

## CHAPTER IV



## RESULTS

### 1. Specimens

All 9 clinical cases described above were positive for RT-PCR in P gene region. The nine cases were from 2 male puppies, 4 female puppies and 3 dogs that had no information. The ages of the dogs were between 2-6 months (table 2).

Table 2 General information of the samples.

Dog No.	Breed	Age	Sex	CDV Vaccination
1	Mongrel	2 months	Male	MD
3	Shih Tzu	6 months	Female	Vaccinated 2 times
5	Siberian Husky	2 months	Female	MD
12	Mongrel	2 months	Male	MD
14	MD	MD	MD	MD
15	MD	MD	MD	MD
16	MD	MD	MD	MD
270	Golden Retriever	2 months	Female	MD
290	Mongrel	4 months	Female	MD

Note: MD: Missing data

Clinical signs such as coughs and purulent nasal discharge were found in 3 dogs, diarrhea was found in 4 dogs, 2 dogs experienced convulsion, fatigue and vomiting, anemia found in one dog and hard foot pad found in one dog. Gross lesion showed pneumonia in 5 dogs, enteritis in 4 dogs, fatty change of liver in 3 dogs and lymphadenitis in one dog (Table 3).

Table 3 Clinical signs and gross lesions

Dog No.	Clinical signs	Gross lesion
1	Fatigue, purulent nasal discharge, anemia, diarrhea	Pneumonia, enteritis
3	Cough, purulent nasal discharge, diarrhea	Pneumonia, hydrothorax(10 ml), catarrhal enteritis
5	Convulsion, cough, bloody diarrhea, hard foot pad	Pneumonia, fatty change of liver, parasitic infection
12	Convulsion, cough, vomiting, fatigue, epistaxis	MD
14	MD	MD
15	MD	MD
16	MD	MD
270	Vomiting and bloody diarrhea	Pneumonia, lymphadenitis, enteritis
290	Purulent nasal discharge, increase lung sound	Pneumonia, enteritis, fatty change of liver

Note: MD: Missing data

## 2. Histopathological examination

### Histopathological and immunohistochemical study

The histopathological and immunohistochemical of CDV antigen results are summarized in table 4. In this study, tissue samples were collected retrospectively.

Brain; cerebrum of dog 270 showed moderate neuronal necrosis and cerebrum of dogs number 5, 12, 14, 15, 16, 270 and 290 showed subacute non-suppurative encephalitis in different degree. Cerebellum showed subacute non-suppurative encephalitis. Demyelination was presented in the white matter and eosinophilic intranuclear inclusion nodules was presented in glial cells.

Immunolabeled CDV antigens were diffuse positive in astrocytes and neurons of dog 270 but only a few antigens found in dog 290 (Figure 6).

Lung section, severe interstitial pneumonia (case 270) and bronchointerstitial pneumonia (case 290) was found with eosinophilic cytoplasmic inclusion bodies in alveolar macrophages, pneumocyte type 2, bronchial and alveolar epithelium and CDV antigens also found extensively strong positive in those cells (Figure 7).

Gastrointestinal tract section showed catarrhal enteritis in 4 samples (case 1, 3, 270 and 290). CDV antigens were broadly strong positive in mucosal epithelial (Figure 8). Liver showed severe diffuse vacuolar degeneration (case 270) and mild focally degeneration (case 290). Immunolabeled CDV antigens were focally mild positive in both cases (Figure 9).

Spleen samples showed severe lymphoid depletion with hemosiderosis and histiocytic splenitis (case 270 and 290) necrosis of the white pulps of spleen and eosinophilic intracytoplasmic inclusion bodies (case 290). Immunohistochemistry showed strong broadly positive of CDV antigens especially within white pulps (case 270 and 290) (Figure 10). Lymph nodes showed severe lymphoid depletion with hemorrhage and CDV antigens were focally moderate positive mononuclear cell (Figure 11).

Table 4 Results of histopathology and immunohistochemistry of the CDV cases.

Dog No.	Sample name	Organ	Histopathology	Immunohistochemistry
1	1 IN	Intestine	Severe catarrhal enteritis.	Strong positive in mucosal epithelial cells.
	-	Lung	Suppurative bronchopneumonia with intracytoplasmic inclusion bodies in bronchial epithelium.	Strong positive in mononuclear cells and epithelial cells.
	-	Lymph node	Lymphoid necrosis.	ND
3	3 IN	Intestine	Moderate catarrhal enteritis.	Strong positive in mucosal epithelial cells.
	-	Brain	Brain congestion and some neuronal necrosis and hemorrhage.	Mild positive in neurons.
	-	Lung	Necrotizing suppurative pneumonia with intracytoplasmic inclusion bodies.	Strong positive in mononuclear cells and epithelial cells.
	-	Urinary bladder	Intracytoplasmic inclusion bodies in transitional epithelial cells.	ND
5	5 BR	Brain	Mild non-suppurative encephalitis.	Mild positive in neurons.
	-	Lung	Pneumonia with intracytoplasmic inclusion bodies in bronchial epithelium.	Moderate positive in mononuclear cells and epithelial cells.
	-	Liver	Severe diffuse hydropic degeneration.	ND
12	12 BR	Brain	Mild non-suppurative encephalitis.	Mild positive in neurons.
	-	Lung	Mild alveolar hemorrhage.	Moderate positive in mononuclear cell and epithelial cells.
	-	Liver	Mild panlobular necrosis and activated of Kuffer's cells.	ND
14	14 BR	Brain	Mild non-suppurative encephalitis.	Negative
15	15 BR	Brain	Mild non-suppurative encephalitis.	Negative

Dog No.	Sample name	Organ	Histopathology	Immunohistochemistry
16	16 BR	Brain	Mild non-suppurative encephalitis.	Negative
270	270 BR	Brain	Subacute non-suppurative encephalitis with eosinophilic intranuclear inclusion bodies in glial cells.	Mild positive in neurons and astrocytes.
	270 LU	Lung	Severe subacute interstitial pneumonia with eosinophilic intracytoplasmic inclusion bodies in pneumocyte type 2 and bronchial epithelium.	Strong positive in mononuclear cells and epithelial cells.
	270 SP	Spleen	Severe histiocytic splenitis with lymphoid depletion.	
	270 LI	Liver	Severe congestion and mild fatty degeneration.	Strong positive in mononuclear cells.
	-	Intestine	Severe catarrhal enteritis.	Mild positive in mucosal epithelial cells.
	-	Lymph node	Lymphoid necrosis, histiocytic lymphadenitis.	Strong positive in mononuclear cells.
290	290 BR	Brain	Subacute non-suppurative encephalitis with eosinophilic intranuclear inclusion bodies in glial cells.	Strong positive in neurons and astrocytes.
	290 SP	Spleen	Severe histiocytic necrotic splenitis, lymphoid depletion with eosinophilic intracytoplasmic inclusion bodies in splenocytes.	Strong positive in mononuclear cells.
	290LI	Liver	Severe congestion and mild fatty degeneration.	Mild positive in hepatocytes.
	290LN	Lymph node	Lymphoid necrosis, histiocytic lymphadenitis.	Strong positive in mononuclear cells.
	-	Lung	Severe subacute bronchointerstitial pneumonia with eosinophilic intracytoplasmic inclusion bodies in pneumocytes type 2 and bronchial epithelium.	Strong positive in mononuclear cells and epithelial cells.
	-	Intestine	Severe catarrhal enteritis.	Strong positive in mucosal epithelial cells.

