members of the subfamily *Alphaherpesvirinae*. Infection with CaHV-1 results in different clinical pictures depending on the age of infected dogs (Decaro et al., 2008). In pregnant bitches, the virus is able to induce foetal death, abortion, mummification or stillbirth. In adult dogs CaHV-1 is responsible for genital lesions and/or ocular involvement (Evermann et al., 2011). CaHV-1 is assuming an emerging role in the etiopathogenesis of canine infectious respiratory disease (CIRD), a multifactorial respiratory syndrome, which is frequently observed in densely housed environments, such as kennels and animal shelters (Buonavoglia and Martella, 2007). However, the most severe clinical forms are observed in neonatal pups that may be infected during passage through the birth canal or by contact with oronasal discharges of other dogs (Decaro et al., 2008). As a consequence of systemic spreading of the virus, newborns develop a fatal generalized necrotizing and haemorrhagic disease, which is characterised by anorexia, dyspnoea, abdominal pain, incoordination, diarrhoea, serous or haemorrhagic nasal discharge and petechial haemorrhages on the mucous membranes (Decaro et al., 2008). Although there are several reports of

CaHV-1 neonatal infection (Decaro et al., 2002b; Larsen et al., 2015; Muro et al., 2015), little is presently known about the extent and duration of the viral shedding by infected pups.

In this paper, we report the evaluation of the viral shedding in two pups of a litter of 11 pointers with CaHV-1 infection. The two pups survived to the fatal outcome and were monitored virologically displaying a long-term viral shedding through the faeces, ocular and nasal secretions.

The clinical outbreak occurred in April 2016 and involved a litter of 11 pointers housed in a breeding kennel in Apulia, southern Italy. The bitch was regularly vaccinated against the main canine infectious diseases (canine distemper, parvovirus, infectious canine hepatitis, leptospirosis) and treated against ecto- and endoparasites, but she had not been administered any specific CaHV-1 vaccine. She had no problem during pregnancy and was healthy at the moment of delivery. At 2–3 days of age, 9 pups displayed signs of systemic disease, including loss of appetite, lethargy, enteritis, and respiratory distress, and died at 6–9 days after the onset of these clinical signs. Two other pups (331/16-O, 331/16-S) were only lethargic and inappetent, thus progressively improving their clinical conditions until complete recovery at

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15–20 days of age. One of the surviving two pups displayed a monolateral keratitis that recovered after two weeks. One dead pup and the vaginal swab of the bitch were submitted to our laboratory for routine virological and bacteriological investigations. At necropsy, the dead pup displayed haemorrhages on the surface of the main internal organs, enlargement of the spleen and liver with scattered necrotic areas, catarrhal pneumonia and enteritis.

After RNA and DNA purifications using the QIAamp cadof® Pathogen Mini Kit (QIAGEN S.p.A., Milan, Italy), internal organs (kidney, liver, lung and brain) of the dead pup and the vaginal swab of the bitch were tested for molecular detection of the main viral pathogens of dogs, i.e., CaHV-1 (Decaro et al., 2010), canine minute virus (Decaro et al., 2002a), canine coronaviruses (Decaro et al., 2004, 2005c), reoviruses (Leary et al., 2002; Decaro et al., 2005a), rotaviruses (Gouvea et al., 1994), caliciviruses (Jiang et al., 1999), canine parvovirus type 2 (Decaro et al., 2005b, 2006), canine adenoviruses type 1 and type 2 (Dowgier et al., 2016), and canine distemper virus (Elia et al., 2006). Standardised procedures were carried out on tissue samples for in vitro isolation of pathogenic bacteria potentially responsible for pup mortality, including *Enterobacteriaceae*, *Brucella* spp., *Staphylococcus* spp., *Streptococcus* spp. Samples were plated out on 5% sheep blood agar and cultured aerobically at 37 °C for 24 h for detection of aerobic pathogens. Bacteria were identified by standard biochemical procedures and analytical profile index (API, BioMérieux Italia S.p.A., Rome, Italy).

