**FULL PAPER** *Virology*

# Serological and biomolecular survey on canine herpesvirus-1 infection in a dog breeding kennel

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ABSTRACT. Canine herpesvirus-1 (CaHV-1) is a globally distributed pathogen causing reproductive, respiratory, ocular and neurological disorders in adult dogs and neonatal death in puppies. This pathogen is considered poorly immunogenic, and neutralizing antibodies are found for only a short time following exposure. Further, seroprevalence can be affected by several epidemiological factors. A virological survey was conducted in a high-density population breeding kennel in Central Italy. There were several factors predisposing animals to CaHV-1 infection, such as age, number of pregnancies, experience with mating and dog shows, cases of abortion, management and environmental hygiene. Serum neutralization (SN) and nested PCR assays were used to estimate prevalence of CaHV-1. None of the submitted samples tested positive for nested PCR, and none of the sera tested CaHV-1 positive. No association was observed between antibody titers and risk factors, and no sign of viral reactivation was detected in either males or females. These results suggest that CaHV-1 is not circulating within this kennel and that further studies are needed in order to better understand the distribution of the virus within Italy.
KEY WORDS: canine, canine herpesvirus-1, PCR, serum neutralization

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In 1965, three different research groups described for the first time a fatal hemorrhagic disease in domestic puppies and fetuses [10, 44, 45] and subsequently identified the etiological agent as canine herpesvirus-1 (CaHV-1). This virus belongs to the genus *Varicellovirus*, subfamily *Alphaherpesvirinae*, family *Herpesviridae*. Host range seems to be limited to domestic and wild canids (red fox, gray fox and coyotes) [13, 15, 17, 48], but neutralizing antibodies have been found in North American river otters [24]. The virus has a global distribution in canine populations, especially in breeding kennels, with prevalences varying amongst different countries: 80% in Norway and Finland [12, 27], 39% in Turkey [53], 40–88% in Belgium, U.K. and the Netherlands [37, 38, 41], and 21.7% in Japan [23].

Puppies become infected through direct contact of infected oronasal secretions from their mother, *in utero*, or during parturition and passage through the birth canal [2, 19, 20]. Transmission in adult dogs occurs through venereal, oronasal and ocular contact [16, 34].

CaHV-1 is typically inactivated by exposure to temperatures above 40°C, explaining viral localization in the cooler external mucous membranes of both nasal and genital tracts of adult canines. In neonates, normal body temperature is 1 to 1.5°C lower than an adult dog. This, in conjunction with an immature body-thermoregulation-system, allows for generalized systemic viral replication [1, 2]. CaHV-1 can cause subclinical to mild upper respiratory, genital, ocular and neurological disorders both in puppies older than three weeks and adult dogs [1, 8].

Nowadays, the primary involvement of CaHV-1 as the cause of an upper respiratory tract infection (tracheobronchitis) is controversial [3, 5, 26, 51, 52]. According to studies, CaHV-1 is not the most prevalent respiratory pathogen in dogs [4, 6, 22]. However, Ronsse and colleagues [40] noticed that the virus is primarily maintained and distributed among dogs through respiratory infections. As it pertains to the genital tract, CaHV-1 is a known agent for vesicular vulvovaginitis and vesicular balanoposthitis [4, 18, 21]. In pregnant females, CaHV-1 may cause systemic infection that results in embryo resorption, fetal death, abortion with or without mummification, premature parturition and stillbirth [19, 20, 34]. Neonatal puppies of antibody negative dams exposed to the CaHV-1 develop an acute, systemic infection. Symptoms include anorexia, dyspnea, abdominal pain, incoordination, yellowish soft feces, serous or hemorrhagic nasal discharge, petechial hemorrhage of the mucous membranes, hypothermia, coma and death [1, 14]. On the contrary, puppies from antibody positive dams are protected from the infection through maternal antibodies received via the placenta during gestation and maternal milk [40].

