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Full Length Research Paper

Isolation of canine distemper viruses from domestic dogs in South Africa using Vero.DogSLAM cells and its application to diagnosis

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Studies comparing canine distemper virus (CDV) strains from Africa with that of previously described lineages have been hampered due to a lack of field strains for *in vitro* experiments. Since the attenuation of the Onderstepoort strain in the 1940s, there have not been any reports of CDV isolations in southern Africa. Clinical specimens consisting of whole blood, spleen, lungs, brain and cerebrospinal fluid from dogs with clinical signs compatible with distemper diagnosis were obtained from private veterinary practices and diagnostic laboratories. The animals all displayed various catarrhal, systemic, or nervous signs of the disease. One hundred and sixty two specimens from 124 dogs were inoculated into Vero cells expressing canine signalling lymphocyte activation molecule (Vero.DogSLAM). Cytopathic effects (CPE) in the form of syncytia formation and cell necrosis were observed in 33 (20.4%) specimens within 24 h of inoculation and the presence of CDV was confirmed with the aid of the direct fluorescent antibody test and electron microscopy (EM). Four of the CDV-positive dogs had a history of canine distemper vaccinations. Seventy three percent of all positive dogs were less than 12 months old. Local isolates of wild-type CDV were generated and we conclude that isolation of CDV using Vero.DogSLAM is a specific and practical method for the antemortem diagnosis of distemper in dogs.

Key words: Isolation, CDV, dogs, South Africa, Vero.DogSLAM.

INTRODUCTION

Canine distemper virus (CDV) is a highly contagious viral pathogen causing lethal disease in both domestic and wild, land- and sea-living animals. It is classified in the *Morbillivirus* genus of the family *Paramyxoviridae* (Griffin, 2001; Murphy et al., 1999). Studies on the characteristics of CDV strains prevailing in the field are scanty, perhaps due to difficulties in isolation and titration of the virus (Lan et al., 2005b). Canine distemper virus isolates detected in naturally occurring cases cluster according to geographical distribution (Bolt et al., 1997; Carpenter et al., 1998). Further studies have reported genetic variation between vaccine strains and current circulating wild-types (Lan et al., 2006; Martella et al., 2006). Since the attenuation of the Onderstepoort vaccine strain in the 1940s (Haig, 1956), there has not been any reports of CDV isolations in southern Africa, justifying the need to isolate field strains from clinical specimens to provide a pool of local strains for characterization.

Canine distemper virus occurs as virulent (wild-type) and laboratory-modified strains that have different target cell tropisms (Appel et al., 1992). Attenuated virus grows readily in monolayers of a variety of epithelial and fibroblast cell lines and in primary cell cultures from different species. However, isolation of virulent CDV in these cells is difficult (Appel and Gillespie, 1972; Appel, 1978). Canine distemper virus has been isolated from animals by co-cultivation of infected tissues with mitogen-stimulated lymphocytes derived from healthy dogs (Appel et al., 1992), or canine alveolar macrophages (Appel, 1978;

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Appel and Jones, 1967). The virus has also been isolated with the aid of ferret peritoneal macrophages obtained from specific pathogen free (SPF) ferrets (Poste, 1971; Whetstone et al., 1981). However, isolation using these methods tends to be prolonged and requires multiple blind cell-passages before cytopathic effects (CPE) are noticed. Moreover, many laboratories no longer keep SPF dogs or ferrets due to animal welfare concerns. These methods are therefore not user-friendly for diagnostic laboratories processing clinical specimens. Less commonly, CDV isolation on the chorioallantoic membrane of embryonated chicken eggs has been used (Ezeibe, 2005; Haig, 1956). This technique requires multiple weekly passages in fresh eggs before viral CPE becomes evident and is relatively expensive. Lednicky et al. (2004a) reported effective primary isolation of CDV from naturally infected free-ranging raccoons using cell lines such as MDCK (canine epithelial kidney cells), MV1 LU (mink lung), and Vero (African green monkey kidney cells). However, a minimum of 10 days was required for isolations in addition to the multiple passages. Cells of the B95a type have also been reported to be highly effective for the isolation of CDV (Kai et al., 1993). Unfortunately, B95a cells are derived from marmosets, which are endangered animals and the purchase and possession of B95a cells in some countries, e.g. the U.S.A., requires a government permit. Signalling lymphocyte activation molecule (SLAM) has been reported to be the principal cellular receptor for morbilliviruses *in vivo* (Tatsuo et al., 2001), and Vero.DogSLAM cells have been shown to aid the isolation of CDV from clinical material within 24 h of inoculation, with a significant reduction in the costs associated with the isolation of wild-type CDV (Seki et al., 2003). This study, therefore, explored this faster and cheaper alternative to isolate field strains of CDV from dogs in South Africa, and to obtain a pool of viruses that will enable molecular comparisons with vaccine and other published strains.

