

A CANINE DISTEMPER VIRUS ISOLATED FROM AN AUTOPSIED DOG IN HANOI, VIETNAM

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ABSTRACT

The fresh samples of lung taken post mortem from an autopsied dog with evidence of canine distemper was used for isolation of canine distemper virus (CDV). The lung of the dog was congested by hemorrhagic and pneumonic, with much fluid or exudate. Severe interstitial pneumonia was found with some eosinophilic cytoplasmic inclusion bodies in alveolar epithelium, and CDV antigens strongly positive among alveoli. The CDV was isolated by using Vero cells with CDV receptor and was confirmed by RT-PCR of the 429 bp fragment P gene. The isolated strain CDV-HN1 had the titer of 3.16×10^5 TCID₅₀ / 25 µl. The results indicated that the dog population in Hanoi was infected with CDV.

Key words: Canine distemper; dog; isolation; Hanoi, Vietnam.

1. INTRODUCTION

Canine distemper disease is a fatal disease in dogs, ferrets and wild carnivores caused by Canine distemper virus (CDV). The first target of CDV is lymphoid tissues, causing immunosuppression, later the virus spreads to epithelial and nervous tissues respectively. CDV is a member of genus *Mobillivirus* in the *Paramyxoviridae* family and closely related to measles virus and Phocine distemper virus (PDV). The *Mobillivirus* is composed of 6 protein genes. Fusion (F) and Hemagglutinin (H) glycoprotein are envelope proteins. H protein is a receptor binding site and F protein induces the fusion between viral envelope and host cell membrane. Nucleocapsid protein (N), Phosphoprotein (P) and Large protein (L) are a nucleocapsid core, cooperating in viral replication. Matrix (M) protein assembles between the envelope proteins and the nucleocapsid core (Griffin and Bellini, 1996).

CDV can be isolated by using co-cultivation of lymphocytes from SPF dogs with mitogen-stimulated canine lymphocytes (Appel

et al., 1992) or ferret peritoneal macrophages from SPF ferrets (Poste et al., 1971; Whetstone et al., 1981). MDCK (Canine epithelial kidney) cells were used to isolate CDV from clinical samples successfully but the development of CPE was delayed (Lednicky et al., 2004). B95a cells derived from marmoset B lymphoblastoid cell line were susceptible to CDV (Kai et al., 1993). Field isolates are not propagated easily in Vero cells derived from African green monkey kidney cells (Appel et al., 1992). In Vero cells, no virus was isolated from dogs (Seki et al., 2003). It was shown that Vero cells with CDV receptor were established and very sensitive for isolation of wild CDV strains compared to B95a cells (Seki et al., 2003). So far, we believe no reports exist of CDV in Vietnam. Therefore, in this study, we reported the pathological findings of a distemper dog and isolated the CDV from this autopsied dog in Hanoi, Vietnam. After isolation of virus, we will examine the characteristics of virus for further purposes and further molecular and vaccine studies.

2. MATERIALS AND METHODS

Samples

Tissues of lung, pulmonary lymph nodes, cerebellum, cerebrum and brain stem selected for this study were collected at necropsy from a three month-dog having the pathological changes consistent with canine distemper, supported by the immunohistochemical demonstration of CDV antigens. These tissues were stored at -80°C.

Cell lines and cultures

Vero cells with CDV receptor generated by transfecting Vero cells with pCXN2 and pCAGDogSLAMtag (DST) (Seki et al., 2003) supplied by Kuyshu University. They were grown in DMEM with 10% FCS, 10% sodium carbonate, 100 units/ml of penicillin and 100µg/ml of streptomycin and 0.4 mg of geneticin (G418) per ml in incubator at 37°C under 5% CO₂.

Pathological examination and immunohistochemistry

After necropsy, samples were fixed with 10% formalin and were embedded in paraffin wax. Sections (4 µm) were cut and stained with haematoxylin and eosin. Immunohistochemistry was done by using a mouse monoclonal antibody specific for CDV-nucleoprotein and an Envision polymer reagent (DAKO Corporation, USA) as described previously (Kumagai *et al.*, 2004).

Virus isolation

The homogenized and sonicated fresh sample of lung treated to obtain only virus suspension was inoculated into petri-dishes seeded with Vero cells with CDV receptor. After adsorption for 30 min, the cells were incubated in 5% CO₂ at 37°C. CPEs were daily observed by phase contrast microscope.

