CHAPTER III

MATERIALS AND METHOD

1. Animals

Total 9 specimens came from different clinical cases. Seven cases were provided by division of Virology (sample number 1, 3, 5, and 12 were collected between October 2001 and December 2002, and number 14, 15 and 16 were collected in year 2004). Eight tissue specimens were collected from 2 necropsy cases (number 270 and 290) that submitted between July 2006 and November 2006 at division of Pathology, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand.

The 9 cases of dogs showed clinical signs of respiratory, digestive, nervous system and clinical diagnosed as CDV infection. After the dogs died, they were submitted to the division of Pathology for routine necropsy examination, history such as vaccinations and important clinical signs were noted. During necropsy, gross lesions were taken and sample specimens including lymph nodes, lung, spleen, intestine and brain were collected either fresh or fixed in 10% formalin. Those fresh tissue samples were kept at -80°C for virological study.

2. Histopathological examination

2.1 Histopathological study

Samples were fixed in 10% buffered formalin and routine histologic processed, embedded in paraffin wax, sectioned at 4 µm thickness and stained by Hematoxylin and Eosin (H & E). Sections were observed under light microscope for observed eosinophilic intracytoplasmic and/or intranuclear viral inclusion body in epithelium cells, mononuclear cells, neurons and other lesions in lymph nodes, lung, spleen, intestine and brain.

2.2 Immunohistochemical study

Immunohistochemical staining was performed for detecting CDV antigen using a labeled streptavidin-biotin (LSAB) method, section slides were deparaffinized in xylene for 30 minutes and antigen retrieved by autoclaved at 121°C for 5 minutes in distilled water. Followed by blocking endogenous peroxidase reaction with 3% hydrogen peroxide (H_2O_2) in methanol for 5 minutes, and slides were washed with phosphate buffer saline (PBS) for 5 minutes, 3 times. Primary antibody, Monoclonal mouse anti – CDV antibody (Monotope Verostat®, USA) at dilution 1: 200 was applied onto the section and incubated at 37°C for 60 minutes, after that washed in PBS for 5 minutes, 3 times. Biotinylated anti-mouse IgG antibody and envision polymer (Envision Polymer DAKO®, Denmark) was used as secondary antibody and section was incubated at 37°C for 30 minutes, washed by PBS 5 minutes for 3 times. The sections were stained with 3, 3-diaminobenzidine tetrahydrochloride (DAB) for 2 minutes, running water for 5 minutes and counterstained with Mayer's hematoxylin. Positive control was a brain section from CDV previously diagnosed necropsy case (Kumakai et al., 2004).

3. Virus isolation

3.1 Virus isolation and titration

0.1 gram of fresh tissue samples; kept in -80°C (as described above), were homogenized with 1 ml of Dulbecco's Modified Eagle's medium (DMEM) with 10% antibiotic (penicillin 100 units/ml, streptomycin 100 µg/ml) in homogenizer (Figure 2). Homogenized samples were sonicated for 7-8 minutes, centrifuged at 4°C for 4 minutes and supernatant was used for virus isolation in monolayer Vero-DST.

Vero-DST cell line was grown in DMEM with 5% fetal calf serum (FCS), penicillin 100 units/ml, streptomycin 100 µg/ml and geneticin (G418) 0.4 mg/ml and incubated at 37°C in a 4% CO_2 incubator.

For virus isolation, monolayer of Vero-DST (Figure 3) was prepared in 24-well culture plates (Figure 4), previous growth medium was aspirated and various concentrations of viral suspensions were overlaid on the cells. After 30 minutes incubation, maintenance medium of 10% tryptose phosphate broth (TPB) was added to each well (1ml/ well). Cytopathogenic effect (CPE) was observed daily by phase – contrast microscope (Figure 5). If CPE was observed, each well viral suspension was kept in -70°C, propagated in Vero-DST cell line. The virus titration was performed in 96 well culture plates by using a 50% tissue culture infectious dose ($TCID_{50}$) assay and calculated by Behrens-Karber method (Yamaguchi et al., 1998).

3.2 Reverse transcriptase-polymerase chain reaction (RT–PCR)

Total RNA was extracted from CDV-infected Vero-DST cells and supernatant from homogenized samples. Five hundred microlitres of sample was lyzed with 500 µl of RNA extraction solution (Trizol®, Invitrogen, USA), kept in -70°C overnight, then mixed with 140 µl of chloroform and centrifuged at 13,200 rpm for 15 minutes at 4°C. The aqueous phases was transferred into new tube, add 340 µl of isopropyl alcohol and 0.5 µl of glycogen, mixed and kept in room temperature for 15 minutes. The solution was centrifuged at 13,200 rpm for 15 minutes at 2-8°C, RNA pellet was washed by 70% ethanol 500 µl, centrifuge at 13,200 rpm for 15 minutes 2-8°C, discarded supernatant, dried at room temperature for 20-30 minutes, dissolved in 20 µl of deionized water without shaking and kept in -20°C until used.

