Serological Analysis of Canine Distemper Virus Using an Immunocapture ELISA

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ABSTRACT. As a rapid and sensitive method to detect canine distemper virus (CDV), an immunocapture enzyme-linked immunosorbent assay (ELISA) was performed. The sensitivity and specificity of the immunocapture ELISA were considered to be high enough. Virus neutralizing (VN) test was also established using the immunocapture ELISA. By using this test, the different cross VN titers between sera of dogs experimentally infected with the Onderstepoort strain and those with a field isolate of CDV were observed. — KEY WORDS: canine distemper virus, immunocapture ELISA, virus neutralizing test.


Canine distemper (CD) is a pantropic viral disease of dogs and of their close relatives. It manifests itself as an acute contagious disease with clinical signs of respiratory, gastrointestinal and/or central nervous system (CNS) [1, 2, 11]. Canine distemper virus (CDV) is the causal agent of CD. For the diagnosis and clarifying the prevalence of CD, isolation and characterization of CDV are essential. However, it is difficult to isolate the virus from affected dogs using cell cultures [11]. In addition, Kai et al. reported the presence of CDV that does not show a characteristic cytopathic effect (CPE) of fusion formation [8]. Thus, alternative rapid and sensitive method for detection of CDV is still required. The immunocapture enzyme-linked immunosorbent assay (ELISA) is known as a sensitive and rapid method that has been used for detecting various virus antigens [12, 15, 18]. In the present study, the immunocapture ELISA was employed for detection and characterization of CDV.

Two different field isolates (Hamamatsu and Yanaka isolate) of CDV were obtained from affected dogs using B95a cells because the cells were highly susceptible to morbillivirus including CDV [8–10]. The Onderstepoort strain of CDV, the Edmonston strain of measles virus (MV), the DL1 strain of canine parainfluenza virus (CPIV), and the Cp49 strain of canine parvovirus (CPV) were also used in the present experiments for testing specificity of the immunocapture ELISA. Both MV and CPIV were maintained and titrated in Vero cells. CDV was propagated and titrated in B95a cells. CPV was grown in Crael feline kidney (CRFK) cells.

The titration of CDV, MV and CPIV were performed using a 96-well microplate. Fifty μl of a cell suspension of B95a cells infected with CDV or Vero cells infected with MV or CPIV was added to each well and cultured for 24 hr. After the supernatant was removed, 50 μl of 10-fold dilution of samples to be titered was added to each well. After incubation for 7 days at 37°C, the cultures were examined under a light microscope, and the titer was expressed as 50% tissue culture infectious dose (TCID50)/50 μl. CPV was titrated by hemagglutination (HA) test as described previously using both borate-buffered saline and phosphate-NaCl buffer with porcine erythrocytes [16]. The HA titer was expressed as the reciprocal of the highest dilution showing complete HA. A hyperimmune serum from a rabbit immunized with the CDV-nucleocapsid protein (NP), which was expressed by a recombinant baculovirus with the NP gene of the Onderstepoort strain (Tsuchiya et al., to be published), was used to detect CDV antigen for the immunocapture ELISA. For capturing CDV, serum from a dog experimentally infected with the Onderstepoort strain (anti-Onderstepoort) was used. This serum was also used in virus neutralizing (VN) test.

For immunocapture ELISA, the ELISA plate (Nunc Maxisorp, Denmark) was precoated with the anti-Onderstepoort serum diluted at 1:2,000 in 50 mM carbonate buffer (pH 9.6) as a capture antibody at 4°C overnight. After preincubation with 100 μl of blocking reagent, Block Ace, (Dainihon-Seiyaku, Japan) followed by washing with phosphate-buffered saline containing 0.05% Tween-20 (PBST), 50 μl of specimen solubilized by radioimmuno-precipitation assay (RIPA) buffer (1% Triton X-100, 1 mM iodoacetamide, 0.2 trypsin-inhibition-unit/ml of Aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1% sodium deoxycholate, 0.14 M NaCl, and 10 mM Tris-HC1 at pH 8.0) was distributed into each precoated well. After incubation for overnight at 4°C, wells were washed with PBST and incubated with 50 μl of rabbit anti-CDV-NP serum diluted at 1:2,000 for 1 hr at room temperature (RT). The wells were washed with PBST again and incubated with anti-rabbit immunoglobulin-biotinylated antibody from a donkey (Amersham, UK) diluted at 1:2,000 for 1 hr at RT followed by washing with PBST. Then, 50 μl of streptavidin-biotinylated horseradish peroxidase complex (Amersham, UK) diluted at 1:1,000 was added to each well and reacted for another 1 hr at RT. The wells were then washed with PBST and reacted with 100 μl of substrate containing 3, 3′, 5, 5′-tetramethylbenzidine (BIO-RAD, U.S.A.) for 30 min at RT. After reaction, optical density (OD) was measured at 655 nm with ELISA reader (BIO-RAD, Model 3550, U.S.A.). As a negative control, uninfected B95a cells solubilized by RIPA buffer were used. The positive/negative cut-off OD was taken as twice the mean OD of the negative control, which was 0.003 ± 0.002