MATERIALS AND METHODS

Clinical specimens

Clinical specimens were obtained from veterinary practices and diagnostic laboratories in South Africa. Specimens obtained from living dogs were limited to sick animals showing clinical signs suggestive of canine distemper and included blood in heparin–containing tubes and cerebrospinal fluid (CSF).

Necropsy specimens were collected from dogs that were euthanized or died with signs of canine distemper and included spleen, lung and brain. All specimens were chilled during transport to the laboratory. One-hundred-and-sixty-two clinical specimens from 124 living and dead animals were received for virus isolation. A litter of 10 puppies was euthanized and the spleen and whole blood from all 10 puppies were submitted. For economic reasons, three blood samples were selected randomly, and the 10 spleen samples were pooled as 2 samples.

Peripheral blood mononuclear cells (PBMCs) were extracted from blood samples as previously described (Appel et al., 1992). Briefly, heparinized blood collected from dogs tentatively diagnosed with canine distemper by clinical examination was diluted with an equal part of RPMI 1640 medium with L- glutamine. The diluted blood was overlayed on an equal volume of Histo-Paque® and centrifuged for 30 min at 500 × g. The cell band was removed and washed three times in RPMI 1640 with L-glutamine plus 2% foetal bovine serum in polypropylene tubes. Cells were resuspended at a concentration of 5 x 10^6 cells/ml in RPMI 1640 with L-glutamine plus 2% foetal bovine serum and 50 µl gentamycin. Cerebrospinal fluid was used directly to inoculate Vero.DogSLAM cells, since they were collected in a sterile manner by clinicians and thus suitable for viral isolation in cell cultures. Necropsy tissues were processed on receipt, as described by Seki et al. (2003), with some minor modifications. Sterile sand was used to grind 0.5 g of the organ in a mortar and resuspended in 10 ml phosphate-buffered saline with calcium and magnesium (PBS plus). The suspension was centrifuged at 1800 × g for 5 min and the supernatant filtered through a 0.22 µm filter to remove bacterial and fungal contaminants. The filtrate was used for inoculation into cell cultures and the remaining fluid poured into 2 ml freezing tubes (Nunc) for storage. The remaining unprocessed tissues were stored at -80ºC for future use.

Vero.DogSLAM cells

Vero.DogSLAM cells were obtained from Dr Yusuke Yanagi, Kyushu University, Fukuoka, Japan and were grown in 25 cm^2 tissue culture flasks with minimum essential medium (MEM) supplemented with 7% foetal bovine serum and incubated in a 37°C incubator. The preparation of Vero.DogSLAM cells was described by Tatsuo et al. (2001) and Seki et al. (2003). Briefly, total RNA from canine PBMCs stimulated with 2.5 µg of phytohaemmaglutinin per ml was extracted and amplified using various combinations of the primers for human and marmoset SLAMs to yield dog SLAM cDNA. The dog SLAM cDNA was then subcloned into the eukaryotic expression vector pCAGGS, and the resulting construct was named pCAGDogSLAM. Vero.DogSLAM cells were then generated by transfecting Vero cells with the eukaryotic plasmid called pCXN2 and pCAGDogSLAM. The Vero.DogSLAM cells was then grown in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum with 0.5% sodium carbonate, 0.5 mg/ml gentamycin, 100 unit/ml penicillin and 100 µg streptomycin.

