A Nosocomial Outbreak of Serious Canine Infectious Tracheobronchitis (Kennel Cough) caused by Canine Herpesvirus Infection

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Abstract. Canine herpesvirus (*canid herpesvirus 1*) (CHV) is principally a perinatal pathogen of pregnant bitches and newborn pups, and secondarily a respiratory tract pathogen of older pups and dogs. Infectious disease of the canine respiratory tract frequently occurs among dogs in groups, in which it is called “infectious tracheobronchitis (ITB)”. Mortality from ITB is generally negligible and the clinical importance of CHV as an ITB pathogen is considered to be low. In the present study a novel ITB outbreak accompanied by deaths among aged dogs in an animal medical center is described. Most inpatient dogs had received medications that could induce immunosuppression. CHV was the only pathogen identified and several CHV isolates were made in cell culture. No other viral pathogens or significant bacterial pathogens were found. Molecular and serological analyses revealed that the causative isolates of CHV were from a single source but none was a peculiar strain when compared with previous CHV strains. Presumably the virus had spread among the predisposed dogs in the center. The present result serves as a warning to canine clinics that, under the specific set of circumstances, such serious CHV outbreaks may be expected wherever canine ITB occurs.
Introduction.

Canine herpesvirus (CHV) (canid herpesvirus 1) is classified in the Varicellovirus genus of the Alphaherpesvirinae subfamily of the Herpesviridae. CHV was first described in 1965 as a pathogen responsible for a fatal generalized hemorrhagic disease of newborn pups (5). The host range of CHV is generally restricted to domestic and wild Canidae (10), and a worldwide distribution of CHV infection in domestic dog populations was shown by virus-isolation (2, 9, 11, 24, 27, 28) and sero-epidemiological (6, 16, 25, 29, 31, 32) studies. It is now well recognized that the pathogenic potential of CHV is influenced mostly by the age of the host (10). Pups infected in the postnatal period show the typical fatal hemorrhagic syndrome. Older pups manifest less severe clinical syndromes upon infection and the respiratory disease called “infectious tracheobronchitis” (ITB) or “kennel cough” may be the most frequent clinical disorder in the field. Ocular disorders such as conjunctivitis and keratitis, either with or without ulceration, were also observed in young pups (17). Although the pathogenic potential of CHV for older dogs is apparently low and adult dogs often do not show any clinical signs following CHV infection, CHV may become an important perinatal pathogen for pregnant bitches, causing papulovesicular genital lesions and reproductive disorders such as embryonic resorption, abortion and stillbirth (10).

CHV is spread mainly by oronasal and venereal transmission of viruses in the respiratory and genital secretions of acutely infected dogs, and fetuses are infected in utero (12, 13). Following the initial productive infection, in most cases CHV is not fully cleared by acquired immunity so that latency becomes established in several
tissues including the sensory ganglia (4, 19) and may persist for life. Latent viruses are sometimes reactivated by factors that alter immunity such as stress, immunosuppressive therapy, or pregnancy, with subsequent excretion of virus.

The significance of CHV in the etiology of canine ITB has been considered to be rather low compared with that of other agents such as canine parainfluenza virus (CPIV), canine adenovirus (CAV) type 2, or Bordetella bronchiseptica (B.b.) (8).

Experimental infection of older pups or adult dogs with CHV isolates often resulted in either no clinical signs or mild upper respiratory symptoms (1, 14, 24, 33). Consequently CHV has not been generally regarded as a primary cause of canine ITB (8, 10). On the other hand, CHV has been repeatedly detected in dogs with ITB (3, 15, 24, 36) and a longitudinal study on respiratory diseases of dogs housed in a rehoming kennel in the United Kingdom (7) indicated that CHV should be re-evaluated as a significant agent responsible for ITB.

This report is an account of a novel outbreak of canine ITB which was accompanied by deaths among dogs hospitalized in an animal referral medical center near Tokyo. It was concluded that CHV alone was responsible for the nosocomial outbreak. All of the dogs had been vaccinated fully before entering the hospital, and during hospitalization most had received steroid medication, surgical operation or radiation therapy. It was considered that such medical treatments, in addition to hospitalization itself, might induce stress in the patients leading to a condition of low immune resistance, and cause recrudescence of latent CHV. Canine herpesvirus might then have spread contagiously among the dogs in the same clinic. The present result
cautions that CHV previously thought to be an agent of low pathogenicity for adult dogs should not be overlooked in specific circumstances.

