The Isolation of Large and Small Plaque Canine Distemper Viruses which Differ in their Neurovirulence for Hamsters

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SUMMARY

Large and small plaque-forming viruses were isolated from the Onderstepoort strain of canine distemper virus (CDV). Small plaque virus, which was released more slowly from infected cells than large plaque virus, readily established persistent infections in Vero cells, whereas large plaque virus required undilute passage to do so. All persistently infected cultures eventually released small plaque virus. No difference was found in the size of polypeptides induced by either plaque-purified viruses or virus released from persistent cultures. Both dilute and undilute passage, large plaque virus produced an acute neurological illness in weanling hamsters, whereas small plaque virus failed to produce any clinical signs of disease for 3 months after inoculation. After this period 50% of the animals infected with small plaque virus showed a general deterioration in their condition and lesions were observed in the brain which resembled those found in cases of large plaque virus infection. Serum-neutralizing antibody titres to CDV rapidly increased after infection with small plaque virus, whereas animals infected with large plaque virus had low or undetectable levels. All hamsters infected with small plaque virus and a small number which survived large plaque virus infection had elevated titres of antibody over a test period of 15 months.

INTRODUCTION

Old dog encephalitis (ODE) is a relatively rare disease of middle-aged dogs which has been compared with the human slow progressive central nervous system (CNS) disease of subacute sclerosing panencephalitis (SSPE; Lincoln et al., 1971). Canine distemper virus (CDV) and measles virus have been implicated in these diseases and each virus can cause two distinct neurological conditions: an immediate acute encephalitis and a delayed degenerative disease (Hadlow, 1962; Connolly et al., 1967; Lincoln et al., 1971; ter Meulen et al., 1972). However, it is not known whether the degenerative type of disease is the result of infection with an atypical virus or due to an altered immunological host response to a common virus.

Plaque-purified isolates of measles and SSPE viruses differ in their biological properties (Gould, 1974, Gould et al., 1976), although differences in their biochemical properties cannot be correlated with the virus origin (Rima et al., 1979). Also, different isolates of CDV vary in their pathogenicity for ferrets and dogs (Reculard & Guillon, 1972; Confer et al., 1975) and recently Appel (1978) reported a reversion to virulence of attenuated CDV following series passage in dogs which he attributed to the selective replication of virulent virus clones in the host.

In this paper we describe the isolation of stable large and small plaque-forming viruses from the Onderstepoort strain of CDV and assess their biological and biochemical properties with respect to their neurovirulence for weanling hamsters.
METHODS

**Virus.** The Onderstepoort strain of CDV was supplied by Professor M. J. G. Appel (New York State Veterinary College, Cornell University, Ithaca, N.Y., U.S.A.). The passage history of this strain in this laboratory was previously described by Campbell *et al.* (1980).

**Cells and medium.** Vero cells obtained from Flow Laboratories were subcultured in Eagle’s medium (Glasgow modification) containing 8% foetal bovine serum (FBS) and maintained in Eagle’s medium containing 1% FBS.

**Antisera.** Rabbit hyperimmune sera were prepared against the Onderstepoort strain of CDV (grown in Vero cells) as follows: initially 1 ml purified virus was mixed with 1 ml complete Freund’s adjuvant and the rabbit was injected intramuscularly. Four further inoculations of virus alone were given intradermally at 2-week intervals. The serum was collected 2 weeks after the final injection.

**Immunofluorescence.** Vero cells infected with CDV were treated for 45 min at 37 °C with a 1:80 dilution of rabbit hyperimmune serum (prepared against CDV and adsorbed with non-infected Vero cells) which had a neutralizing titre of 1280. The cells were washed and treated for a further 45 min with sheep anti-rabbit immunoglobulin conjugated with fluorescein isothiocyanate (Wellcome Reagents). Cells were either examined live or after acetone fixation to determine the respective amounts of surface and intracellular virus antigen. Uninfected Vero cells showed no fluorescent staining.

**Plaque assays and plaque selection.** Assays and selection of plaques were performed as described by Gould (1974) and Gould & Linton (1975).