(Note: : referred from Keawcharoen, 2002; ND: not done )



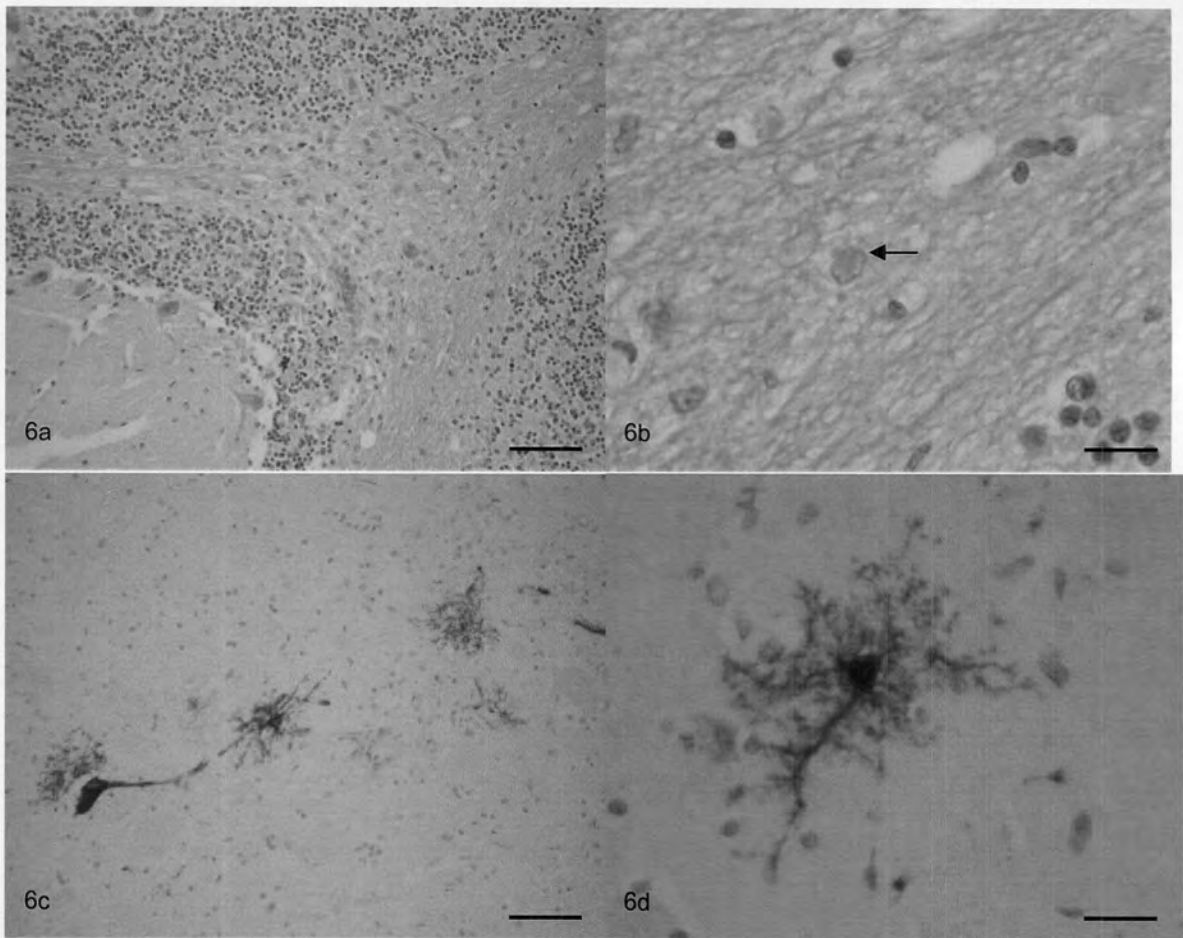


Figure 6a: This picture shows focal demyelination in cerebellum (case 290), (H&E staining, bar = 100  $\mu$ m).

Figure 6b: The intranuclear inclusion bodies were found in glial cell (arrow), (case 290), (H&E staining, bar = 10  $\mu$ m).

Figure 6c: The infected astrocyte in brain tissue (case 290), (LSAB method, counterstained with Meyer's Hematoxylin, bar = 40  $\mu$ m).

Figure 6d: The infected astrocyte in brain tissue (case 290), (LSAB method, counterstained with Meyer's Hematoxylin, bar=20  $\mu$ m).