MATERIALS AND METHODS

Clinical specimens for virus detection. Swabs from two populations of dogs were investigated. The first population comprised dogs which were hospitalized in a referral animal medical center. In retrospect it was considered that an ITB outbreak started among dogs in the center around 20 May 2008 and was over after six weeks, following the application of quarantine measures. At least 15 dogs showed typical signs of respiratory disease and five dogs died during the period. After the first two dogs died on 9 June, nasal and pharyngeal swab specimens from 10 dogs were collected immediately (Table 1) and submitted for examination for viruses and bacteria. Serum samples were taken from 5 dogs (71995, 71802, 71927, 71982, and 71579) in the middle of the epidemic (11 and 13 June). An external commercial laboratory conducted bacterial examination of the swabs: no significant respiratory pathogens such as mycoplasma spp. or Bordetella spp. were detected. A further three dogs, (71917, 71947, and 71927 in Table 1) died on 13, 18 and 23 June, respectively. Necropsy of the dogs was not performed.

The other samples were swab specimens cryopreserved after primary examination in our laboratory and comprised a total of 176 respiratory clinical specimens (81 oropharynx, 63 nasal cavity, 28 conjunctiva, and 4 others) from 137 dogs brought to
animal hospitals in various areas of Japan between 1998 and 2008. These samples had been tested for CAV, CPIV, canine coronavirus (CCoV), canine respiratory coronavirus (CRCoV), canine distemper virus (CDV), minute virus of canines (MVC) and influenza A virus (IAV). Some of the results of this examination were published previously (21, 23, 38). However, detection of CHV had not been attempted in these samples, mainly because of the classical impression that CHV was not important in canine IBT (10).

**Reference CHV strains and cells.** Canine herpesvirus strains GCH-1 (11) and D004 (ATCC no. VR-552) (3) were used as reference CHV in PCR, restriction length polymorphism (RFLP) and serum-neutralization. Madin-Darby canine kidney (MDCK) (20), A-72 (ATCC no. CRL-1542), primary dog kidney (DK), and *Felis catus* whole fetus-4 (fcwf-4) (ATCC no. CRL-2787) (26) cells were used either for culturing CHV or virus detection from clinical specimens. The cells were grown with Dulbecco’s modified Eagle’s medium (Eagle’s MEM) supplemented with 10% fetal calf serum (FCS) and antibiotics.

**Detection of viral agent from clinical specimen.** Swabs were placed in 2 ml of Eagle’s MEM and extracts were clarified by centrifugation at 15,000 rpm for 20 min. The resulting supernatant was examined for viruses. For general virus isolation, the supernatant was inoculated into both MDCK and fcwf-4 cell cultures at 37°C. The cultures were examined for cytopathic effect (CPE) and were blind-passaged a further two times when no CPE was apparent. The following viruses were specially examined
molecularly by methods described previously (21, 23, 38); CDV, CPIV, CCoV, CRCoV, MVC, and IAV.

CHV detection was specifically performed by both virus isolation with either MDCK or primary BK cells and two kinds of PCR: one was the PCR designed for CHV (19) (CHV PCR) and another was for diverse herpesvirus species by consensus primers (34) (consensus primer PCR). They were performed precisely following the methods originally described; the CHV D004 strain was used as a positive reference.

Molecular characterization of CHV isolates. For viral DNA extraction, A-72 cells were infected with CHV at low multiplicity of infection. When CPE was prominent, the cells were collected and treated with a 0.1 M Tris-HCl (pH 9.0) solution containing 0.1M NaCl₂, 5 mM EDTA, 1% sodium dodecyl sulfate, and 0.1 mg/ml of proteinase K at 37°C overnight. DNA was extracted with a mixture of phenol and chloroform-isoamylalcohol (25:24:1), precipitated with ethanol, and then dissolved in water.

To examine whether the CHV isolates originated from either single or multiple sources, viral DNA was digested with restriction endonucleases HindIII and XbaI for RFLP analysis (37). Digested fragments were separated by electrophoresis on a 0.7% agarose gel for 18.5 hrs at 20V. The gels were stained with ethidium bromide.