**Growth curves.** Vero cell monolayers were prepared in 16 mm wells of plastic trays (Linbro multi-dish dispenser trays: Flow Laboratories) and inoculated with virus at a multiplicity of 3. After incubation for 4 h the medium was removed and the cultures were washed six times with PBS and 1 ml maintenance medium was added. Supernatant and intracellular virus titres were estimated after the adsorption period and the cultures were re-incubated. At appropriate time intervals the medium was removed from cultures and spun at 3000 g for 5 min and the supernatant titrated for infectivity. Infected cells were scraped into 1 ml fresh maintenance medium and the cell pellet obtained from the original medium added. Virus was harvested by sonication of the cells for 2 × 10 s by probe using an MSE 100 W ultrasonic disintegrator (operated at an amplitude of 7 μm) and cell debris was removed by centrifuging at 3000 g for 5 min. This supernatant was titrated for infectivity. Infectivity assays were performed as follows. Vero cell monolayers were prepared in the individual wells of plastic microtitre plates (Sterilin). Serial 10-fold dilutions of virus were made in maintenance medium and 0.2 ml quantities of each were delivered to four monolayer cultures. Cultures were incubated at 37 °C in 5% CO₂ and were examined for up to 5 days for evidence of c.p.e. TCID₅₀ was calculated by the method of Reed & Muench (1938).

**Plaque reduction assays.** An equal volume of each virus preparation was mixed with a 1:10² dilution of a standard virus stock. Quantities (0.2 ml) of each mixture were delivered to three Vero monolayer cultures prepared in 16 mm wells of plastic trays. The virus yield from individual wells was determined 1 to 3 days post-inoculation (p.i.) when c.p.e. had either reached completion or become limited.

**Polyacrylamide gel electrophoresis and autoradiography.** Virus was grown and labelled as previously described (Rima & Martin, 1979) with the following modifications. L-³⁵S-methionine (5 μCi/ml; 1000 to 1200 Ci/mmol) or ³H-glucosamine (5 μCi/ml; 38 Ci/mmol) and ³H-mannose (5 μCi/ml; 5 Ci/mmol) were used to label cultures.

**Inoculation of weanling hamsters and preparation of brain homogenates.** Outbred weanling golden hamsters, 20 to 25 days old, were anaesthetized lightly with ether and inoculated intracerebrally with 0.1 ml vol. stock virus. Hamster brain tissue was suspended in 2 ml vol. maintenance medium with sterile glass beads (1.5 to 2 mm; Searle Laboratory
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Reagents, Chicago, Ill., U.S.A.) and vortexed until a homogenate was formed. The samples were spun at 3000 g and the supernatant removed and stored at -70 °C.

Perfusion techniques and blocking procedure. Following pentobarbitone anaesthesia the animals were perfused through the ascending aorta with warm isoionic saline (0.5 min pre-wash) followed for 15 min by warm modified Karnovsky fixative (Karnovsky, 1965) comprising 2.5% glutaraldehyde, 3.5% formaldehyde (EM-grade, low methanol content) and 10% sucrose in 0.1 M sodium cacodylate buffer pH 7.4. Following perfusion, the brain and spinal cord were left in situ for at least 1 h, before their removal and transfer to cold fixative. Sections (1 μm in size) were stained with toluidine blue for light microscopy. Blocks were embedded in paraffin and 5 μm sections were stained with haematoxylin and eosin, and haematoxylin/luxol fast blue.

Neutralization tests. Individual sera from hamsters were tested for neutralizing antibody titres against the parent Onderstepoort virus. The microtitre method described by Confer et al. (1975) was used with the following modifications: (i) sera were diluted in twofold increments, (ii) cultures were examined for c.p.e. after 5 days and (iii) the 50% neutralization endpoint was calculated by the formula of Reed & Muench (1938).

RESULTS

Isolation of large and small plaque-forming viruses

The large plaque (1 to 2 mm) and the small plaque (0.1 to 0.5 mm) viruses were isolated by three sequential plaque selections and each virus retained its plaque characteristic after four passages in Vero cells at an m.o.i. of 0.01.