The pooled internal organs of the dead pup and the vaginal swab of their mother were homogenized in Eagles minimal essential medium (E-MEM), treated with antibiotics (penicillin 5000 IU/ml, streptomycin 2500 µg/ml) at 37 °C for 30 min and inoculated onto confluent canine A-72 cells. The inoculated cells were observed daily for the occurrence of cytopathic effect (cpe). After three days of incubation the cells were tested by an immunofluorescence (IF) assay using a 1:100 dilution of dog polyclonal serum specific for CaHV and a 1:60 dilution of rabbit anti-dog IgG conjugated with fluorescein isothiocyanate (Sigma Aldrich srl, Milan, Italy).

The two surviving pups were sampled at 3–4 days of distance from 11 (0–3 days after the death of the other pups) to 36 days of age by collecting nasal, ocular and rectal swabs. Nucleic acid was extracted from the collected swabs using the QIAamp cadof® Pathogen Mini Kit (QIAGEN S.p.A.) and subjected to real-time PCR for monitoring the CaHV-1 shedding.

Real-time PCR for simultaneous detection and quantitation of CaHV-1 DNA (Decaro et al., 2010) was performed on a 7500 Real-time PCR System (Applied Biosystems, Foster City CA) with iTaq™ Supermix added with ROX (Bio-Rad Laboratories Srl, Milan, Italy). Ten-fold dilutions of a plasmid containing a CaHV-1 DNA fragment, representing  $10^0$ – $10^9$  copies of DNA/10 µl of template, were used to generate the standard curve for absolute quantification. The TaqMan assay was carried out in duplicate for each unknown and standard sample and a template control was included in each assay.

CaHV-1 DNA was detected by real-time PCR in tissue samples of the dead pup and vaginal swab of its mother. Viral DNA loads were  $1.41 \times 10^5$ ,  $7.72 \times 10^5$ ,  $4.60 \times 10^4$ , and  $5.12 \times 10^3$  DNA copy numbers mg<sup>-1</sup> of template in kidney, liver, lung and brain of the dead pup, respectively. The vaginal swab of the bitch contained only small amounts of virus, equal to  $5.09 \times 10^2$  CaHV-1 DNA copies µl<sup>-1</sup> of DNA extract.

After inoculation on A-72 cells, the internal organs of the dead pups induced cpe, represented by cell rounding and detachment from the cell monolayer, testing positive by the CaHV-1 IF assay. The vaginal swab tested negative by virus isolation, likely due to the low viral load. No other pathogen was identified by means of molecular or traditional assays in the tested samples.

The two surviving pups tested positive for CaHV-1 in their nasal, ocular and rectal swabs at 11 days of age and were monitored every 5 days until 36 days of age in order to evaluate the viral shedding in

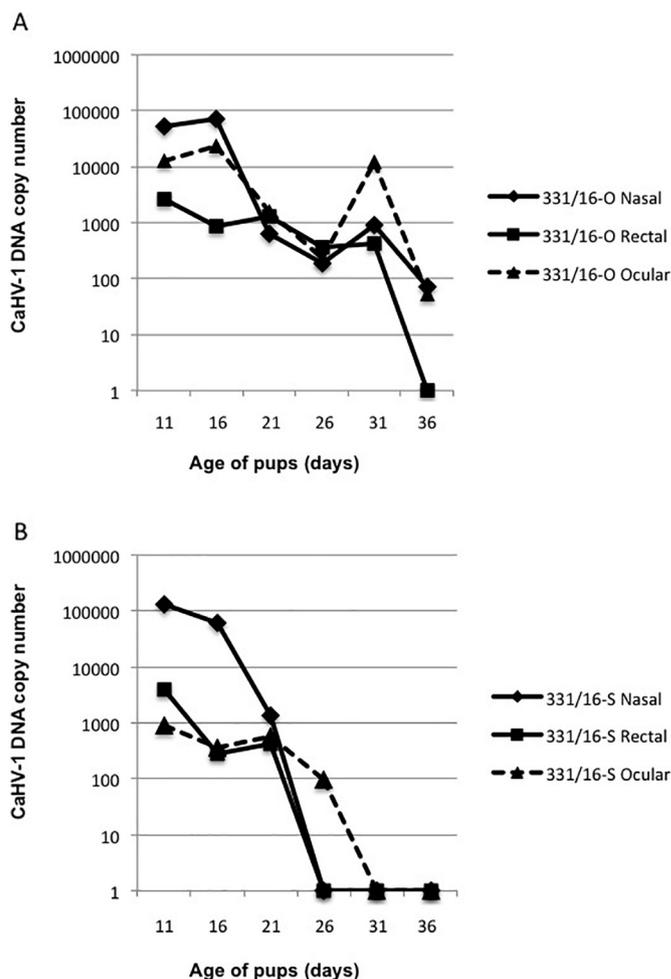


Fig. 1. CaHV-1 shedding in pups surviving to neonatal infections and showing (A) or not (B) ocular involvement. Viral loads are expressed as DNA copies µl<sup>-1</sup> of template.