Recovery from infection is associated with lifelong latency and recrudescence with subsequent mucosal replication followed by viral shedding. CaHV-1 travels through sensory nerves to sensory ganglia neurons (trigeminal, lumbosacral and vestibular ganglia), tonsils, thymus and lymph nodes (retropharyngeal, hypogastric and pulmonary) [30], where it establishes latency [11, 36]. Reactivation is thought to

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be the product of several factors including age, pregnancy, stress, immunosuppressive therapy and concomitant disease [7, 9, 47]. After reactivation, it is supposed that the lumbosacral ganglia play an important role in venereal infection after viral reactivation [8].

CaHV-1 is poorly immunogenic. Neutralizing antibodies are produced 7 to 14 days following primary infection and circulate for approximately 8 months. In cases of viral reactivation, antibody titers increase 7 days post-recrudescence and then wane in a few weeks [29]. This makes serological diagnosis complicated. In large kennels, however, there is a constant presence of circulating virus as a consequence of frequent reactivation and reinfection. As a result, high antibody titers may be detected for more than two years [49]. There are many assays for serological investigation of CaHV-1, such as immunofluorescence, hemagglutination inhibition and ELISA; however, Serum neutralization [33] is a generally accepted technique. This widely adopted assay, known for its excellent specificity [41], represents one of the methods of choice for CaHV-1 serology.

*The aim of this survey was to analyze immunological changes in a dog population in order to*: (1) evaluate possible signs of viral reactivation in bitches during estrus, before mating, and immediately after parturition, and (2) to investigate the effect on antibody titer in breeding males. In addition, possible viral shedding from animals was investigated using a nested PCR assay.

## MATERIALS AND METHODS

*Dogs and samples*: For this survey, a breeding kennel in Central Italy was chosen. The kennel contained 243 dogs (160 breeders) of different sex, age, breed, origin, experiences of mating, number of pregnancies, performance of current and previous pregnancies. Furthermore, the kennel participated in canine exhibitions and competitions. Dogs had never been vaccinated against CaHV-1. Sixty–80 bitches were coupled every year, but for the aim of this work, only females that came into estrus at the same time were included, a total of 27 bitches. Blood samples and vaginal swabs were collected from each of the 27 bitches during estrus, when progesterone values were between 2 and 20 *n*g/m*l*. One week after parturition, 14 of the bitches were re-sampled (blood and vaginal swabs). [Samples from 13 bitches, which did not get pregnant and consequently excluded from this survey, were analyzed as well.] Blood samples were collected from 9 studs prior to mating and then again approximately 4 weeks post-mating. All animals were apparently healthy at physical examination. Blood was collected from the cephalic vein in vacutainer tubes without additives and stored in the refrigerator until centrifugation. Samples were centrifuged at 2,200 rpm for 15 min, and serum was separated from the clot. Serum was stored at −80°C until serological analysis. Vaginal swabs were collected as follows: the lips of the vulva were parted, and then, the swab was gently inserted at a relatively steep angle. When the swab was fully inserted: it was rotated through 2–3 revolutions, allowing the cotton tip to pick up an adequate amount of material. Vaginal swabs were collected in sample medium (Dulbecco Minimal Essential Medium, DMEM). Any aborted fetuses, stillborn pups, umbilical cords and placentas from all the bitches of the breeding kennel were collected and stored at −80°C together with vaginal swabs until tested.

*Serum neutralization*: For antibody analysis, virus strain CaHV-1 ATCC VR-552 was used. SN titration was performed through serial dilutions in Madin-Darby Canine Kidney (MDCK) cells supplemented with 10% fetal calf serum and EMEM (Eagle's minimal essential medium) to obtain an antibody titer. The viral concentration, calculated through the Spearman-Karber method, was $10^{2.5}$ 50% tissue culture infective dose ($TCID_{50}$) in 100 $\mu l$. All sera were analyzed at the same time. Sera were inactivated by heating at 56°C for 30 min. Fifty $\mu l$ of sera, placed in 96-well microplates, were diluted twofold starting at 1:4. Fifty $\mu l$ of virus was added, corresponding to 150 $TCID_{50}$. After incubating at 37°C for 2 hr with 5% $CO_2$, 100 $\mu l$ of suspension containing 15,000 MDCK cells was added to each well. Virus, serum and cell controls were included, and serum cell toxicity was assessed as well. Two sera from known infected dogs with a titer of 1:64 and 1:128 respectively, have been used as positive controls for the test. Plates were incubated at 37°C with 5% $CO_2$. After 3 days, the final reading was carried out through Spearman-Karber method.