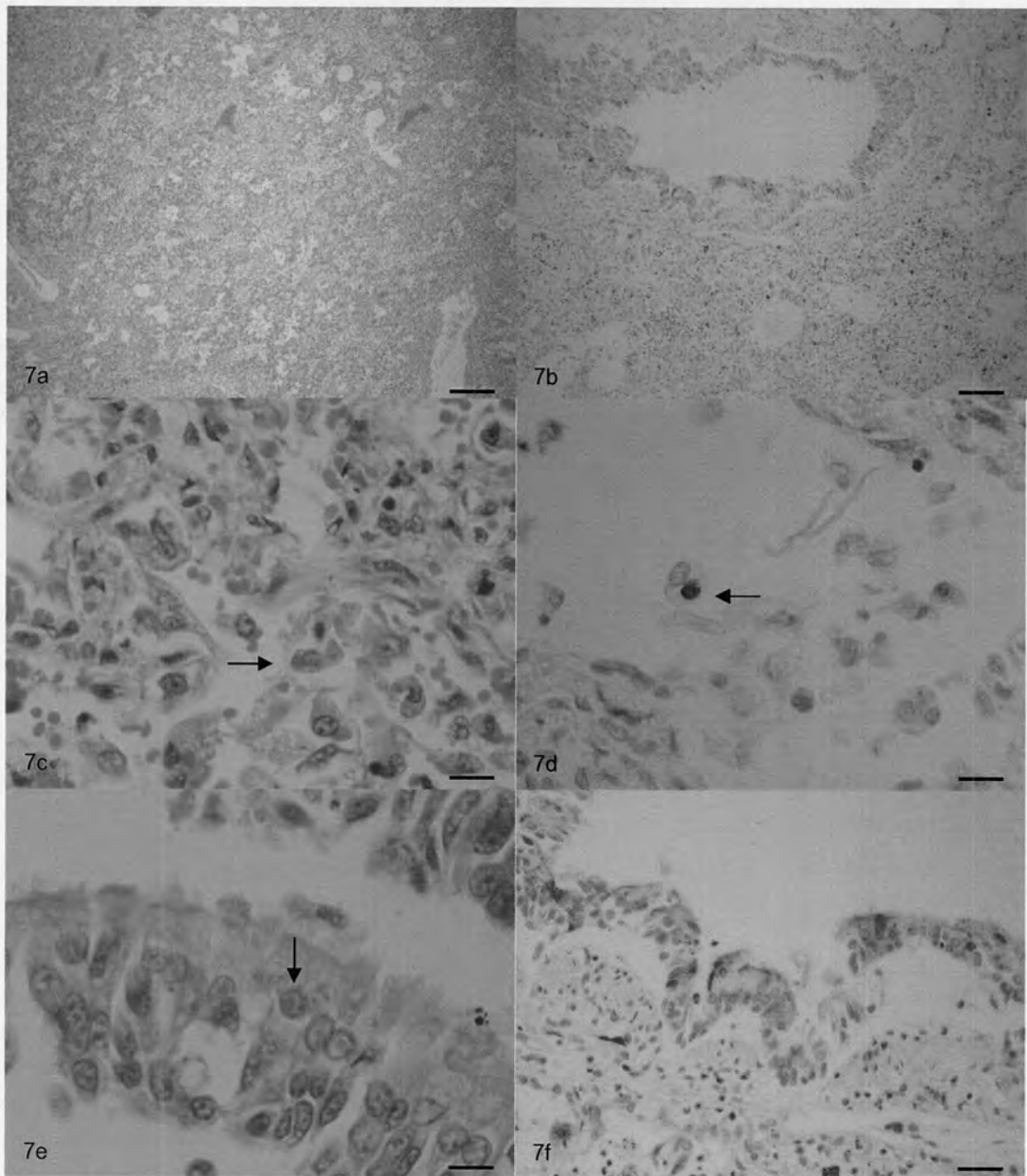


Figure 7a: This picture shows moderate interstitial pneumonia (case 290), (H&E staining, bar = 200 um).

Figure 7b: The CDV antigens were broadly positive in lung tissue (case 290), (LSAB method, counterstained with Meyer's Hematoxylin, bar = 200 um).

Figure 7c: Intracytoplasmic inclusion bodies were found in mononuclear cells (arrow) (case 290), (H&E staining, bar= 20 um)

Figure 7d: CDV antigens were positive in some mononuclear cells (arrow) (case 290), (LSAB method, counterstained with Meyer's Hematoxylin, bar= 20 um).

Figure 7e: Intracytoplasmic inclusion bodies were found in bronchial epithelial cells (arrow) (case 290), (H&E staining, bar= 20 um).

Figure 7f: CDV antigens were positive in bronchial epithelium (case 290), (LSAB method, counterstained with Meyer's Hematoxylin, bar= 100 um).

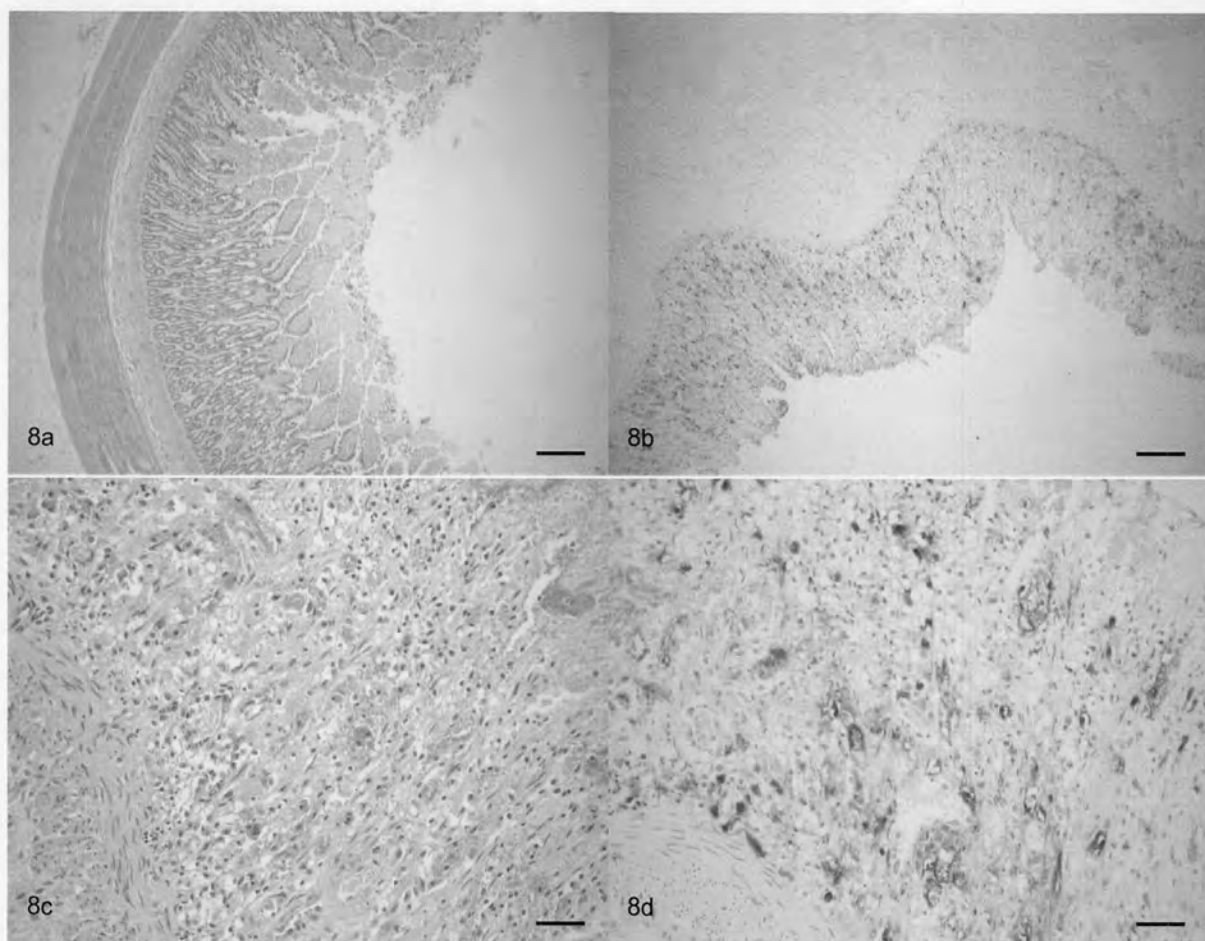


Figure 8a: This picture shows mild catarrhal enteritis (case 270), (H&E staining, bar=250um).

Figure 8b: CDV antigens were positive in the mucosal epithelium (case 270), (LSAB method, counterstained with Meyer's Hematoxylin, bar=200um).

Figure 8c: This picture shows mucosal epithelium hemorrhage and mononuclear cells infiltrated in the mucosal epithelium (case 270), (H&E staining, bar=100um).

Figure 8d: CDV antigens were positive in the mucosal epithelium (case 270), (LSAB method, counterstained with Meyer's Hematoxylin, bar=100um).