Cytopathic effect

Vero cell monolayers infected at a multiplicity of 0.01 with each type of plaque-forming virus showed two distinct c.p.e.s. The large plaque virus produced large spreading syncytia by 16 h p.i. which gradually fused the entire monolayer by 24 to 36 h p.i. The small plaque virus initially produced foci of rounded cells which gradually fused to give small irregular syncytia. Complete fusion of the monolayer rarely occurred and by 48 h p.i. the cell sheet began to disintegrate. At an m.o.i. of 3, both viruses developed the same type of c.p.e. more rapidly. However, the small plaque virus did not initially produce foci of rounded cells.

Growth curves

Growth curves were carried out on three separate occasions and triplicate cultures of each virus were used. De novo intracellular virus was detected between 8 and 12 h p.i. in both cultures infected with the large and small plaque viruses and in each case intracellular virus titres of 100 TCID₅₀/cell were eventually obtained between 24 and 28 h p.i. However, at this time although cultures released 20 TCID₅₀/cell of large plaque virus only 1 TCID₅₀/cell of extracellular small plaque virus was detected. Maximum release of small plaque virus did not occur until 32 h p.i. Fusion of the cell monolayer was complete in cells infected with the large plaque virus by 20 to 24 h p.i., whereas single cells could still be observed in monolayers infected with the small plaque virus at 28 to 32 h p.i.

Establishment of persistently infected cultures with dilute passage virus stocks

We have compared the abilities of the large, small and non-plaque-purified virus stocks to establish persistent infection in Vero cells.

Dilute passage (DP) virus was prepared by serial passage at an m.o.i. of 0.01 and had an approx. titre of 10⁷ TCID₅₀/ml. Vero cell monolayers were infected at multiplicities of 1, 0.01 and 0.0001 with either the parental or the large or small plaque viruses. After incubation at
Table 1. Comparison of the ability of DP stocks of parent, large plaque and small plaque viruses to persistently infect Vero cell cultures

<table>
<thead>
<tr>
<th>Subculture number</th>
<th>M.o.i. of parent virus</th>
<th>M.o.i. of small plaque virus</th>
<th>M.o.i. of large plaque virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>0.01</td>
<td>0.0001</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
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</tr>
<tr>
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</tr>
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<td>24</td>
</tr>
<tr>
<td>6</td>
<td>39</td>
<td>40</td>
<td>27</td>
</tr>
</tbody>
</table>

* D, All cells dead.

37 °C for 48 h the surviving cells in the infected monolayers were subcultured. The procedure was designed thereafter to give each infection the maximum opportunity to establish a confluent-infected monolayer, i.e. in cultures which reached confluence quickly the cells were subcultured but in bottles which showed extensive c.p.e. the medium was replaced and the cells reincubated without subculture.

Table 1 shows a typical set of results for the early passages of each type of culture. Although the parent and small plaque viruses established persistently infected cultures at all m.o.i.s, only a few cells survived in initial passages of cultures infected with the parent virus (most apparent at an m.o.i. of 1) and these took several weeks to reach confluence. A large percentage of cells infected with the small plaque virus survived even at the first passage and these were confluent within 4 days. However, the large plaque virus failed to establish a persistent infection regardless of the input multiplicity and the cultures showed extensive c.p.e. and no cells survived after the second passage.

Establishment of persistently infected cultures with undilute passage virus stock

Undilute passage (UP) pools of the large and small plaque viruses were prepared by infecting cultures of Vero cells with undiluted virus harvests from the previous passage. Table 2 shows the infectivity titres obtained after each undilute passage, the interference produced by each of these pools in a plaque reduction assay and the ability of the virus to produce lytic and persistent infections at each passage level.

After the first passage the large plaque virus titre was reduced by 2 logs and this pool readily established a persistently infected culture as the surviving cells were confluent after 2 days. By the second undilute passage the virus titre was further reduced to $10^{2.7}$ TCID$_{50}$/ml and this stock failed to produce a lytic infection in Vero cells. Interference was further demonstrated at both passage levels of this virus in plaque reduction assays.

Although all UP pools of the small plaque virus readily established persistent infections, stocks from passages 1 to 5 were also able to produce lytic infections and interference was not detected until the 6th passage.