Virus isolation

Each specimen was individually inoculated at a volume of 0.5 ml into a 25 cm^2 plastic tissue culture flask containing a sub-confluent monolayer of Vero.DogSLAM cells. Each sample was inoculated in duplicate and uninoculated flasks used as negative controls were included in each run. The flasks were incubated in a 37°C humidified incubator and examined daily for cytopathic effects (CPE). CPE was observed as giant multinucleated syncytium formation and detachment of cells. Blind passages were done for those not showing CPE after 4 days. The presence of CDV was confirmed with a direct fluorescent antibody test and electron microscopy (EM), as described below.

Direct fluorescent antibody test

A direct fluorescent antibody test, as described by Maes et al. (2003), was used for canine distemper virus antigen detection. Ten µl of scraped cells from inoculated flaks showing CPE was placed on a blank 12-well Teflon slide and allowed to air dry. It was fixed in an acetone-alcohol mixture (75:25) for 10 min at room temperature and 10 µl of CDV-specific FITC antibody conjugate (VMRD, Inc) was added to each well and incubated in a humid chamber for 30 min at 37°C. A 0.16 M sodium carbonate solution was used as

Table 1. Samples analysed by viral isolation in Vero.DogSLAM cells.

Specimen	Whole blood	Spleen	Lung	Brain	CSF	Total
Total number tested	102	53	3	2	2	162
Number positive	21	10	0	2	0	33
Number negative	81	43	3	0	2	129

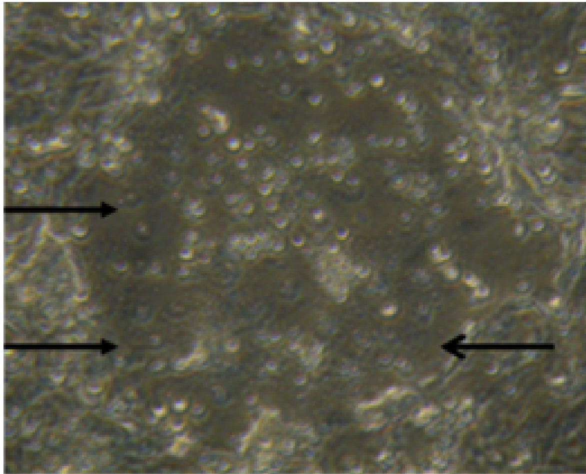

Figure 1a. Vero.DogSLAM cells infected by CDV from clinical samples, showing formation of giant cells [syncytia] (arrows).

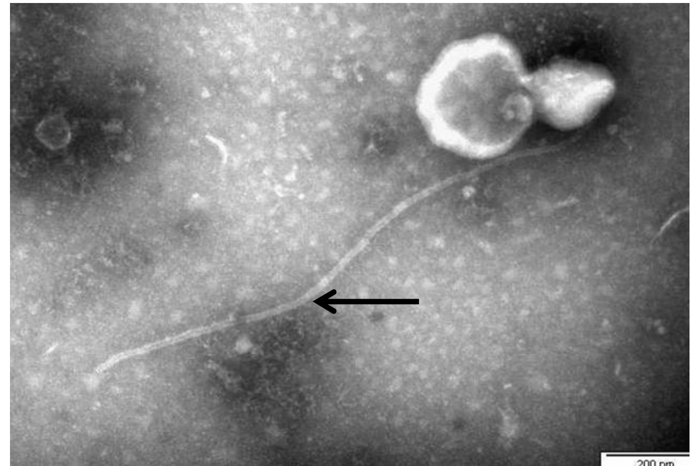

Figure 2. Herring bone appearance of the CDV nucleocapsid (arrow), as revealed by electron microscopy