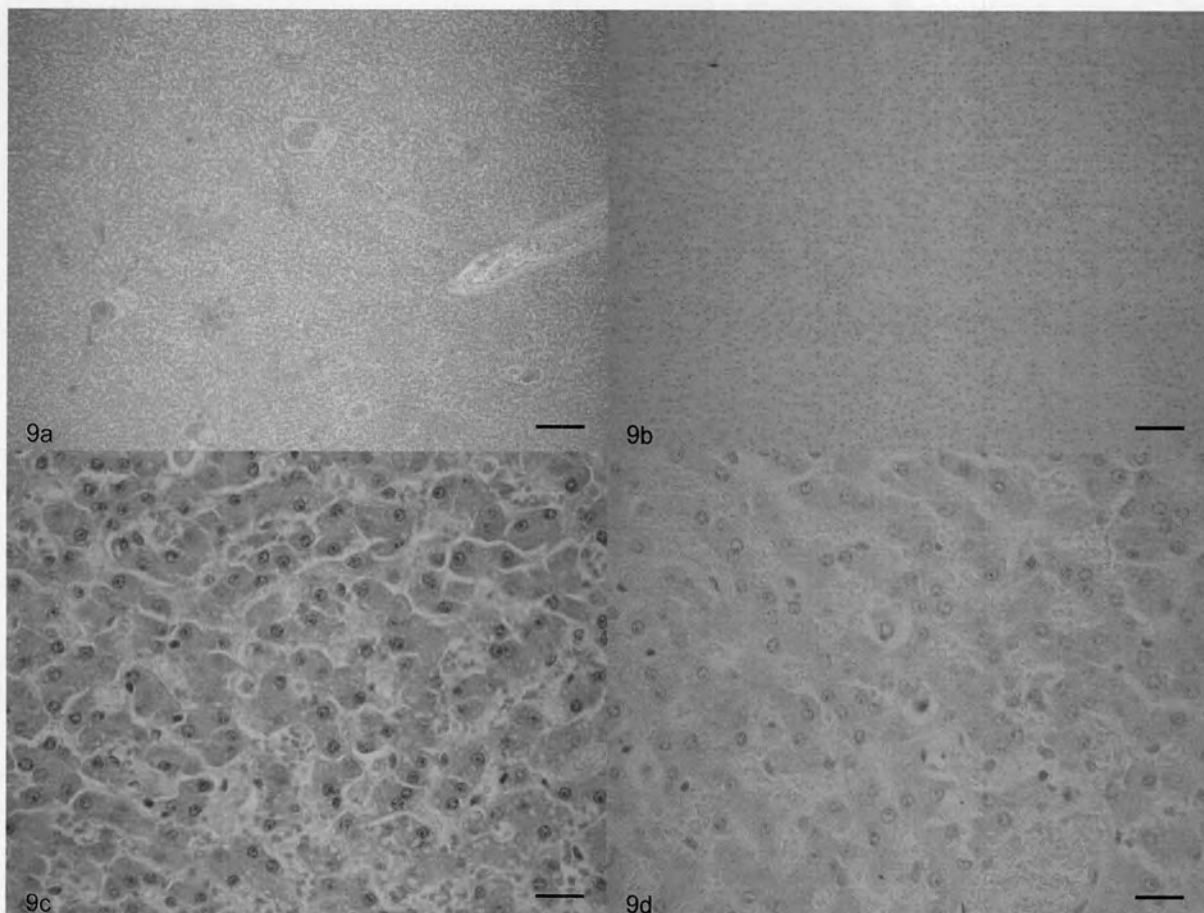


Figure 9a: This picture shows hepatic congestion (case 270), (H&E staining, bar=200um).

Figure 9b: CDV antigens were focally mild positive in hepatocytes (case 270), (LSAB method, counterstained with Meyer's Hematoxylin, bar=200um).

Figure 9c: This picture shows mild focally hepatic degeneration (case 270), (H&E staining, bar=20um).

Figure 9d: CDV antigens were focally mild positive in hepatocytes (case 270), (LSAB method, counterstained with Meyer's Hematoxylin, bar=20um).

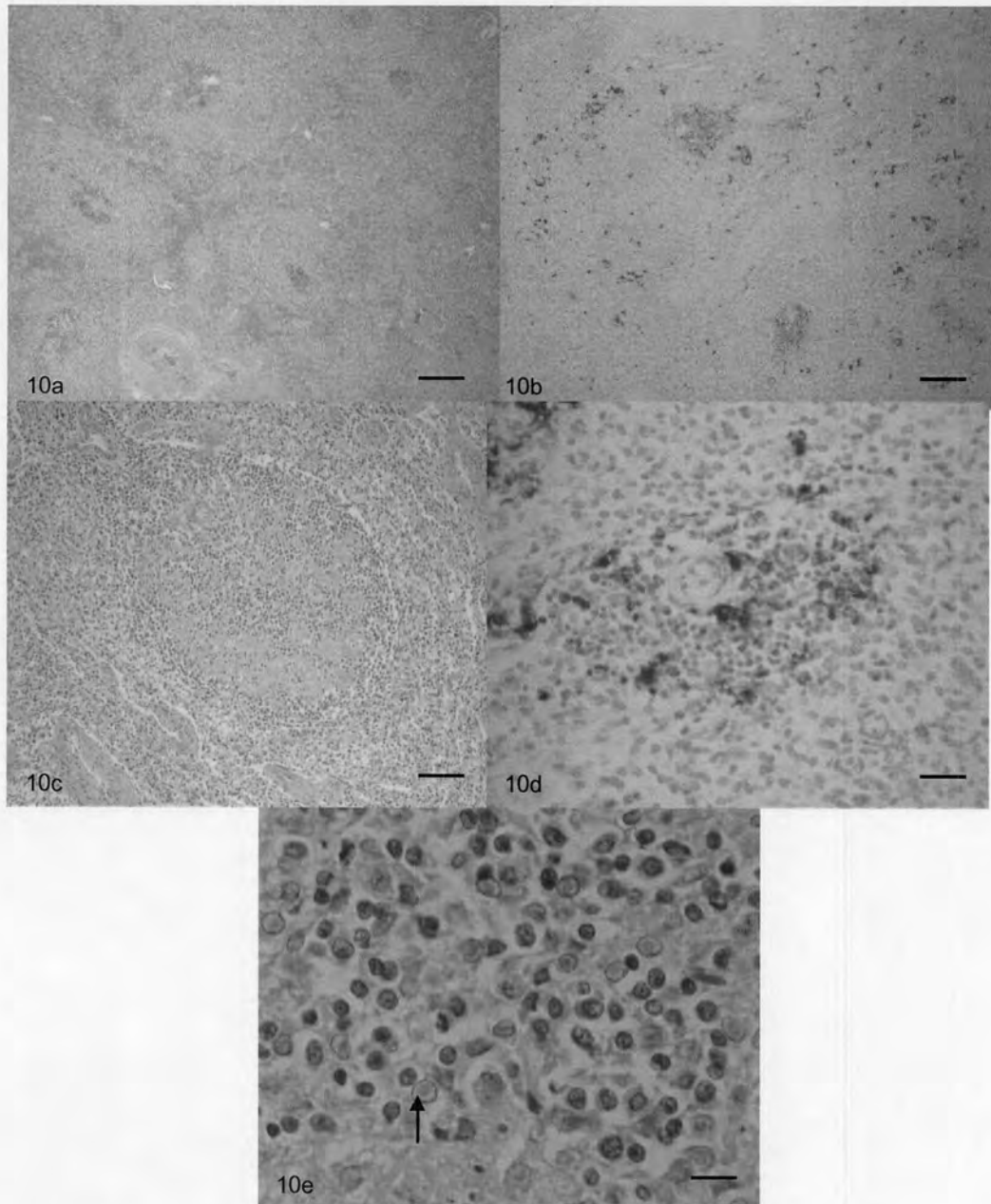


Figure 10a: This picture shows mild splenic depletion and necrotic splenitis (case 290), (H&E staining, bar=200um).