For nucleotide sequencing, viral DNA (a portion of DNA polymerase gene) was amplified from the isolate 08/005 by the PCR using herpesviral consensus primers TGV (5’-TGTAACCTCGGTGTAYGGNTTYACNGGNGT-3’) and IYG
(5’-CACAGAGTCCGTRTCNCCRTADAT-3’) described previously (34). The PCR product was purified with QIAquick PCR purification kit (QIAGEN, Germany) and sequenced with primers TGV and IYG using BigDye® terminator v3.1 cycle sequencing kit (Applied Biosystems Inc., CA).

Serology. Blood samples collected from both the inpatient dogs and experimentally infected dogs were titrated for CHV neutralization (NT) antibodies. Neutralization was performed with complement supplementation and the titer was determined by a plaque-reduction method. Twofold serial dilutions of heat-inactivated sera were made in Eagle’s MEM and transferred to 96-well plates at 60 µl per well. Approximately 100 PFU of CHV strain D004 in 30 µl and complement (15% concentration of fresh guinea-pig serum in Eagle’s MEM) in 30 µl were added to each well and the plates were incubated at 37°C for 1 hr in 5% CO₂. One hundred µl of the mixture was then inoculated onto a MDCK cell sheet formed in 24-well plates and left for adsorption at 37°C for 1 hr in the chamber containing 5% CO₂. After unabsorbed viruses were washed out, all wells received 1 ml of Eagle’s MEM containing 2% FCS and 1% agar and the plates were incubated at 37°C for 4 to 5 days. The plaques were visualized by staining with 0.005% neutral red. The antibody titer was defined as the reciprocal of the highest dilution of serum that reduced the number of plaques to less than half of the virus control without serum.

Field serum samples. The serum samples that had been cryopreserved in our
laboratory were screened for NT antibodies against CHV with complement supplementation. The samples had been collected from dogs presenting at veterinary teaching hospitals located in Yamaguchi (western Japan; 48 dogs), Tokyo (eastern Japan; 60 dogs), and Sapporo (northern Japan; 30 dogs) during the period 2000 to 2001 for a canine calicivirus study (22). One hundred and thirty-eight samples were available for the present examination. The reason for visiting the hospital was not limited to respiratory disease.

Experimental infection of SPF dogs with the CHV isolate. Four 26-week-old SPF Beagles (dog nos. 2, 8, 12, and 16) were used for experimental infection. The dogs did not possess anti-CHV NT antibody before challenge. Isolate 08/005, passaged twice in MDCK and once in A-72 cell cultures, was used as inoculum. Each dog received 3 ml of culture fluid containing $10^5$ TCID$_{50}$ of infective virus, as 1 ml orally and 2 ml intranasally. The clinical condition of each dog was observed for 2 weeks after inoculation. Oral swab samples were taken daily to detect virus excretion by the CHV PCR and blood samples were obtained every other day for the first week and additionally on the tenth and fourteenth days.

The virulence of CHV strain GCH-1 was also examined for comparison. The strain was isolated from the typical fatal hemorrhagic syndrome of newborn pups in 1975 (11) and had been passaged 31 times in DK cell culture. Two 28-week-old seronegative SPF Beagles (dog nos. 13 and 14) were challenged and observed in the same conditions as those for isolate 08/005.
RESULTS

Viruses detected from the hospitalized dogs with ITB. No CAV, CPIV, CCoV, CRCoV, CDV, MVC, or IAV was detected in the respiratory specimens, except for specimen 08/026 in which a CPIV RNA fragment was detected by the RT-PCR. In contrast, as shown in Table 1, CHV was isolated from 4 nasal specimens 08/005, 08/011, 08/015 and 08/021 in MDCK cell culture. The viruses showed typical herpesvirus-like CPE in the primary culturing at 37°C as described previously (11). Since this result was unexpected, all specimens were then screened by the CHV PCR. Ten dogs, including the four dogs that harbored infectious CHV in the nasal cavity, were found to be positive for the CHV DNA fragment in either nasal, oropharyngeal or both specimens (data not shown).

Antibodies in the serum samples taken from inpatient dogs in the middle of epidemic. Serum samples from 5 dogs were examined for NT antibodies against CHV strain D004 (Table 2). No NT activity was detected in 3 samples (dogs 71995, 71927, and 71982); however, NT titers of 64 and 2,048 were found in the two others (71802 and 71579, respectively). Although all dogs possessed antibody titers sufficient to prevent infections of CPIV, CAV-2, and CPV-2, anti-CDV titers of some dogs were comparatively low (Table 2).
Cross neutralization test between reference CHV D004 strain and the CHV 08/005 isolate. In the test, anti-CHV D004 rabbit hyper-immunized serum and CHV 08/005-infected dog serum taken at 4 weeks post-challenge were used. Both isolate 08/005 and the D004 strain were neutralized by the homologous and heterologous serum samples with no significant difference in NT titer (data not shown), indicating that there was no antigenic difference between the viruses.