RT-PCR was done by using a one-step RNA PCR Kit (AMV) (Takara Bio Inc., Japan). Oligonucleotide primers were specific to the region on H gene and P gene (Table 1). For 1824 base pair (bp) fragment of H gene, the forward primer was CDV-HS1 and the reverse primer was CDV-HS2. For 390 bp fragment of P gene, the forward primer was Upp1 and the reverse primer was

Upp2. Each PCR mix was 50 µl in volume containing 5 µl of x10 Buffer, 10 µl of $MgCl_2$, 5 µl of dNTP, 1 µl of RANse inhibitor, 1 µl of AMV RTaseXL, 1 µl of AMV-taq, 2 µl of forward primer, 2 µl of reverse primer, 2 µl of RNA sample and 21 µl of RNAse-free water. The PCR mixes was placed in thermoregulator PC-802 (Astec®, Japan), thermocycling conditions for amplification was 50°C for 30 minutes and 94°C for 2 minutes. Followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C 1 minute for P gene and 4 minutes for H gene, final extension was done at 72°C for 5 minutes. The PCR products were applied in 1.2% agarose gel (Invitrogen, USA) and electrophoreses in 1x Tris borate EDTA (TBE) buffer, pH 7.6 at electric gradient 100 volts for 30-40 minutes in minigel apparatus, gel were stained with 10% ethidium bromide and observed under an UV illuminator.

Table 1 Primers for RT-PCR and sequence analyzes of CDV P and H genes

Gene	Primer	Sequence (5'-3')	Nucleotide position
P	Upp1	ATGTTTATGATCACAGCGCGGT	2132-2149
P	Upp2	ATTGGGTTGCACCACTTGTC	2560-2541
H	CDV-HS1	AACTTAGGGCTCAGGTAGTCC	7054-7074
H	CDV-HforD	GACACTGGCTTCCTTGTGTGTAG	7948 -7970
H	CDV-hf1	TGTGTGTAGAAGAGAGCACTGT	7962-7983
H	CDV-Hr2	GTTCTTCTTGTTTCTCAGAGG	8198 -8178
H	CDV-HS2	ATGCTGGAGATGGTTTAATTCAATCG	8994-8969

3.3 Sequence and Phylogenetic analyses

3.3.1 Sequencing

Positive PCR products were purified by using QIA quick® PCR purification kit (Qiagen, Netherland) according to the manufacturer's

instructions. Briefly, buffer PBI 200 µl was added with 40 µl PCR products, mixed and put in spin column and centrifuged 13,000 rpm for 1 minute. The mixture was discarded flow-through, pellet was washed with 750 µl buffer PE and centrifuged 13,000 rpm for 1 minute, discarded flow-through and placed column in 1.5 ml microcentrifuge tube. Eluting DNA was done by added 50 µl buffer EB in the center of column waited for 1 minute and then centrifuged 13,000 rpm for 1 minute. Purified samples were kept at -20°C. The purified DNA fragments were directly sequenced by using Big Dye® Terminator V3.1 cycle sequencing kit (Applied Biosystems Inc., CA). Each mixture was 20 µl in volume; contain 2 µl of premix, 3 µl of x5 buffer, 0.32 µl of each primer, 3 µl of DNA and 11.68 µl of distill water. Primer Upp1 and Upp2 primer were used for P gene PCR products and CDV-HS1, CDV-HS2 primer were used for H gene and primer CDV-Hf1, CDV-HforD and CDV-Hr2 as an internal H gene sequence primer (Table 1). All mixtures were put in thermoregulator, thermocycling conditions were 96°C for 1 minute and then 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. Sequence products were added with 50 µl 99% ethanol and 2 µl of sodium ACE 3 M, spin down and incubated at room temperature for 20 minutes. The products were centrifuged at 13,200 rpm for 10 minutes, completely aspirated, added 200 µl of 99% ethanol, centrifuged 13,200 rpm for 10 minutes, completely aspirated and dried at room temperature for 20-30 minutes. The products were mixed with 25 µl Hi-Di, mixed, spin down, incubated at 95°C for 2 minutes then immediately put in ice. The samples were transferred to sequence plate and performed by DNA core facility (Faculty of Medicine, University of Miyazaki, Japan).