Figure 10b: CDV antigens were positive especially around the white pulp area (case 290), (LSAB method, counterstained with Meyer's Hematoxylin, bar=200um).

Figure 10c: This picture shows mild splenic depletion and necrotic splenitis (case 290), (H&E staining, bar=100um).

Figure 10d: CDV antigens were positive especially around the white pulp area (case 290), (LSAB method, counterstained with Meyer's Hematoxylin, bar=20um).

Figure 10e: Intracytoplasmic inclusion bodies were found in splenocytes (arrow) (H&E staining, bar=10um).

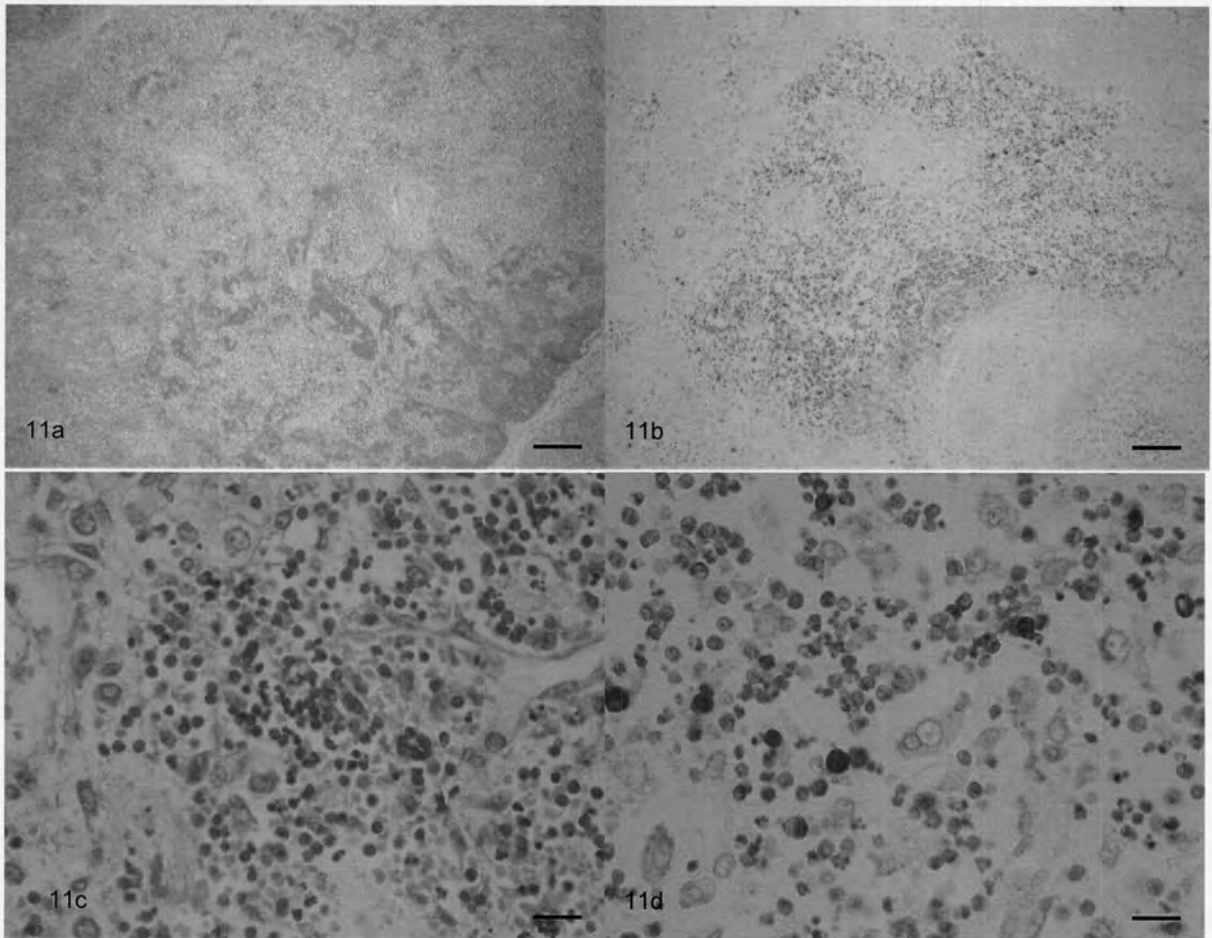


Figure 11a: This picture shows severe lymphoid depletion with hemorrhage (case 270), (H&E staining, bar=200um).

Figure 11b: CDV antigens were focally moderate positive in mononuclear cells (case 270), (LSAB method, counterstained with Meyer's Hematoxylin, bar=200um).

Figure 11c: This picture shows severe lymphoid depletion with hemorrhage (case 270), (H&E staining, bar=10um).

Figure 11d: CDV antigens were positive in mononuclear cells (case 270), (LSAB method, counterstained with Meyer's Hematoxylin, bar=10um).

### 3. Virus isolation

#### 3.1 Virus isolation and titration

Each isolated sample was observed daily for CPE daily by using a phase contrast microscope. Only sample 12BR showed typical CPE within the first inoculation and virus titer was  $1.48 \times 10^5$  TCID<sub>50</sub> /ml. Later, samples number 270 LU and 270 BR showed both typical and few different appearance of CPE after third passage in Vero-DST cell (Figure 12), virus titers were  $1.48 \times 10^5$  and  $3.16 \times 10^5$  TCID<sub>50</sub> /ml, respectively (Table 5). Supernatant of these samples were collected to performing RT-PCR. The other 10 samples didn't show CPE but we presumed that virus still existed in the supernatant, thereafter, supernatant without CPE was also collected and inspected by RT-PCR, together with the homogenized samples.