Sequencing and RFLP analysis of CHV isolates. Nucleotide sequence of the PCR product obtained from isolate 08/005 was compared with those of herpesviruses deposited in the databases. The product was 234 bp long and was a fragment of the DNA polymerase gene. Perfect identity in 185 bp, excepting both end primer sequences, to that of CHV reported previously (GenBank accession no. EU531507) was obtained (data not shown), indicating that the PCR product was of CHV origin.

The RFLP of viral DNA obtained from the isolates was compared with those of the reference CHV strains D004 and GCH-1 (Fig. 1). The migration pattern of the digested fragments were almost identical among all 4 isolates (08/005, 08/011, 08/015 and 08/021), although some subtle differences were observed between the present isolates and the reference strains. In the HindIII-digested pattern, several differences were detected: a gain of 4.4 kbp and loss of 3.1 kbp fragments were clear for the present 4 isolates. In XbaI-digested pattern, it was clear that the present 4 isolates showed a gain of a 10 kbp fragment instead of a loss of an 11.4 kbp fragment.
Virulence of the CHV isolate to SPF dogs. In the experimental dogs infected with isolate 08/005 the prominent clinical signs were pyrexia and serous nasal discharge. Dog no. 12 had a high fever (39.9°C - 40.1°C) from the second day after challenge, which continued for 5 days, and a serous nasal discharge for 5 days from the fifth day. The other 3 dogs, nos. 2, 8, and 16, also showed similar clinical signs although these were less serious and of shorter duration than those of dog no. 12. A slight lachrimation was observed in dog no. 16. Virus excretion was detected in the nasal cavity of all dogs for 5 days between the third and seventh days after challenge.

Serum NT titers of the samples taken 2 weeks after challenge were elevated to 32 ~ 1,024.

Of the dogs infected with strain GCH-1, dog no. 13 had a fever of 39.4°C on the fourth day and had a serous nasal discharge for 2 days from the fifth day. Virus excretion was detected in the nasal cavity on the fifth day. Dog no. 14 had a fever of 39.5°C on the third day and a serous nasal discharge for 5 days from the third day. The feces of this dog changed from loose to diarrheic during the same period. Virus excretion was detected in the nasal cavity for 3 days between the third and fifth days.

Serum NT titers of the samples taken 2 week after challenge were elevated to 1,024 ~ 2,048 in both dogs.

CHV detection from the cryopreserved clinical specimens. Herpesvirus DNA was detected in the oropharyngeal swabs of two dogs by the CHV PCR, with a positive rate of 1.46%. However, the specimens were negative by the consensus primer PCR.
No CAV, CPIV, CDV or CRCoV was detected in the same specimens. The samples were from 2-3 month-old pups that had not received canine combined vaccine, and their principal clinical signs were coughing, nasal discharge, and dyspnea. Slight conjunctivitis was observed in one pup. Attempted isolation of CHV from the CHV DNA-positive specimens using primary DK cell culture at 34°C did not succeed.

**Survey for CHV NT antibodies of the field serum samples.** In all, 21.7% of dogs had NT antibodies to CHV with a mean positive antibody titer of 19.1. However, a regional variation was noted. There was higher seroprevalence among dogs in the Tokyo area: the antibody-positive rate was 40% and mean positive antibody titer was 29.6. In contrast, seroprevalence rates of Yamaguchi and Sapporo areas were 10.4% and 3.3%, respectively, and the antibody titer of seropositive dog in these areas was 10 for all dogs.