Release of virus from persistently infected cells

A persistent culture established by infecting Vero cells with uncloned virus which had undergone three undilute passages has been maintained for over 100 passages. During early passages the cells produced large syncytia and released both large and small plaque viruses. By passage 17 the culture produced areas of rounded cells and small irregular syncytia and released only small plaque virus. Cultures established with small plaque virus only released this type of virus, whereas release of both large and small plaque virus was detected at the
Table 2. Effect of undilute passage of the small and large plaque viruses on infectious yield, interference and type of infection produced

<table>
<thead>
<tr>
<th>Passage number</th>
<th>Small plaque virus</th>
<th>Large plaque virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infectivity titre (log TCID₅₀/ml)</td>
<td>Interference plaque reduction titre (log p.f.u./ml)</td>
</tr>
<tr>
<td>0</td>
<td>6.2</td>
<td>—</td>
</tr>
<tr>
<td>1</td>
<td>4.95</td>
<td>—2.8*</td>
</tr>
<tr>
<td>2</td>
<td>5.45</td>
<td>—3.4</td>
</tr>
<tr>
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<td>5.45</td>
<td>—3.3</td>
</tr>
<tr>
<td>4</td>
<td>6.2</td>
<td>—3.7</td>
</tr>
<tr>
<td>5</td>
<td>5.7</td>
<td>0.0</td>
</tr>
<tr>
<td>6</td>
<td>4.7</td>
<td>2.4</td>
</tr>
</tbody>
</table>

* Negative plaque reduction due to high titre of test virus.

10th passage of a persistent culture established with the large plaque virus ('large plaque UP')

Temperature sensitivity of viruses

Virus released from these persistent cultures was compared for its efficiency of plaque formation (e.o.p.) at 37 and 40 °C. The e.o.p. at 40 °C was between 50 and 100% of that at 37 °C for both the plaque-purified small and large plaque viruses and virus released from persistent cultures.

Production of virus antigens in persistently infected cells

All cultures were confirmed to be persistently infected by examination for the presence of virus antigens by immunofluorescence. Antigen production was monitored for over 40 passages of a culture established with parent virus ('parent UP'). In the initial passages of these cells large brightly staining syncytia were observed, whereas by passage 9 the majority of cells produced large cytoplasmic and nuclear inclusions. During the 20th passage no c.p.e. was observed and no virus antigen was detected in any cells when the culture was examined by immunofluorescence. Twenty-four h after the next subculture over 90% of the cells contained virus antigen, mainly at the cell surface and by 96 h after subculture extensive c.p.e. of an irregular syncytial type was observed. Further passage of the culture at 37 °C resulted in cyclic fluctuations in c.p.e. and antigen production but total disappearance of antigen has not reoccurred. Attempts to induce either an increase or decrease in antigen production of cultures by incubation at 32 or 40 °C were unsuccessful.

Virus-induced polypeptide synthesis

To compare the size of virus-induced polypeptides monolayers of Vero cells were infected with either the large or small plaque virus at an m.o.i. of 1 as described, labelled with ³⁵S-methionine or ³H-glucosamine and mannose and analysed on 8 to 15% polyacrylamide gradient slabs.

The polypeptides and glycoproteins induced by virus released from the 'parent UP' (small plaque) and 'large plaque UP' (large and small plaque) persistent cultures were analysed in a similar manner. In each case a pool of virus was prepared by infecting Vero cells with the
undiluted supernatant from the persistent culture and this was used to reinfect new cells which were labelled as before. No differences were found in the size of polypeptides induced by virus released from the persistent cultures and the plaque-purified large and small plaque viruses.

Neurovirulence of the small and large plaque viruses for weanling hamsters

Twenty- to 25-day-old hamsters were inoculated intracerebrally with $10^6$ p.f.u. of either large or small plaque virus.

Animals were sacrificed when they became moribund. Twenty-five out of 37 hamsters inoculated with the large plaque virus developed clinical signs of ataxia, convulsions and paralysis, typical of an acute encephalitis and were sacrificed between 5 and 20 days after infection. In initial experiments the parent virus also produced a similar disease. However, all 25 animals inoculated with the small plaque virus failed to produce any clinical signs of illness for 3 months post-inoculation. After this period, 50% of animals developed a general deterioration in condition over the next 9 months. Symptoms included loss of weight, drowsiness and susceptibility to infection. No neurological signs were apparent.