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Table 1. Sensitivity of the immunocapture ELISA

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<tr>
<th>CDV strain</th>
<th>Log minimum virus titer (TCID&lt;sub&gt;50&lt;/sub&gt;/50 µl)</th>
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<tr>
<td>Onderstepoort</td>
<td>1.9</td>
</tr>
<tr>
<td>Hamamatsu</td>
<td>2.1</td>
</tr>
<tr>
<td>Yanaka</td>
<td>2.0</td>
</tr>
</tbody>
</table>

(n=6). Thus, OD of 0.006 was used for the positive/negative cut-off OD. The sensitivity of the immunocapture ELISA was compared among the Onderstepoort strain and 2 field isolates of CDV by limiting dilution. The lowest virus titers detected by this method were expressed as the reciprocal of the highest dilution showing cut-off OD (Table 1). The three viruses showed titers of around 10<sup>2</sup> TCID<sub>50</sub>/50 µl as the detectable amount by this method, which suggested no difference in sensitivities among CDV strains used. To determine the specificity of the immunocapture ELISA, the cells infected with MV, CPIV, or CPV were also tested (Table 2). A slight cross reactivity was observed against MV, which belongs to the same genus as CDV, while no cross reactivities were observed against both CPIV and CPV.

To evaluate the usefulness of the immunocapture ELISA for titration of CDV, a stock solution of CDV was assayed both by the standard titration method and by the immunocapture ELISA. CDV titers of TCID<sub>50</sub> and OD obtained by the immunocapture ELISA were shown in Fig. 1. A high correlation between the CDV titers of TCID<sub>50</sub> and OD was observed (r=0.94).

The growth curve of the Onderstepoort strain of CDV in B95a cells was shown in Fig. 2. It was observed that OD obtained by the immunocapture ELISA corresponded to CDV titers kinetically. This result suggested that the growth of CDV could be assayed by the immunocapture ELISA. These results demonstrated the usefulness of the immunocapture ELISA for CDV titration, rapid diagnosis and antigenic analysis of CDV.

In the present study, the VN test was also performed using the immunocapture ELISA to determine the VN titer against field isolates of CDV, which showed ambiguous CPE in the cultured cells by a standard virus titration. Briefly, B95a cells were seeded into a microplate and cultured for 24 hr at 37°C and the supernatant in the wells was discarded. The cultured B95a cells were then washed with serum-free RPMI1640 medium. Five-fold dilutions of sera were mixed with 100 TCID<sub>50</sub> of CDV and incubated for 1 hr at 37°C. Then, the mixture was added to the B95a cells in each well. After incubation for 1 hr at 37°C, the cells were washed with serum-free RPMI1640 and cultured in RPMI1640 supplemented with 3% fetal calf serum for 4 days. The cultured B95a cells were solubilized with RIPA buffer and assayed for CDV growth by the immunocapture ELISA. Neutralizing titer was determined by 50% inhibition of CDV growth. The inhibition of CDV growth by neutralizing antibodies was evaluated as the percentage of reduction of the OD by following formula:

\[
\text{percentage inhibition} = \left(1 - \frac{\text{sample OD}}{\text{positive control OD}}\right) \times 100
\]

The reciprocal of the highest sample dilutions with a percent inhibition showing 50% were considered as VN titers. For confirming the correspondence, the titers of 5 field sera from dogs suspected of CDV infection were assayed by both the standard VN test [6] and the method using the immunocapture ELISA. As shown in Fig. 3, a high correlation (r=0.97) between the titers was observed.