Table 5: Positive samples and their 50% tissue culture infectious dose (TCID<sub>50</sub>) assay

Positive samples	Organ	CPE showed	TCID <sub>50</sub> /ml
12 BR	Brain	1 <sup>st</sup> time inoculation (36 hours p.i.)	$1.48 \times 10^5$
270 BR	Brain	3 <sup>rd</sup> time passage (36 hours p.i.)	$3.16 \times 10^5$
270 LU	Lung	3 <sup>rd</sup> time passage (36 hours p.i.)	$1.48 \times 10^5$

Note: p.i. = post inoculation



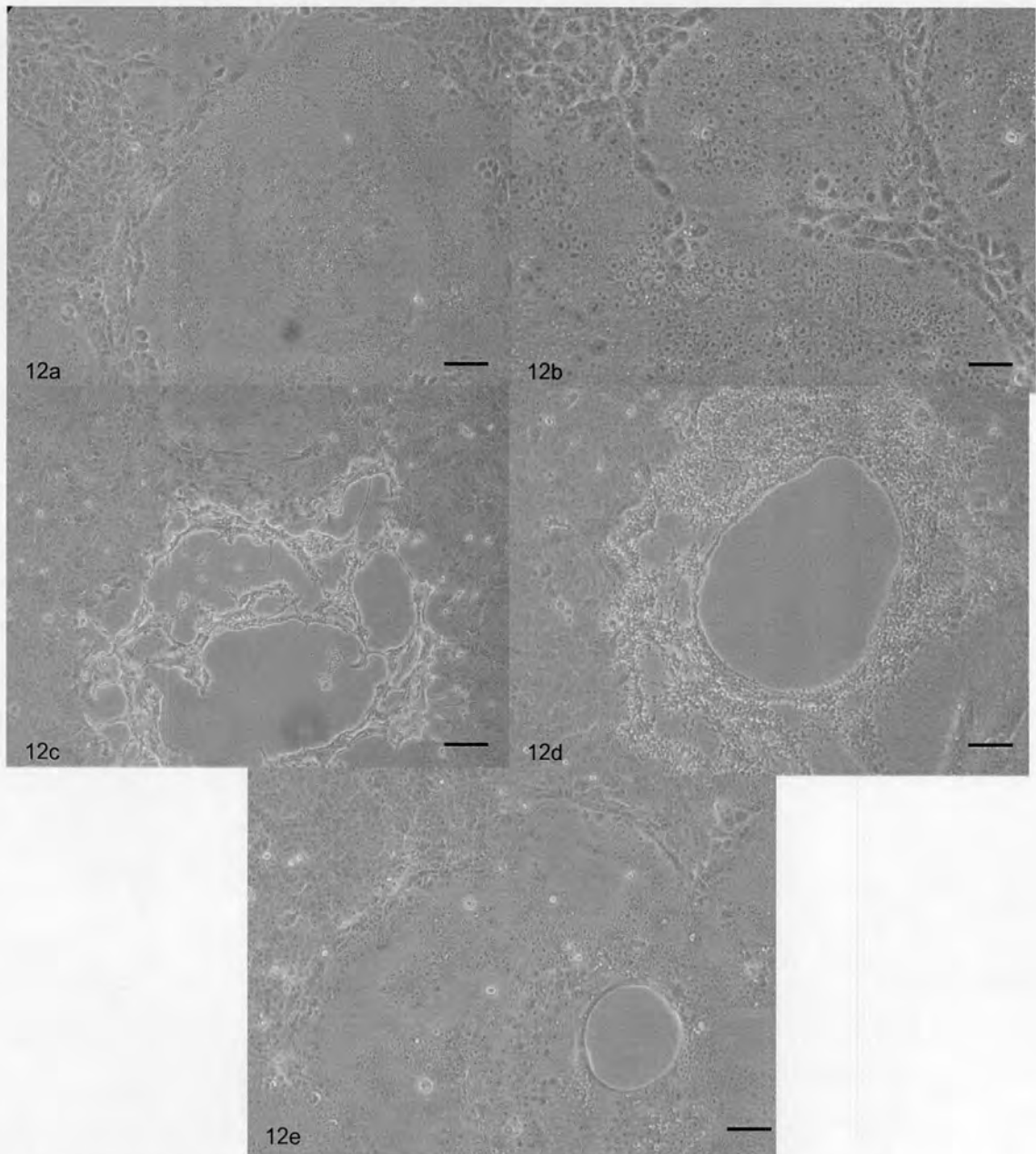


Figure 12a: Vero-DST cell was inoculated with the Onderstepoort strain, observed by phase contrast microscope, 36 hours after first inoculation (bar=200um).

Figure 12b: Vero-DST cell was inoculated with sample number 12BR, observed by phase contrast microscope, 36 hours after first inoculation (bar=100um).

Figure 12c: Vero-DST cell was inoculated with sample number 270LU, observed by phase contrast microscope, 36 hours after the third inoculation (bar=200um).

Figure 12d: Vero-DST cell was inoculated with sample number 270LU, observed by phase contrast microscope, 36 hours after the third inoculation (bar=200um).

Figure 12e: Vero-DST cell was inoculated with sample number 270BR, observed by phase contrast microscope, 36 hours after the third inoculation (bar=200um).

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

All samples were divided in 2 categories; homogenized and supernatant from the isolation in Vero-DST cell. All 14 homogenized samples (1 IN, 3 IN, 5 BR, 14 BR, 15 BR, 16 BR, 270 SP, 270 LI, 270 LU, 270 BR, 290 SP, 290 LI, 290 LN and 290 BR) were positive with P gene (at 390 bp) and negative with H gene (at 1824 bp) (primer as described above). The supernatant from the isolation in Vero-DST cell were divided into; the 3 samples that showed CPE (sample number 12 BR, 270 BR and 270 LU) and other 12 samples that did not showed CPE (1 IN, 3 IN, 5 BR, 14 BR, 15 BR, 16 BR, 270 SP, 270 LI, 290 SP, 290 LI, 290 LN and 290 BR). The 12 supernatant samples that did not showed CPE had 3 samples (sample number 270 SP, 290 SP and 290 BR) that positive with P gene but negative with H gene. Then, the other 9 supernatant samples without CPE (1 IN, 3 IN, 5 BR, 14 BR, 15 BR, 16 BR, 270 LI, 290 LI and 290 LN) were negative for both P and H gene. The sample number 12 BR which showed CPE was positive for P gene but negative for H gene. Samples number 270 BR and 270 LU were positive with both P and H gene (Figure 13 and 14). The positive samples were directly used for nucleotide sequencing in the P gene region and only sample number 270 BR and 270 LU had nucleotide sequence performed in H gene region. Data is concluded in table 6.