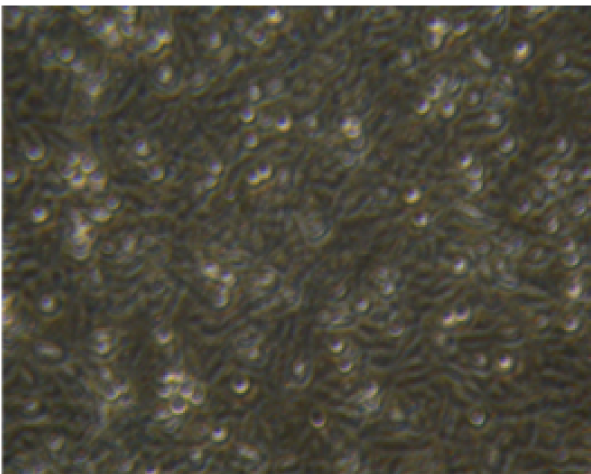

Figure 1b. Vero.DogSLAM cells uninoculated negative control.

wash buffer for 10 min in a magnetic stirrer. Mounting fluid, consisting of a buffer-glycerol mixture (50:50), was added to the slide and viewed with an epi-fluorescence microscope.

Electron microscopy

Two ml of tissue culture fluid from each flask with CPE was centrifuged at 2000 × g for 15 min and the supernatant further centrifuged at 14000 × g for 45 min. The supernatant was discarded and the remaining pellet rinsed with deionised water. One drop of deionised water was added to the pellet and mixed vigorously with a pipette. A drop of 3% phosphotungstic acid and pellet mixture was individually mixed in a tissue culture plate well and left to stand for 15 s. A formvar carbon coated grid (coated side under) was floated on top of the mixture and left for 15 s. The excess fluid on the grid was blotted onto a paper towel and allowed to dry. The grid was then examined using a Philips CM10 transmission electron microscope operated at 80 kilovolt.

RESULTS

The number of specimens processed, inoculated in tissue culture and the results are indicated in Table 1. Thirty three out of 162 (20.4%) samples were positive for CDV. The 33 specimens were obtained from 26 dogs. Thus, virus was isolated from 26 of 124 (21%) dogs studied. Cytopathic effects (CPE), seen as giant multinucleated syncytium formation (Figure 1a), was observed in positive specimens between 24 and 48 h of inoculation. Uninoculated negative controls included in each run did not show any effects (Figure 1b). The characteristic apple green fluorescence seen with the aid of the direct fluorescent antibody test confirmed the presence of CDV antigen. The herring bone appearance of the viral nucleocapsid observed with transmission electron microscopy (Figure 2) provided further confirmation of the presence of CDV. Of the 26 dogs that were positive in this study, eight sho-

wed systemic signs only (diarrhoea, vomiting, anorexia, emaciation, ocular discharges, depression, dehydration, leukopaenia, fever), three showed nervous signs only (jaw clamping, clonic-tonic/ temporal muscle contraction, muscle fasciculations) and two showed respiratory signs only (cough, purulent nasal discharges, dyspnoea, increased lung sounds). A combination of systemic and nervous (four dogs), systemic and respiratory (three dogs), nervous and respiratory (two dogs) signs were also noticed. One case of digital hyperkeratosis (so called 'hard pad' disease) was noted in a dog that also presented with bilateral uveitis, blepharospasm and ocular discharge. Three dogs displayed a combination of the catarrhal, systemic and nervous forms of the disease.

The two brain samples studied only yielded CPE after 48 h, while spleen samples from the same dogs yielded CPE within 24 h post-inoculation. However, the two CSF samples studied did not yield any CPE, despite the detection of CDV IgG in the CSF by the indirect fluorescent antibody test. Table 2 shows the vaccination history, clinical presentation, age, sex and breed distribution of the 26 positive dogs. The spleen sample from dog 7 did not yield CPE, but clear CPE was detected in the PBMCs from the same dog. Four (15.4%) of the 26 dogs had a history of prior vaccination to CDV. Others had no known vaccinetion history, or were never vaccinated. Expectedly, 19 (73%) of the positive dogs in our study were aged 12 months and below. Two (7.7%) were 24 months old, two were between 25 and 36 months old, while three (11.5%) were between 60 and 72 months old. Twelve (46.2%) of the positive dogs in our study were males, while 14 (53.8%) were females.