*PCR and Nested PCR analysis*: DNA was extracted from vaginal swabs using QIAmp® DNA mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's directions. DNA extraction from lung, liver, and spleen of the stillborn pups and the aborted fetuses, as well as from placentas and umbilical cords was performed using GenElute™ Mammalian Genomic DNA Miniprep Kit (SIGMA, St. Louis, MO, U.S.A.). A PCR assay targeting the CaHV-1 thymidine kinase (TK) gene and a nested PCR were performed [43]. Two primer pairs were used (Table 2). Four hundred and ninety three bp and 168 bp amplicons were obtained with the first and the second primer pair, respectively. The first PCR reaction-mix consisted of 150 *n*g of template DNA, 2.5 $\mu l$ of buffer 10X, 2.5 $\mu l$ of bovine serum albumin (0.1 mg/m*l*), 3 $\mu l$ of $MgCl_2$ 25 mM, 0.5 $\mu l$ of dNTPs 20 mM, 1 $\mu l$ of CaHV-1 primer 1 10 pmol/$\mu l$, 1 $\mu l$ of CaHV-1 primer 2 10 pmol/$\mu l$ and 0.1 $\mu l$ of 5 U/$\mu l$ Taq DNA polymerase (Microtech). Water was added to the mixture in order to obtain a final volume of 25$\mu l$. The PCR amplification was carried out after initial DNA denaturation at 94°C for 5 min, then 35 cycles of 94°C for 30 sec, an annealing step for 30 sec at 55°C, the elongation at 72°C for 45 sec and a final elongation at the same temperature for 5 min. Nested PCR was performed with the same reaction mixture, using 1 $\mu l$ DNA of the previous PCR product. The second PCR reaction-mix consisted of 150 *n*g of template DNA, 2.5 $\mu l$ of buffer 10X, 2.5 $\mu l$ of bovine serum albumin (0.1 mg/m*l*), 3 $\mu l$ of $MgCl_2$ 25 mM, 0.5 $\mu l$ of dNTPs 20 mM, 1 $\mu l$ of CaHV-1 primer 3 10 pmol/$\mu l$, 1 $\mu l$ of CaHV-1 primer 4 10 pmol/$\mu l$ and 0.1 $\mu l$ of 5 U/$\mu l$ Taq DNA polymerase (Microtech). Water was added to the mixture in order to obtain a final volume of 25 $\mu l$. The PCR cycling protocol was carried out as previously reported, using an annealing temperature of 56°C instead of 55°C. In

Table 1. Dogs belonging to the study group and risk factors predisposing to CaHV-1 infection