Selected brains from animals infected with the large plaque virus were harvested at the time of sacrifice and a homogenate of the tissue tested for the presence of infectious virus by observing the development of c.p.e. and in selected cases examining infected cultures by immunofluorescence. Infectious virus was recovered from all animals tested and in each case produced c.p.e. in Vero cells typical of the large plaque virus (i.e. large spreading syncytia). Approx. $10^4$ TCID$_{50}$ of virus were recovered from the brains of these animals irrespective of the time of sacrifice after inoculation.

A further 13 weanling hamsters were inoculated with the small plaque virus as before. These animals were sacrificed at various times after inoculation and the brain homogenate tested for infectious virus by both development of c.p.e. and immunofluorescence in Vero cells. Virus was recovered up to 7 days p.i.

Neurovirulence of undilute passage large plaque virus for weanling hamsters

To examine the effect of virus interference on the outcome of infection in weanling hamsters, animals were inoculated with either 100 p.f.u. of large plaque virus which had two undilute passages in Vero cells (and did not produce a lytic infection in these cells) or 100 p.f.u. of dilute passage virus (several passages at an m.o.i. of 0-01). All seven animals inoculated with UP large plaque virus and six out of seven animals inoculated with DP virus showed clinical signs typical of an acute encephalitis over a similar period of time. Infectious virus was recovered from the brains of all these animals which produced a lytic infection in Vero cells.

Histology

To determine the neuropathological changes induced by large and small plaque virus infection, two mock-infected, seven large plaque-infected and seven small plaque-infected animals were examined. The latter group included animals from 9 days to 7 months p.i. Brains and spinal cords of animals were removed after perfusion and prepared for histological studies as described in Methods.

In the control animals no significant histological lesion was recognized. All of the large plaque-infected animals examined developed clinical signs of an acute encephalitis. In each case some histological abnormality, usually at the site of injection and in the brain stem, was observed. This was characterized by some meningeal inflammatory infiltration, inflammatory cuffing and neuronal degeneration with some glial reaction. Structures suggestive of virus inclusions were seen in a few cells in both the nuclei and cytoplasm. These inclusions were not, however, of the classical Cowdry A type. Of the cases of small plaque virus infection
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examined three showed histological abnormality. In two animals (4 weeks p.i.) these changes were similar to those seen in cases of large plaque virus infection but much less severe. In one case (7 months p.i.) the inflammation and neuronal degeneration in the brain stem was severe and equal in intensity to that seen in large plaque virus infection. This animal was in a general state of deterioration with symptoms which included loss of weight, eye infection and drowsiness.

Serum neutralizing antibody to CDV

Sera from individual animals were collected at intervals up to 15 days p.i. and titrated for neutralizing antibody. Animals inoculated with large plaque virus were sacrificed when they became moribund. The results are shown in Fig. 1. Neutralizing antibody titres of between <2 and 4 were observed in the sera from all animals inoculated with large plaque virus, up to 12 days p.i. except for one sample taken after 9 days which had a titre of 16. However, when sera from animals inoculated with small plaque virus were examined, neutralizing antibody was detectable from 4 days p.i. and reached levels of between 64 and 256 by 15 days (Fig. 1). The difference observed in titres between animals inoculated with large and small plaque virus was statistically significant \( P < 0.001 \) by \( \chi^2 \) analysis.

The sera from a further 17 hamsters were examined from 1 to 15 months p.i. This group consisted of 12 animals inoculated with small plaque virus and five animals which had survived large plaque virus infection and remained symptom-free up to the time of sacrifice (in one case 15 months p.i.). Neutralizing antibody levels of between 64 and \( >4096 \) were present in all sera tested and titres were independent of the clinical state of the animal and type of virus infection. Maximum titres (1024 to \( >4096 \)) were observed between 4 and 8 months p.i.