DISCUSSION

Canine distemper is endemic in Africa as in other parts of the world. Underreporting is a characteristic of almost every infectious disease in most developing countries. The clinical signs of distemper in some dogs are not typical, emphasising the need for a definitive diagnostic test that is rapid and reliable. Diagnosis of canine distemper in the live animal, although difficult, is important because of the global distribution, broad host range, diverse signs and history of mass mortality events associated with the disease.

Several approaches have been used for the diagnosis of canine distemper. The use of haematology has been reported (Cornwell et al., 1965), but changes in haematological parameters are non-specific and sometimes absent. Canine distemper virus-specific inclusion bodies may be present only during the viraemic stage and only in a small percentage of dogs (Shell, 1990). The use of serum chemistry has also been reported (Weisbrode and Krakowka, 1979), but biochemical changes are usually non-specific and may reflect other pathological changes such as dehydration or azotaemia (Shell, 1990). Increased protein levels and lymphocytic pleocytosis in CSF may support the diagnosis of CDV infection of the nervous system (Gorham, 1966; Gossett et al., 1982). However, not all dogs infected with CDV will show the latter changes.

The use of direct immunofluorescence for CDV diagnosis is not sufficiently sensitive and specific. It is prone to false negative results (Guy, 1986). Immunohistochemistry for CDV diagnosis may also produce false negative results, especially in cases of subacute and chronic infection because the virus is often fleetingly present in certain tissues (Vandevelde et al., 1985; Vandevelde and Zurbriggen, 1995).

The use of techniques such as the reverse transcription polymerase chain reaction (RT-PCR) represents a marked improvement on the classical CDV detection methods (Rima et al., 1985; Rzezutka and Mizak, 2002). However, direct RT-PCR of some tissues couldn't detect CDV, despite virus isolation from the same tissues in Vero cell cultures (Lednicky et al., 2004a; 2004b). Moreover, some laboratories in resource-poor parts of Africa lack the sophistication and technical expertise of present-day molecular diagnostic facilities.

The gold standard for the diagnosis of virus infections has for a long time been virus isolation in cell cultures (Bussell and Karzon, 1965; Cosby et al., 1981; Greene and Appel, 2006; Guy, 1986). The findings of this study confirmed that Vero.DogSLAM cells facilitate the isolation of virulent CDV from infected dogs as early as 1 day post-inoculation with clinical material, and there is a significant reduction in the costs associated with the isolation of wild-type CDV. The efficiency, ease and cost-effectiveness of using Vero.DogSLAM cells for confirmatory diagnosis (by viral isolation) of CDV should encourage wider use, especially in third world countries where cost and technical expertise may limit the use of present-day molecular diagnostic tools. The ability of laboratories to diagnose, isolate and store current field isolates of CDV has become more important since the host range of CDV appears to have broadened and interspecies transmission occurs, leading to epizootics with high mortality (Barrett et al., 1992; Kennedy et al., 2000; Van Moll et al., 1995). Characterization of wild-type field strains from Africa will assist with studies on the epidemiology of the virus. Virus isolation is important not only to confirm a diagnosis and provide material for direct sequence analysis, but also for investigation of the pathogenesis in animal experiments and vaccine improvements (Lednicky et al., 2004a).

Virus was not isolated from all submitted tissue specimens obtained from virus-positive dogs. The two brain samples studied (Table 2) yielded CPE only after 48 h, while spleen samples from the same animals yielded CPE within 24 h. The spleen sample from dog No. 6 (Table 2) did not yield CPE even though CPE was detected from the PBMCs of the same animal. Similarly, the CSF samples studied did not yield CPE, despite detection of CDV-specific IgG in the CSF by indirect fluorescent

Table 2. Description of the animals and samples positive for CDV by viral isolation in Vero.DogSLAM cells.