Table 6: RT-PCR results

Sample	Organ	Sample	CPE in Vero-DST cell	RT-PCR (P gene)	RT-PCR (H gene)
1 IN	Intestine	Homogenized	ND	+	-
		Isolated	-	+	-
3 IN	Intestine	Homogenized	ND	+	-
		Isolated	-	-	-
5 BR	Brain	Homogenized	ND	+	-
		Isolated	-	-	-
12 BR	Brain	Isolated	+	+	-
14 BR	Brain	Homogenized	ND	+	-
		Isolated	-	-	-
15 BR	Brain	Homogenized	ND	+	-
		Isolated	-	-	-
16 BR	Brain	Homogenized	ND	+	-
		Isolated	-	-	-
270 SP	Spleen	Homogenized	ND	+	-
		Isolated	-	+	-
270 LI	Liver	Homogenized	ND	+	-
		Isolated	-	-	-
270 LU	Lung	Homogenized	+	+	-
		Isolated	+	+	+
270 BR	Brain	Homogenized	+	+	-
		Isolated	+	+	+
290 SP	Spleen	Homogenized	-	+	-
		Isolated	-	+	-
290 LI	Liver	Homogenized	-	+	-
		Isolated	-	-	-
290 LN	Lymph node	Homogenized	-	+	-
		Isolated	-	-	-
290 BR	Brain	Homogenized	-	+	-
		Isolated	-	+	-

(Note; +: positive, -: negative, ND: not done)

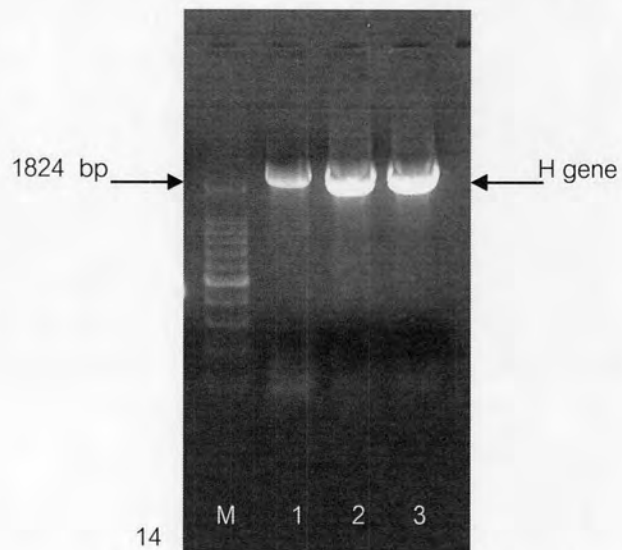
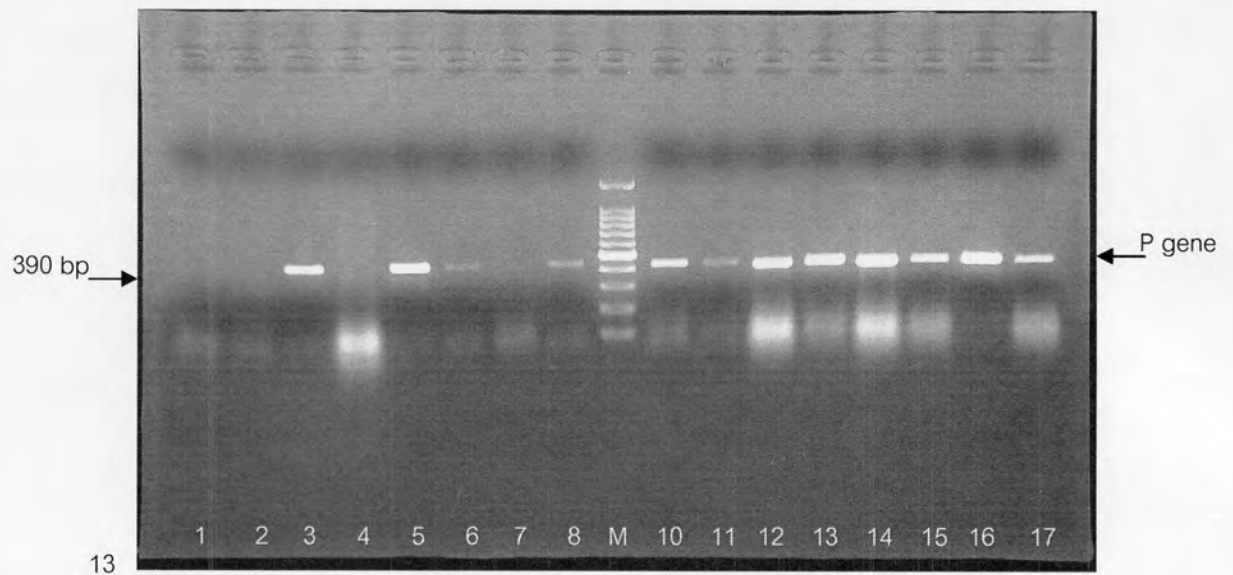


Figure 13: RT-PCR of 390 bp of P gene; lane 1: sample number 1 IN, lane 2: sample number 2\*, lane 3: sample number 3 IN, lane 4: sample number 4\*, lane 5: sample number 5 BR, lane 6: sample number 14 BR, lane 7: sample number 15 BR, lane 8: sample number 16 BR, M: 100 bp DNA ladder, lane 10: sample number 270 SP, lane 11: sample number 270 LI, lane 12: sample number 270 LU, lane 13: sample number 270 BR, lane 14: sample number 290 SP, lane 15: sample number 290 LI, lane 16: sample number 290 LN and lane 17: sample number 290 BR (\*: the sample showed negative result and not included in the experiment).

Figure 14: RT-PCR of 1824bp of H gene; M: 100 bp DNA ladder, lane 1: positive control, lane 2: sample number 270 LU and lane 3: sample number 270 BR.



### 3.3 Sequence and Phylogenetic analyses

#### 3.3.1 Sequences analyses

An alignment of 390 bp P gene demonstrated that nucleotide sequences among sample number 1IN, 3 IN, 5 BR, 12 BR, 14 BR, 15 BR and 16 BR varied between 98.46 – 100% homology. Four samples from the brain, lung, spleen and liver of dog 270 and supernatant of 270 SP showed the nucleotide varied between 99.23 – 100% homology. Samples from the brain, lymph node, spleen, liver of dog 290 were 100% homology (Table 7).

From these results, sample number 12 BR, 15 BR, 270 BR (from the homogenize samples) and 290 BR were chosen to represent each group, to compare with other reference strains in Genbank (Figure 15).

Sample number 12 BR and 15 BR were 99.23% identical. Sample number 12 BR was 95.90% identical to both number 270 BR and 290 BR. Then number 15 BR was 96.67% identical to both number 270 BR and 290 BR. Nucleotide sequence of sample number 12 BR and 15 BR were 98.97 and 98.27% identical to the S124C strain which was represented the Asia1 lineage. Then, they were 95.89 and 96.67% identical to the Onderstepoort strain (represented the vaccine lineage), 96.41 and 97.81% identical to the 007 Lm (represented the Asia2 lineage), 95.90% and 96.67% identical to the P012689 strain (represented the American lineage), and 96.95 and 98.21% identical to the P5804 strain (represented the Europe lineage) (Table 8).

Samples number 270 BR and 290 BR were 99.49% homology. They were 94.87 and 95.39% homology to the Onderstepoort strain, 95.65 and 95.64% homology to the S124C strain, 95.79% homology to the 007 Lm strain, 94.87% homology to the P012689 strain and 96.41% homology to the P5804 strain (Table 8).

The nucleotide sequences and percentage of homology were shown in figure 15. Sample number 270 BR and 270 LU with CPE

showed nucleotide sequences of P gene 100% homology to their origin samples (Figure 16).