| Subject No. | Breed | F/M[a)] | Age (years) | Origin | Number of pregnancies | Reproductive disorders | Participation in canine exhibitions and competitions | Experiences of mating outside the kennel |
|---|---|---|---|---|---|---|---|---|
| 1 | Poodle | F | Six | Bought adult | Multiparous | None | No | No |
| 2 | Yorkshire Terrier | F | Four | Bought at 1 year | Multiparous | None | No | Yes |
| 3 | Dachshund | F | Two and a half | Born in kennel | Multiparous | None | Yes | No |
| 4 | Poodle | F | One and a half | Born in kennel | Primiparous | Abortion | Yes | No |
| 5 | German Spitz | F | One and a half | Born in kennel | Primiparous | None | No | No |
| 6 | German Spitz | F | One and a half | Born in kennel | Primiparous | Stillbirth | Yes | No |
| 7 | German Spitz | F | One and a half | Bought puppy | Primiparous | Stillbirth | No | No |
| 8 | Poodle | F | Two and a half | Bought puppy | Multiparous | Premature parturition | No | No |
| 9 | Poodle | F | Eight | Born in kennel | Multiparous | None | No | No |
| 10 | Poodle | F | Two | Bought puppy | Multiparous | None | No | No |
| 11 | Pug | F | Four | Bought puppy | Multiparous | Stillbirth | No | No |
| 12 | Poodle | F | Five | Bought puppy | Multiparous | None | No | No |
| 13 | German Spiz | F | Two | Born in kennel | Multiparous | Pseudopregnancy | No | No |
| 14 | Poodle | F | Four | Bought puppy | Multiparous | NN | No | No |
| 15 | Yorkshire Terrier | M | Five | Bought puppy | | None | No | Yes |
| 16 | Dachshund | M | Two | Bought puppy | | None | Yes | Yes |
| 17 | Poodle | M | Four | Bought puppy | | None | Yes | No |
| 18 | Poodle | M | Two | Bought puppy | | None | Yes | Yes |
| 19 | German Spitz | M | Four | Bought adult | | None | No | No |
| 20 | German Spitz | M | Four | Bought puppy | | None | No | Yes |
| 21 | Poodle | M | Six | Bought adult | | None | No | No |
| 22 | Poodle | M | Five | Bought puppy | | None | No | No |
| 23 | Poodle | M | Two | Bought puppy | | None | No | No |

a) F: Female, M: Male.

Table 2. Primer orientation, length and nucleotide sequence

| Primer Pairs | Designation of Primers | Orientation | Length | Nucleotide Sequence |
|---|---|---|---|---|
| 1 | CaHV-1 primer 1 | sense | 19 mer | TGC CGC TTT TAT ATA GAT G |
| | CaHV-1 primer 2 | antisense | 20 mer | AAG CGT TGT AAA GTT CGT |
| 2 | CaHV-1 primer 3 | sense | 19 mer | CGT GGT GAA TTA AGC CTA A |
| | CaHV-1 primer 4 | antisense | 19 mer | ATG CTA TTG GGG TGT CTA TC |

each PCR, both positive (CaHV-1 ATCC VR 552 strain) and negative (double-distilled water) controls were included.

## RESULTS

None of the dogs under examination showed any clinical sign related to CaHV-1 infection during the study period, and none of the dogs demonstrated the presence of antibodies for CaHV-1 by SN assay. Subject N°4 had an abortion, and N° 6, 7 and 11 gave birth to stillborn pups (Table 1). The aborted fetuses, umbilical cords, placentas and vaginal swabs (collected during estrus and after parturition), as well as stillborn pups all tested negative by nested PCR assay, while the virus template, used as a positive control, was successfully amplified.

## DISCUSSION

In this survey, 59 serum specimens were tested using an SN assay, including acute and convalescent samples. None of the dogs had primary antibody titers to CaHV-1 by SN, including bitches that had abortions and stillbirths. Nor did any dog seroconvert, suggesting that these animals are not generating an immune response to CaHV-1 either because the virus is not being reactivated during breeding or these dogs are not being exposed. Even though it is known that antibody titers against CaHV-1 considerably decrease quickly after infection, which could explain why none of the dogs tested positive, it has been reported that antibodies in seropositive animals from kennels may be detected for up to 15 months [25]. Latent infection by CaHV-1 has been detected in the lumbosacral ganglion [8] and in vaginal swabs after the immunosuppressive state caused by an administration of prednisolone [32] and subsequent stress conditions, but not as frequently detected as in samples from the upper respiratory tract [31]. Because it has been hypothesized that the carrier mother spreads the virus to her young because of viral reactivation due to the immunosuppressive state caused during pregnancy and parturition [27, 32], the authors decided to take blood samples and vaginal swabs at two criti-

cal points in order to find any evidence of viral reactivation due to stress: during estrus before mating and immediately after parturition. Since the survey was performed as a cross-sectional virological study of bitches simultaneously in heat, the dogs were influenced by the same conditions at the time of the survey making the study group homogeneous. Animals were previously allowed to travel outside of the kennel and do various normal activities−such as contests or breeding−which could have exposed them to other infected dogs, increasing the possibility of circulation of CaHV-1 among the 160 breeders in the kennel. Because this was not a closed population of animals, it was anticipated that at least some percent of animals would be exposed and would be seropositive or PCR positive. Surprisingly, this was not the case.