**DISCUSSION**

When dogs are housed in a group into which newcomers are constantly introduced, canine ITB is typically caused by a complex of pathogens (8). CHV has been regarded as a secondary agent in ITB because of infrequent association with the syndrome. Actually, in the present study, the detection rate of CHV from the dogs with respiratory disease in the field was low (1.46%) compared with those for CPIV (7.4%) and B.b. (10.3%), revealed by our recent study in which the same clinical
specimens had been examined (23). The detection rate of CHV was similar to those for CRCoV and CDV (1.5%), suggesting that CHV is of secondary significance as a pathogen, as previously regarded. However, from a recent epidemiological study in a rehoming center in England, Erles et al. (7) proposed a different scenario for the pathogenic role of CHV in canine ITB. They proposed that: 1) CHV latently harbored in some dog(s) can become reactivated during the course of ITB due to stress by other viral and bacterial infections; hence, 2) CHV infection is associated with ITB in which more severe respiratory signs are present; therefore, 3) CHV may have an exacerbating effect on other viral or bacterial infections. There are both common and different epidemiological features in the ITB outbreaks involving CHV between the present animal medical center and the rehoming center described previously (7). The present study introduced a new concept about the role of CHV in canine ITB and served as a warning to facilities where dogs are kept in a group and are subject to stress.

Most inpatient dogs of the animal medical center were aged and had been vaccinated with combined core vaccines containing primary ITB agents such as CPIV and CAV-2 (Table 1). The dogs were very likely to be immune to infections with such canine pathogens since they had high antibody titers against each virus (Table 2). Indeed, excepting one dog (71947 in Table 1) in which CPIV was detected, neither primary viral nor bacterial ITB pathogens were associated with the clinical syndrome. In the end, it was unexpectedly found that almost all cases were solely attributable to CHV. Unfortunately some dogs died during the epidemic but no conclusive pathological reasons for the outcome were obtained since no postmortem examination
was performed in accordance with dog owners' wishes. Although most inpatient dogs had received some medication before as well as during the CHV epidemic, it was considered very unlikely that such medical treatments were responsible for the fatalities.

To elucidate the reasons of the ITB outbreak in the animal medical center, the following points should be considered: first, the origin and virulence of the causative CHV, and secondly, the factor(s) which made the situation very serious.

Previous studies showing very low antigenic and genomic divergence of global CHV isolates indicates that CHV is a monotypic virus (7, 30). In the present study, no significant antigenic difference between the reference CHV strain and the present CHV isolate was observed by the cross neutralization test. Sequence analysis of the DNA polymerase gene of isolate 08/005 indicated 100% identity of the sequenced region with those of the reference CHV strain. Although these results were indeed useful for antigenic and molecular identification of the isolate as CHV, they were not helpful for discriminating between isolates. Xuan et al. (37) described the value of RFLP analysis of genomic DNA to differentiate between CHV isolates by using endonucleases HindIII, XbaI and PvuII. They differentiated unrelated individual CHV isolates from each other. In contrast, CHV isolates from the same origin showed almost identical restriction cleavage pattern. Indeed, the present RFLP results (Fig. 1) were sufficiently clear to show that the present four CHV isolates are of the same origin because they showed the same migration pattern. Therefore, it was hypothesized that the ITB outbreak in the animal medical center was caused by a CHV strain of a single origin.

Although the analysis of anti-CHV NT antibodies was performed for a limited
number of the inpatient dogs (Table 2), it provides a hint about the suspect dog that
introduced CHV into the center. The necessary conditions to identify the dog are to
have been hospitalized before the epidemic as well as to have anti-CHV antibodies, and
therefore to have been infected with CHV. For example, dog no.71579 met this
requirement. However the evidence may not be enough to definitely identify the
suspect dog since we could not examine all dogs hospitalized before the outbreak.

The virulence of the present CHV isolates remains unresolved. Some previous
animal experiments showed that CHV induced mild upper respiratory disease in pups
aged less than 12-week-old (1, 14, 34) while other CHV isolates were avirulent in
young pups (24, 35). However, it was confirmed experimentally that the present
isolate 08/005 possesses some pathogenic potential for the respiratory tract of
adolescent dogs. In addition, the reference CHV strain GCH-1 that was isolated from
the fatal hemorrhagic syndrome of pups 30 years ago in Japan, also showed similar but
rather less virulence to the respiratory as well as alimentary tracts of the dogs. These
results lend support to the recent proposal of Erles et al. (7) that CHV is a more
significant pathogen than previously believed especially when evaluating canine ITB
occurring among dogs stressed in a group. Further experiments will be required to
clarify the pathogenic diversities of different CHV strains, especially for
immunosuppressed dogs.