**DISCUSSION**

In the present work we resolved two plaque types when the Onderstepoort strain of CDV was plated on Vero cells and subsequently isolated a large and small plaque virus each with stable biological and biochemical characteristics. Our investigation confirms an earlier report by Bussell & Karzon (1965) who observed similar plaque types in a study of plaque formation by the Onderstepoort strain of CDV in AV\(_3\) cells.
Growth curve studies showed that both the large and small plaque viruses had similar replication rates as de novo intracellular virus was detected between 8 and 12 h p.i. and reached similar levels by 24 to 28 h p.i. However, maximum release of small plaque virus from cells occurred 4 to 8 h later than that of large plaque virus. It is possible that the small plaque virus may bud more slowly from the cell, reflecting a decreased fusion ability which may in turn account for the production of a more limited type of c.p.e.

The slower rate of cell destruction by the small plaque virus may also contribute to its ability to readily establish persistent infection in Vero cells compared to the large plaque virus which requires undilute passage to do so.

Hall et al. (1974) reported that defective interfering (DI) particles accumulate when cells are infected with undilute passage measles virus and Rima et al. (1977) showed that such preparations rapidly established persistent infections in tissue culture. In our study the large plaque virus produced interference more rapidly than the small plaque virus with undilute passage which suggests that DI particles accumulated more readily. The establishment of cultures persistently infected with either parent or large plaque virus eventually released small plaque virus which could either be present in the original virus stock or (in the case of the large plaque virus) must have arisen by mutation during the initial passages of the cells. This indicates that although small plaque virus is not necessary for the establishment of persistent infections it is probably important for the maintenance of such cultures. Temperature-sensitive mutants do not appear to be involved in either the establishment or maintenance of our persistent cultures as none of the virus released from persistently infected cells was temperature-sensitive.

Both DP and UP pools of the large plaque virus produced an acute neurological disease in weanling hamsters which suggests that (in contrast to the situation in tissue culture) DI particles do not play a major role in the establishment of long term persistent infections in vivo. Furthermore, when hamsters were inoculated with a UP stock, which only produced persistent infections in Vero cells, the virus recovered from the brains of these animals produced lytic infections in tissue culture. It is probable that some degree of clearance of DI particles took place in the brains of these animals.

The small plaque virus failed to produce any clinical signs of disease in animals up to 3 months p.i. However, over the following 9 months, 50% of these hamsters developed a general deterioration in their condition, although no neurological signs were apparent. Virus could not be recovered from brain homogenates of animals after 7 days p.i. However, an examination of the neuropathological state of such animals from 9 days to 7 months p.i. showed that approx. 50% of these had histological lesions in the brain similar to those seen in cases of large plaque virus infection. Variation occurred in both the severity of the lesions and clinical state of the animals tested.

When hamster sera were examined for levels of neutralizing antibody to CDV a rapid increase in titre with time was observed in animals inoculated with small plaque virus, whereas very low or undetectable levels of antibody were produced by animals exhibiting acute illness following large plaque virus infection. However, all animals, including a small number which survived infection with large plaque virus, had elevated levels of neutralizing antibody over a test period of 15 months. These studies suggest that the host immune defence mechanism is an important factor in controlling the onset of acute neurological disease and that the difference in immune response to small and large plaque virus partially determines their neurovirulence. It is possible that either small antigenic differences exist between these viruses or they differ in their involvement with the reticulo-endothelial system.

Taken in conjunction, the serological and neuropathological data obtained in this study suggest that small plaque virus persists for long periods of time in the CNS of hamsters (as might be expected from the tissue culture model). In a minority of animals infected with large
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plaque virus the immune defences may protect the animal against acute fatal encephalitis and such cases may also lead to persistent infection.

Further investigation is in progress to examine CNS tissue for the presence of virus antigen and to determine whether or not this relates to variation in the severity of illness induced by small plaque virus infection.

No differences were found in the size of polypeptides induced by either the plaque-purified large and small plaque viruses or the virus released from persistent cultures. Nevertheless, small differences in either the amino acid composition of individual proteins or their degree of glycosylation may be present and we are currently involved in a more detailed study which may resolve this question.

We hope that further analysis of the small and large plaque viruses of the Onderstepoort strain of CDV and their behaviour in vivo may help to elucidate the mechanisms involved in the establishment of degenerative neurological disease associated with morbilliviruses.

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REFERENCES


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