Dog No.	Sex	Age(Month)	Breed	Sample Type	Clinical Signs	Vaccination history
1	M	29	Papilon	Spleen, Brain†	Muscle fasciculation, jaw clamping	N
2	M	7	Afrkanis	Blood	Muscle contraction with ocular discharges	N
3	M	3	Dachshund	Blood	Cough, diarrhoea, vomiting, anorexia	N
4	F	2	Border Collie	Blood, Spleen	ocular discharge, coughing, twitching	N
5	F	12	Boerboel	Blood	Emaciated, cough, oculonasal discharge,jaw clamping	Y
6	M	5	Cocker	Blood, Spleen‡	Bilateral uveitis, blepharospasm, hyperkeratosis of right footpad,bilateral mcopurulent ocular discharge	N
7	F	4	Boerboel	Blood	Anorexia, fever, dehydration,vomiting	N
8	F	72	Dachshund	Blood	Nasal discharge,crusty eyes,lung sounds	N
9	M	3	Great Dane	Blood	Muscle contraction,crusty eyes and nose	N
10	F	36	Border Collie	Blood	Diarrhoea,leukopaenia, fever, temporal muscle contraction	N
11	M	6	Boerboel	Blood	Cough,dyspnoea, jaw clamping	Y
12	M	12	German Sheperd	Blood, Spleen	Muscular weakness, ocular discharge, depressed	N
13	F	4	Labrador	Blood, Spleen	Depressed,vomiting,bloody diarrhea ocular discharge,increased lung sound	Y
14	M	7	Afrkanis	Blood, Spleen	Mucopurnlent ocular discharge, dry crusty nose	N
15	F	12	Doberman	Blood, Spleen	Oculo-nasal discharge,vomting,diarrhoea, increased lung sound	N
16	M	12	Toypom	Spleen	Emaciated, Oculo-nasal discharge,depressed	N
17	F	24	Toypom	Spleen	Emaciated,depressed, mucopurnlent discharge	N
18	F	24	Dachshund	Blood	Cough, vomiting,haemorrhagic diarrhoea	N
19	F	5	Labrador	Spleen	Emaciated, Oculo-nasal discharge	N
20	M	60	Jack Russell/Terrier Cross	Blood	Purnlent nasal discharge, Cough, dyspnea	N
21	M	9	Jack Russell	Blood	Dyspnea, increased lung sound.muscle contraction	N
22	F	3	Border Collie	Blood, Spleen	Crusty Oculo- nasal discharge, depressed, dehydrated	N
23	M	12	Yorkie	Blood	Depressed, fever, dehydration, anorexia	Y
24	F	15	Dachshund	Blood, Spleen	Constantly crying, twitching of facial muscles,salivation	N
25	F	72	Dachshund	Blood‡, Spleen†	Vomiting, anorexia, Muscle fasciculation, mucopurnlent oculo-nasal discharge	N
26	F	12	Chihuahua	Blood	Emaciated, Vomiting, anorexia, fever	N

antibody testing. The inability of the CSF to cause CPE in cell cultures was probably due to the neutralizing effect of the IgG produced by the animal in response to the viral antigen present in the brain. Some specimens stored at 4°C for a period of more than 7days were still positive when tested, supporting the statement by Greene and Appel (2006) that CDV survives in the environment for weeks at near-freezing (0 to 4°C).

Improved vaccination has reduced the frequency and magnitude of canine distemper outbreaks (Chappuis, 1995). However, four of the 26 (15.4%) CDV-positive dogs in our study had been vaccinated against the virus. There are several reports of distemper outbreaks in which affected dogs had received CDV vaccines (Blixenkrone-Moller et al., 1993; Ek-Kommonen et al., 1997; Lan et al., 2006). Speculations to explain these cases are varied but include immuno-suppression, insufficient time after vaccination to develop immunity, improper refrigeration of vaccine, excessive exposure of vaccine to sunlight, maternally derived antibody, and an overwhelming challenge dose of virus (Blixenkrone-Moller, 1989; Blixenkrone-Moller et al., 1992; Iwatsuki et al., 2000). Most of the 124 dogs studied had unknown/unlikely vaccination status against distemper or other canine diseases. Thus, the conditions for sporadic large-scale canine distemper outbreaks are evident and, additionally, places a large wildlife resource at risk.