Virus titration

Virus titration was performed with 50% tissue culture infectious dose (TCID₅₀) assay. Sample of CDV isolated from lung tissue in

Vero cells with CDV receptor were serially diluted in 10 fold steps. Twenty-five µl of each dilution was inoculated into each of 3 wells of 96 well plates with Vero-DST cells (Yamaguchi *et al.*, 1988). TCID₅₀ was calculated by the method of Behres- Karber.

Reverse transcription reaction (RT-PCR)

Total RNA was extracted from CDV - infected Vero cells with CDV receptor by using Trizol reagent (Invitrogen) according to the manufacture's instructions. A mixture of 1 µl (150 ng/µl) of random hexanucleotide primers, 5 µl of RNA and 4 µl of DEPC treated water was denatured at 70°C for 5 min and incubated at room temperature for 10 min. The cDNA was synthesized in 20 µl of reaction mixture containing 10 µl of annealed RNA-random hexamer mixture, 2.5 µl of DEPC treated water, 4 µl of RT buffer, 2 µl of 0.1M DTT, 1 µl of 10mM dNTP (dATP, dCTP, dGTP, dTTP) and 0.5 µl of reverse transcriptase (Superscript II) (Invitrogen). Reverse transcription reaction was performed at room temperature for 5 min and 37°C for 40 min. Primer set used for polymerase chain reaction (RT-PCR) was upp1 and upp2 (Lan *et al.*, 2005). cDNA of Onderstepoort strain propagated in Vero-DST cells was the positive control, and mixture without the matrix was the negative control of the reaction. The electrophoresis of 10 µl of PCR products was performed in a 1.2% agarose gel. The size of amplicons was compared with a 100bp DNA ladder.

3. RESULTS

Pathological findings

The necropsied dog had a history of coughing, nasal, ocular discharge and emeciation.

The lung was congested by hemorrhage and pneumonia, with much fluid or exudate (Fig. 1). Lung section showed severe interstitial pneumonia with some eosinophilic cytoplasmic inclusion bodies in alveolar

epithelium. CDV antigens strongly positive among alveoli (Fig. 2a,b).

Lymph nodes were grossly enlarged. Microscopically, lymph nodes showed lymphoid depletion of the tissue or necrosis. The intestine showed enteritis with eosinophilic

intracellular inclusion bodies in the intestinal crypts and gastric glands. CDV antigens were strongly positive in epithelial cells detected by immunohistochemistry. No significant lesions were observed in the nervous system.



Fig. 1. The lung of autopsied dog was congested by hemorrhage with pneumonia.

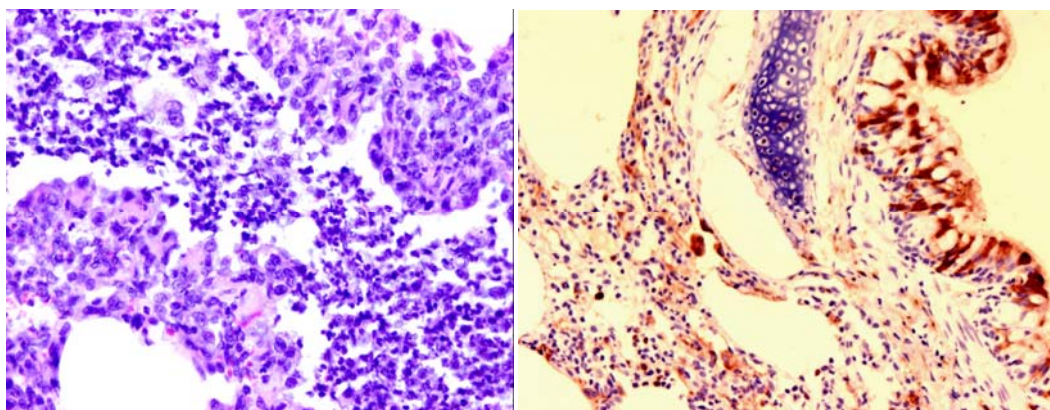


Fig. 2. The histopathological findings of the canine lung. Severe interstitial pneumonia was found with some eosinophilic cytoplasmic inclusion bodies in alveolar epithelium (a) (x40); CDV antigens (shown by brown color) were strongly positive among alveoli (b) (x 10).