3.3.2 Sequences analyses

Sequence analysis was done by using the Clustal W program (DDBJ). The nucleotide sequences of Thai CDV isolates were compared

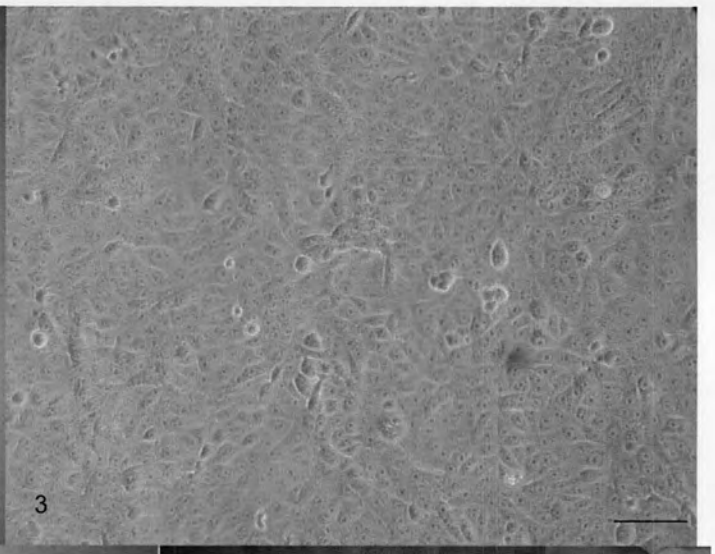
to other reference isolates in the P and H gene regions by the percentage of homologous nucleotides.

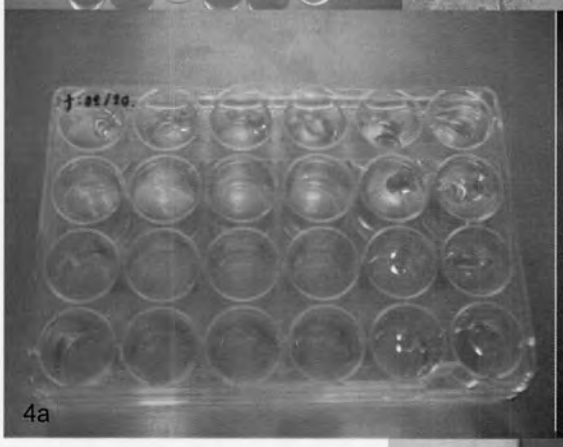
The nucleotide sequences accession numbers in the Genbank database of P gene sequences of reference strains are: Onderstepoort (AF305419), Rockborn (AF181446), SnyderHill (AY286481), Yanaka (AB028915), S124C (AB212961), 007Lm (AB212728), 009L (AB252714), 01-2689 (AY286488), A75/17 (AF164967), Bulgarian (AF259549) and 5804(AY386315).

The nucleotide sequences accession numbers in the Genbank database of H gene sequences of reference strains are: Onderstepoort (AF378705), Convac (Z35493), SnyderHill (AF259552), Yanaka (D85755), Ac96I (AB212963), KDK1 (AB025271), 007Lm (AB212728), 009L (AB252718), 01-2689 (AY649446), DogIsolateCDen (AF478547) and 00-2601 (AY443350).

3.3.3 Phylogenetic analyses

A phylogenetic tree was drawn by using the PHYLIP 95 software package with the neighbor – joining method. The phylogenetic trees were compared with those of the reference strains from Genbank, as described in sequence analysis.





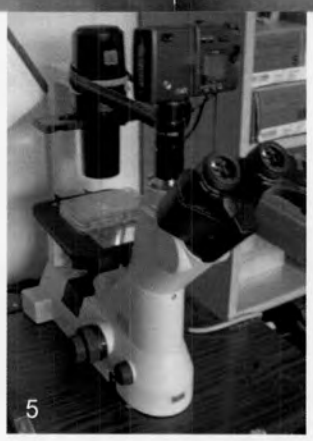


Figure 2: Homogenizer used to homogenize the tissue samples.

Figure 3: This picture shows a monolayer of normal Vero-DST cell line (bar=100um).

Figure 4: This picture shows the 24 well culture plate (4a), the 5 ml culture bottle and 20 ml culture bottle (4b) used for culture Vero-DST cell and CDV.

Figure 5: Phase contrast microscope used for observe the cultured cell.