Headley and Graça (2000) did not find any sex discrimination in an epidemiological study of 250 cases in Brazil. However, Alex and Dhanapalan (1994) found that more males than females (60:40%) were infected in Madras city, India, while the contrary (45:55%) was reported in Texas, USA by Gou et al. (1986). Gorham (1966) did not find any breed predisposition to CDV infection. However, a Norwegian study found the relative distemper mortality rate low in the boxer, Pekingese and Scottish terrier, and high in the Samoyed, German shepherd and cocker spaniel (Erno and Moller, 1961). Other workers (Alex and Dhanapalan, 1994; Gou et al., 1986) have also indicated that a difference exists in breed susceptibility to distemper. Brachycephalic dogs have been reported to have a lower prevalence of disease, sequelae and morstality compared with dolichocephalic breeds (Gorham, 1966; Shell, 1990). Rockborn (1958) reported increased numbers of clinical cases of canine distemper virus infections in the fall and winter months, but other studies reported that the incidence of the disease is highest in the spring and early summer (Eckersley et al., 1992; Erno and Moller, 1961; Van Moll et al., 1995). Leisewitz et al. (2001) did not observe any seasonal incidence of CDV infection in a two-year retrospective study in South Africa. Thus, seasonal variation may or may not affect the incidence of disease.

The severity of canine distemper depends on the virulence of the virus, immune competence and age of the affected dogs. In this study, animals displayed a variety of clinical signs characteristic of canine distemper. This agrees with a two-year retrospective study of 133 CSF samples in South Africa where 34 (25%) were positive, of which 23, 4, and 2 had only nervous, systemic and respiratory signs, respectively. Four had a combination of nervous and systemic signs, 1 had both respiratory and systemic signs and no dogs were observed to have a combination of nervous and respiratory signs (Leisewitz et al., 2001). In Denmark, a distemper outbreak was reported in which half of the cases presented with respiratory signs. However, nervous signs and hyperkeratosis were also observed (Blixenkrone-Moller et al., 1993). In an outbreak amongst sled dogs in northern Greenland, a 33% mortality was reported with classical respiratory and nervous signs, and there was no digital hyperkeratosis but blindness was observed in a few survivors (Bohm et al., 1989). In a Finnish study, which reported 30% mortality, the majority showed classic respiratory signs and a few cases of digital hyperkeratosis (Ek-Kommonen et al., 1997). Lymphadenopathy has been reported among Nigerian dogs with distemper (Ezeibe, 2005). Since CDV invades lymphoid tissues (Zurbriggen et al., 1995), it was postulated that either a stimulation of the lymph nodes by the virus or attempts by host tissues to produce more lymphocytes to replace lost ones may have lead to the observation of lymphadenopathy in Nigerian dogs. There are many other reports of CDV outbreaks of varying severity among dog populations in several European countries. These reports demonstrate that despite the development of effective vaccines, CDV remains endemic in most parts of the world.

The CDV-negative samples in this study were obtained mostly from animals with respiratory and systemic signs. In many cases, animals are incorrectly diagnosed with canine distemper when showing other related clinical signs. These signs are usually due to other viral and/or bacterial agents (Demeter et al., 2007; Leisewitz et al., 2001). The clinical signs of some fungal and parasitic diseases may also be confused with distemper (Greene, 1998). Most early clinical signs of CDV infection can be misleading, necessitating the use of specific diagnostic tests such as PCR (where available) and isolation in Vero.DogSLAM cells for confirmatory diagnosis of CDV.

In conclusion, the results of this study demonstrated that the availability of Vero.DogSLAM cells makes virus isolation a useful adjunct for diagnosis of canine distemper, whereas previously virus isolation in cell cultures was time-consuming, difficult and expensive.

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