Isolation and titration of new isolates

The samples collected from the lung of the autopsied dog were inoculated into Vero cells with CDV receptor. CPE characterized by syncytium formation occurred from 24 hpi. The number and size of syncytia were increased gradually (Fig. 3) The isolated CDV was confirmed by RT-PCR with the 429 bp fragment

P gene and immunocytochemistry (Figure 4) and called CDV-HN1. CDV in fresh sample of lung was also checked by RT-PCR.

After successful isolation in Vero cells with CDV receptor, the titers of strain CDV-HN1 were checked by TCID₅₀ with same cell line. The titer of strain CDV-HN1 was 3.16×10^5 TCID₅₀/ 25 μ l.

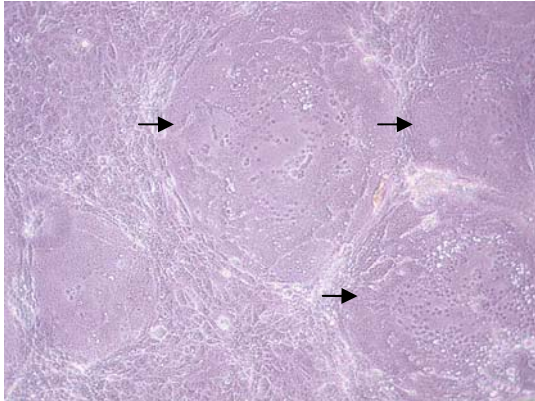


Fig. 3. CPE caused by CDV in Vero cells with CDV receptor was characterized by syncytium formation and occurred at 24 hpi.

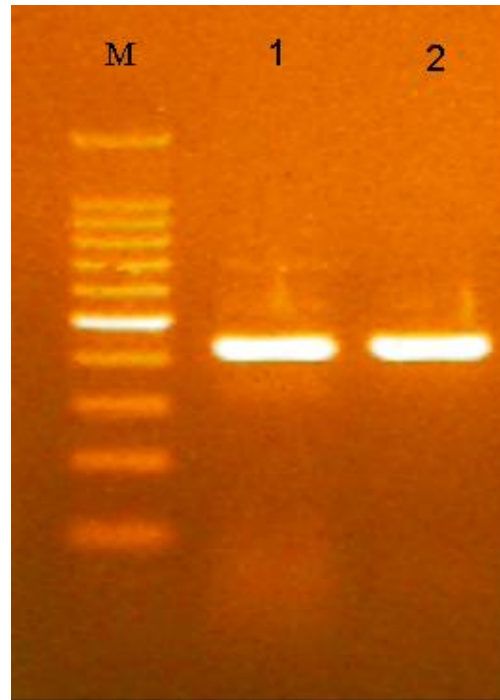


Fig. 4. The isolated CDV was detected by RT-PCR of the 429 bp fragment P gene. Lane M: 100bp DNA Marker; Lane 1: Fresh sample of lung used for RT-PCR; Lane 2: Sample from Vero cells with CDV receptor infected with CDV showing CPE.

4. DISCUSSION

These results confirmed that Vero cells with CDV receptor were extremely efficient for isolation of CDV from clinical samples compared to Vero cells. Mitogen stimulated canine lymphocytes was effective for isolation of CDV from field cases (Appel *et al.*, 1992). However, it is not easy to get lymphocytes from SPF dogs. Recently, B95a cells (Kai *et al.*, 1993) and MDCK cells (Lednicky *et al.*, 2004) have been using for isolation of CDV successfully. However, the isolation rate was low and the development of CPE required a long time. Two amino acids in H protein of the viruses isolated from this cell line are different from those of the isolated viruses from Vero-DST cells (Seki *et al.*, 2003).

It was reported that CDV could be titrated by plaque forming unit (PFU) in Vero cells

(Johnson *et al.*, 1985). We tried to titrate new isolates in Vero cells and Vero-DST cells. Plaques were not produced in Vero cells. In contrast, large, untidy plaques were formed in Vero-DST cells and could not be counted. The adherent nature of Vero-DST cells is probably too sensitive for forming syncytia. In Vero-DST cells, we could not only isolate CDV but also identify the titer of virus from clinical samples easily.

Recently, there are two groups of CDV circulating in Asia (Lan *et al.*, 2006). However, in order to understand which group that the CDV-HN1 strain belongs to, the molecular characteristics of the virus should be done in the further study.

In conclusion, the results of present study indicated that CDV was circulating in the dogs in Hanoi, Vietnam.

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