To compare the antigenicities of field isolates of CDV with the Onderstepoort strain, cross VN test was performed.
Table 3. Results of cross-virus neutralizing tests

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Onderstepoort</th>
<th>Hamamatsu</th>
<th>Yanaka</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-Onderstepoort</td>
<td>657 *</td>
<td>123</td>
<td>74</td>
</tr>
<tr>
<td>anti-Hamamatsu</td>
<td>1725</td>
<td>&gt;6250</td>
<td>&gt;6250</td>
</tr>
<tr>
<td>anti-Yanaka</td>
<td>147</td>
<td>&gt;2500</td>
<td>&gt;2500</td>
</tr>
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</table>

* VN titers obtained by using the immunocapture ELISA.

For cross VN test, sera from dogs experimentally infected with Hamamatsu and Yanaka isolates (anti-Hamamatsu and anti-Yanaka) and anti-Onderstepoort dog serum were used. VN titers of these sera against each isolate and against the Onderstepoort strain of CDV were assayed using the immunocapture ELISA (Table 3). Anti-Onderstepoort serum showed high VN titer against the Onderstepoort strain and rather low titers against both field isolates. Anti-Hamamatsu and anti-Yanaka sera showed high VN titers against both field isolates, but low VN titers against the Onderstepoort strain.

From the diagnostic point of view, one of the most reliable methods for diagnosis of CD is to isolate CDV from affected animals or to detect the CDV antigen in their tissues or cells. However, the virus isolation takes a long time and is frequently unsuccessful. Recently, the detection of CDV-NP gene using the reverse transcription-polymerase chain reaction has been developed [17]. This method can only be carried out in equipped laboratories, though it is highly sensitive and useful method.

In the present study, serum against CDV-NP was used in the immunocapture ELISA and detection of CDV was successfully achieved. The NP is required for virus replication and is the most abundant viral protein among the viral proteins of CDV [4]. In addition, the immunoreactivity of the NP can be preserved even in ambient temperature, despite the fact that the NP is sensitive to intracellular proteolysis [12]. It seemed that no difference in sensitivities to NP was observed between field isolates and the Onderstepoort strain detected by the anti-CDV-NP rabbit serum.

Recently the outbreaks of CD were observed, and their epidemiological [7] histopathological (Okita et al., unpublished data), molecular biological, serological and virological investigations were carried out. In these outbreaks, some atypical characteristics of CDV and CD were observed [6, 8, 17]. Results of the cross VN test in the present study revealed the differences in VN activities between sera from dogs infected with the Onderstepoort strain and those with field isolates of CDV, and suggested that there were antigenic differences between field isolates and Onderstepoort strain of CDV. Antigenic alterations of the recent isolates from “old-type” CDVs were suggested to cause the discrepancy of VN titers. Recently, it was reported that marked differences in VN titers were found when tested with different strains of CDV (Onderstepoort strain and an isolate from a lion) [3]. A possibility of the appearance of CDV in the field which had different properties and contributed to the recent outbreaks could be suspected.

The differences in VN activity of field isolates may be caused by antigenic alterations of the surface glycoproteins (H, F) between the Onderstepoort strain of CDV and the field isolates. It was known that the H protein of CDV was a target for the host immune defense mechanism and induced VN antibodies [5]. Recently, it was observed that the properties of the H protein and H gene of the Yanaka isolate were different from those of the Onderstepoort strain of CDV (Iwatsuki et al., to be published). Similar alterations of recent isolates of MV were also reported and relationship between variation of H protein and prevalence of measles was suspected [13, 14]. Variation of H protein of CDV may be also considered and the results of cross VN tests obtained in this study were in agreement with this hypothesis.

The number of field isolates examined in the present study is too small, however, above observations suggested the existence of the antigenic variations of CDV in the field. In addition, the differences in VN activities in dog sera might implicate the possible increase of CD cases. Extensive and epidemiological investigations on the alterations of neutralizing epitopes on field viruses are necessary, and the immunocapture ELISA could be useful for the investigations.

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REFERENCES