The present research did not show any association between antibody titers and risk factors, such as age, sex, breed, experiences of mating, number of pregnancies, participation in dog shows and origin of the animals. This could be due to a limited number of animals tested during the study period. Although the dogs in the kennel were toy breeds, there is no published scientific evidence of breed disposition for CaHV-1 infection. However, the small number of animals evaluated in this survey limits the analysis of CaHV-1 infections among dogs as it pertains to breed. Our results partially contrast with a survey in Belgium [39] regarding the correlation between risk factors and reproductive disorders. In this survey, Ronsse and colleagues demonstrate supportive evidence for the influence on antibody titers due to several factors including aging, estrous stage, number of pregnancies, experience with mating, cases of abortion, management and environmental hygiene. Similar to a Swedish study published in 2012 [46], our survey had no change in antibody titer in correlation with mating, pregnancy and parturition.

None of the vaginal swabs tested positive via nested PCR analysis. Presumably, there was a low probability of detecting the virus, because there were no vaginal lesions related to CaHV-1 infection on the breeding bitches at the time of sampling [35]. Stillborn pups, umbilical cords, placentas and aborted fetuses from bitches excluded from the present survey were also analyzed and tested PCR-negative for CaHV-1. Negative results of the nested PCR—in association with the outcome of the serological analysis—raise doubts about the circulation of the virus, despite the large size of the breeding kennel examined.

The PCR negative results could be due to the short duration of virus spread and the intermittent elimination of the virus from the animals. Moreover, the probability of detecting the virus was likely very low, because none of the dogs showed vaginal lesions consistent with CaHV-1 [35]. Failure to detect CaHV-1 reactivation in the survey could be due to short, self-limiting reactivations that occurred between sampling and examination periods. Ledbetter and collaborators [28] maintain that viral reactivation varies amongst different species, and recurrent herpetic ocular disease in dogs is less frequent compared to that of cats and humans. Indeed, after human herpesvirus-2 (HSV2) reactivation, asymptomatic patients sometimes exhibit viral shedding for only one day [50]. Similarly, the negative results observed in our survey might be a consequence of the brief viral shedding documented for CaHV-1 under our field conditions, despite the endemic prevalence of the virus in Italy.

Both PCR and SN negative results allow the authors to assume that the virus was not circulating within the breeding kennel studied despite the presence of several risk factors predisposing to CaHV-1 infection, as mentioned above. On the other hand, we can assume that the absence of neutralizing antibodies could be associated with the rapid decline of these following exposure to CaHV-1, because the virus is known to be poorly immunogenic and viral DNA detection via PCR assay may not be possible because of the very short viral shedding period of this pathogen.

Although it is difficult to extrapolate to the general Italian dog population from a survey based on an examination of 23 dogs in a breeding kennel, these findings indicate that CaHV-1 may not occur as widely in the Italian dog population. Albeit too few have been investigated in Italy since only two works are present in literature, Sagazio and colleagues found a lower seroprevalence in Apulia region (11.4%) than other European Countries [42]. Another epidemiological Italian study in 2014 showed a seroprevalence of 14.6% in Southern Italy and, in particular, 12.5% in breeding kenneled dogs [35]. However, it is hard to compare our survey with the other studies mentioned above due to their different experimental scheme. They differ for total sample numerosity, sample homogeneity and samples modality, since in both works, dogs were chosen randomly. So, it can only be said that CaHV-1 is not circulating within this specific kennel. It is generally accepted that CaHV-1 is a major cause of reproductive disorders and stillbirths in dog kennels, and vaccination for CaHV-1 is common practice for disease prevention. However, our results, along with other seroprevalence studies, suggest that further investigation is warranted to better understand the distribution of CaHV-1. Existing studies are limited to geographical regions within Italy, and they are not representative of Italy in its entirety. Additionally, thorough diagnostic work-ups on abortion cases would provide insight as to the major causes of abortion in dog kennels, thereby allowing for future, targeted, preventative medicine.

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