A possible source of infection for the present ITB outbreak is a latently infected
dog that was introduced to the animal hospital some time before the outbreak, as
discussed above. The serological survey conducted in the present study indicated the
dogs in Tokyo area, adjoining the hospital, possessed a higher prevalence of anti-CHV antibodies than the dogs raised in the suburban areas of Yamaguchi or Sapporo.

Almost half of the dogs had NT antibody against CHV, indicating that they harbored latent CHV (4, 19). Following treatment with agents that induce stress, recrudescence of latently infected CHV occurs within a week (10). According to the recent study of adult dogs latently infected with CHV (18), the virus was activated and detected on the tenth day after the systemic administration of prednisolone (3.0 mg/kg/day for 7 consecutive days from the first day). This dose and duration of the treatment is within the range of standard usage in small animal clinics, and most dogs in the animal medical center described here also received similar dose of prednisolone (0.25~5mg/kg/day for 5~29 consecutive days; Table 1). Even without prednisolone treatment the dogs might become immunosuppressed owing to the stress caused by hospitalization itself, surgery, chemotherapy or radiation as listed in Table 1.

The present study demonstrated that CHV was a causative agent for a serious outbreak of canine ITB that occurred in a large animal medical center. Although the virulence of the CHV isolated from cases of disease seemed to be slightly higher than those of the previous CHV strains, the most significant reason for the spread of infection in the center may be immunosuppressive status of the susceptible inpatient dogs. It is logical to assume that the number of immunosuppressed dogs was high enough to perpetuate transmission after the initial introduction of CHV. This situation might develop anywhere canine ITB occurs. Although herpesvirus vaccines are generally not sufficient to prevent infection, vaccination might be one of the choices
applied to animals in such special circumstances. An inactivated vaccine is available in Europe to protect newborn pups from systemic infection. Vaccination of aged dogs may have some effect in reducing virus excretion from infected individuals, consequently controlling the spread of infection in a group.

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FIG. 1. RFLP of viral DNA of the present CHV isolates and reference CHV strains.

DNA extracted from virus-infected cells was digested with endonucleases HindIII (lanes 1 to 6) and XbaI (lanes 7 to 12). Lanes: 1 and 7, reference D004 strain; 2 and 8, reference GCH-1 strain; 3 and 9, 08/005 isolate; 4 and 10, 08/011 isolate; 5 and 11, 08/015 isolate; 6 and 12, 08/021 isolate; M, lambda DNA digested with HindIII as size markers. Arrows indicate the different fragments clearly detected between the present isolates and the reference CHV strains.
<table>
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<th>Dog</th>
<th>Breed*</th>
<th>Sex</th>
<th>Age</th>
<th>Date of Checkup</th>
<th>Clinical Signs</th>
<th>Vaccines*</th>
<th>CSF Status</th>
<th>CSF Virology</th>
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<td>80G52 (%)</td>
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<td>+</td>
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<td>+</td>
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<td>25 yr</td>
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<td>Coughing, sneezing</td>
<td>Yes</td>
<td>Yes</td>
<td>80G52 (5%)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Abbreviations: M/C, mixed; B/S, brachycephalic; T/S, terminal; H/S, heritable; S/S, seasonal.
*Clinical signs at presentation.
*Vaccines: C, canine; H, human; P, PPR; M, modified canary PPR; N/P, no PPR.
*CSF status: 80G52, positive for 80G52.
*Comment: Not specified.
<table>
<thead>
<tr>
<th>Dog</th>
<th>Hospitalization record</th>
<th>Antibody titer* against</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CHV</td>
</tr>
<tr>
<td>71927</td>
<td>May 22nd June 23rd*</td>
<td>&lt;2</td>
</tr>
<tr>
<td>71982</td>
<td>June 9th June 15th</td>
<td>64</td>
</tr>
<tr>
<td>71982</td>
<td>May 31st June 14th</td>
<td>&lt;2</td>
</tr>
<tr>
<td>71995</td>
<td>June 2nd June 19th</td>
<td>&lt;2</td>
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<tr>
<td>71579</td>
<td>May 16th June 14th</td>
<td>2,048</td>
</tr>
</tbody>
</table>

* Serum was taken on June 11th or 13th.

+ Neutralization titer for canine herpesvirus (CHV), canine parainfluenza virus (CPIV), canine adenovirus type 2 (CAV-2) and canine distemper virus (CDV), and hemagglutination-inhibition titer for canine parvovirus type 2 (CPV-2).

+ Left the animal medical center because of death.