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

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ARTICLE INFO

Keywords:
Dog
Canine alphaherpesvirus
Viral shedding
Neonatal infection

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.
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 cador® 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 coronavirus (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 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 cador® 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 1 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 × 10^5, 7.72 × 10^5, 4.60 × 10^5, and 5.12 × 10^4 DNA copy numbers μg⁻¹ 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 × 10^2 CaHV-1 DNA copies μl⁻¹ 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 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 × 10^4 and 2.35 × 10^5 DNA copy numbers μl⁻¹ of template, respectively). In contrast, faecal shedding occurred at lower titres, reaching maximal values at 11 days of age (2.65 × 10^5 DNA copy numbers μl⁻¹ 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 × 10^5 DNA copy numbers μl⁻¹ of template), whereas ocular shedding occurred at very low titres (highest value of 8.94 × 10^2 DNA copy numbers μl⁻¹ 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

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**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⁻¹ of template.
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 quantification 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 was the low number of dogs studied, which are not enough to draw any scientific-based conclusion about the variation in viral titres, so that results should be interpreted with cautious.

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.

Acknowledgments

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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