their secretions. During the observation period, the pup with keratitis (#331/16-O) shed the virus for a longer timespan (26 days) than the other pup (#331/16-S) (16 days, Fig. 1). In pup #331/16-O, CaHV-1 DNA was detected in the nasal and ocular swabs over the entire observation period, with viral loads peaking at 16 days of age ( $7.08 \times 10^4$  and  $2.35 \times 10^4$  DNA copy numbers µl<sup>-1</sup> of template, respectively). In contrast, faecal shedding occurred at lower titres, reaching maximal values at 11 days of age ( $2.65 \times 10^3$  DNA copy numbers µl<sup>-1</sup> of template) and lasting up to 31 days of age. The pup that recovered without developing the ocular involvement shed CaHV-1 at three or four time points, i.e. from 11 to 21 and from 11 to 26 days of age for nasal/rectal and ocular swabs, respectively. Viral titres peaked at 11 days of age for all collected samples. Maximal loads were reached in nasal swabs ( $1.28 \times 10^5$  DNA copy numbers µl<sup>-1</sup> of template), whereas ocular shedding occurred at very low titres (highest value of  $8.94 \times 10^2$  DNA copy numbers µl<sup>-1</sup> of template).

CaHV-1 can cause different clinical forms according to the age and physiological status of the infected dogs, with the most severe disease and outcome in neonatal pups. Usually, infected neonates die within few days after infection irrespective of the treatment administered (Decaro et al., 2008). In the outbreak described in the present report, 9 out of 11 infected pups died, while other two dogs survived to the infection developing a milder clinical form followed by the complete recovery.

Few data are currently available on the CaHV-1 shedding in neonatal pups, whereas several studies have been carried out to assess the

ocular shedding in adult dogs with experimental infection (Ledbetter et al., 2009a,b, 2012). Analogously, nasal shedding in young and adult dogs occurred for about 2 weeks after primary oronasal infection and for up to 32 days after corticosteroid treatment (Okuda et al., 1993). In the present study, the nasal, ocular and faecal shedding was monitored in two pups that survived to CaHV-1 infection. Our findings demonstrated that in neonatal pups, CaHV-1 shedding occurs mainly by the nasal route, especially in the first phase of infection. However, high-titre nasal and ocular shedding was observed in the pup with ocular disease up to 31 days of age and low titres of the virus were detected up to the end of the observation period. In contrast, faecal shedding occurred at low titres in both pups. The difference of viral quantity between nasal, ocular and rectal swabs, and also the change of viral titre over time could have been partially biased by the sampling procedures (i.e., number of scraping times) and quantitation method (i.e., lack of standardisation of template volume). However, even considering some variations between the different sample types and over the time, these variations can unlikely account for the huge differences encountered in the viral DNA titres. Therefore, our findings strengthen the main role of nasal shedding for CaHV-1 transmission among neonatal pups. This is in agreement with the primary respiratory tropism of the virus observed in pups older than 2 weeks, in which CaHV-1 is able to colonise only the respiratory tract, being included among pathogens responsible for canine infectious respiratory disease (Decaro et al., 2008).

The main limitation of this study is that it was not possible to monitor the complete period of the shedding and date back the time of infection, since sampling was started at 11 days of age, when the two pups were being infected from few days. Another drawback of the study is represented by the low number of dogs studied, which are not enough to draw any scientifically-based conclusion about the variation in viral titres, so that results should be interpreted with cautions.

Even considering these study limitations, monitoring of pups that had a natural infection by CaHV-1 allowed us to assess the viral shedding under realistic conditions. Although this study contributes to expand the knowledge about the mechanisms of CaHV-1 transmission, further studies using molecular diagnostic methods are needed to better understand the pathobiology and epidemiology of this virus.

#### Conflict of interest statement

There is no conflict of interest of any authors in relation to the submission.

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