Table 7 Percentage of nucleotide sequences homology between each sample of the P gene region

	1	3	5	12	14	15	16	270 Sp-H	270 Sp-S	270Li	270Lu	270Br	290 Sp-H	290 Sp-S	290Li	290Ln	290 Br-H	290 Br-S	
1 IN																			
3 IN	99.49																		
5 BR	99.23	99.74																	
12 BR	100	99.49	99.23																
14 BR	100	99.49	99.23	100															
15 BR	99.23	99.23	98.97	99.23	99.23														
16 BR	99.23	98.72	98.46	99.23	99.23	98.46													
270 SP-H	95.9	95.9	96.15	95.9	95.9	96.67	95.13												
270 SP-S	95.65	95.4	95.65	95.65	95.65	96.16	94.89	99.49											
270 LI	95.9	95.9	96.15	95.9	95.9	96.67	95.13	100	99.49										
270 LU	95.64	95.64	95.9	95.64	95.64	96.41	94.87	99.74	99.23	99.74									
270 BR	95.9	95.9	96.15	95.9	95.9	96.67	95.13	100	99.49	100	99.74								
290 SP-H	95.9	95.9	96.15	95.9	95.9	96.67	95.13	99.49	98.98	99.49	99.23	99.49							
290 SP-S	99.34	99.23	98.97	99.74	99.74	98.97	98.97	95.64	95.39	95.64	95.39	95.64	95.64						
290 LI	95.9	95.9	96.15	95.9	95.9	96.67	95.13	99.49	98.98	99.49	99.23	99.49	100	95.64					
290 LN	95.9	95.9	96.15	95.9	95.9	96.67	95.13	99.49	98.98	99.49	99.23	99.49	100	95.64	100				
290 BR-H	95.9	95.9	96.15	95.9	95.9	96.67	95.13	99.49	98.98	99.49	99.23	99.49	100	95.64	100	100			
290 BR-S	95.9	95.9	96.15	95.9	95.9	96.67	95.13	99.49	98.98	99.49	99.23	99.49	100	95.64	100	100	100		
Ond	95.9	95.9	96.15	95.89	95.9	96.67	95.13	94.87	94.39	94.82	94.62	94.89	95.39	95.64	95.39	95.39	95.39	95.39	95.39

(Note: SP: Spleen, LI: Liver, LU: Lung, BR: Brain, LN: Lymph node, H: homogenized sample, S: Isolated sample, Ond: Onderstepoort strain)

Table 8 Homology of nucleotide sequences of P gene

	Nucleotides %			
	12 BR	15 BR	270 BR	290 BR
15 BR	99.23			
270 BR	95.90	96.67		
290 BR	95.90	96.67	99.49	
Onderstepoort	95.89	96.67	94.87	95.39
S124C	98.97	98.27	95.65	95.64
007Lm	96.41	97.81	95.79	95.79
P012689	95.90	96.67	94.87	94.87
P5804	96.95	98.21	96.41	96.41

Two H gene sequences of the new isolates consisting of 1824 nucleotides in one open reading frame encoding 607 amino acids were compared with reference strains in the database. Nucleotide sequence of sample number 270 BR and 270 LU was 99.89% identical, whereas, amino acid sequence was 100% identical (table 9). Nucleotide sequence of sample number 270 BR was 91.78% and 270 LU was 91.87% identical to the Onderstepoort strain, 93.91% and 94.02% identical to the Ac96I strain, 92.82% and 92.93% identical to the 007Lm strain, 93.91% and 94.02% identical to the 01-2689 (USA) strain, 95.34% and 95.45% identical to the Dog C (Denmark) strain. Amino acid of both samples showed 91.27% identical to the Onderstepoort strain, 94.73%, 93.41%, 93.74% and 95.55% identical to the Ac96I strain, the 007Lm strain, the 01-2689 (USA) strain and the Dog C (Denmark) strain, respectively. Eight potential N-linked glycosylation sites (N-X-S/T) were identified in the predicted H gene amino acid alignment of both new isolate strains, as



well as the Ac96I strain (represented the Asia1 lineage), the 007Lm strain and the USA01-2689 strain (represented the American lineage). Seven of those sites were found within the Onderstepoort strain, and the Dog C (Denmark) strain. Twelve of twelve cysteine (C) residues, and 33 of 35 proline (P) residues were found as a conserve part among the viruses in studied samples and other CDV isolates (Figure 17).

Table 9 Homology of nucleotide and amino acid sequences of H gene

	270Brain	270Lung	Onderstepoort	Ac96I	007Lm	01-2689	Dog CDen
			Nucleotides (%)				
270Brain		99.89	91.78	93.91	92.82	93.91	95.34
270Lung	100.00		91.87	94.02	92.93	94.02	95.45
Onderstepoort	91.27	91.27		91.50	91.72	91.61	92.83
Ac96I	94.73	94.73	90.61		92.93	93.59	95.23
007Lm	93.41	93.41	90.61	93.41		92.82	94.08
01-2689	93.74	93.74	89.79	92.75	91.93		95.50
Dog CDen	95.55	95.55	91.63	94.89	93.58	94.07	
Amino acid (%)							

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Ondestepoort          1:AAGAGGTAAAGGAATCGAAGATGCTGACAGTCTCGTGGTACCTGCAGGCACTGTCGGTA 60
th_12-sm-uppl.nuc    1:.....G.....A... 60
th_15-homo-uppl.nuc  1:.....G.....A... 60
th_270BR-homo_uppl.nuc 1:.....C.....G.....AA... 60
th_290BR-homo^uppl.nuc 1:.....C.....G.....A... 60
SI24C                1:.....G.....A... 60
007Lm               1:.....G..A..A... 60
P01-2689cut         1:.....G.....A... 60
P5804Gecut          1:.....G.....A... 60
*****

Ondestepoort          61:ATCGAGGATTCGAGAGAGGAGAAGGAAGCCTTGATGATAGCACTGAGGATTCTGGCGAAG 120
th_12-sm-uppl.nuc    61:..... 120
th_15-homo-uppl.nuc  61:..... 120
th_270BR-homo_uppl.nuc 61:G.....C.....GA.....C..... 120
th_290BR-homo^uppl.nuc 61:G.....C.....GA.....C..... 120
SI24C                61:.....C..... 120
007Lm               61:.....A.....A..... 120
P01-2689cut         61:.....G..... 120
P5804Gecut          61:..... 120
*****

Ondestepoort          121:ATTATTCGAAGGAAATGCTTCATCTAACTGGGGATATTCTTTCGGCCTTAAACCGGACA 180
th_12-sm-uppl.nuc    121:.....G.....A... 180
th_15-homo-uppl.nuc  121:.....G.....A... 180
th_270BR-homo_uppl.nuc 121:.....G.....A... 180
th_290BR-homo^uppl.nuc 121:.....G.....A... 180
SI24C                121:.....G.....A... 180
007Lm               121:.....A..... 180
P01-2689cut         121:.....G.....A... 180
P5804Gecut          121:.....G.....A... 180
*****

Ondestepoort          181:GAGCAGCTGATGTGAGCATGCTGATGGAAGAGGAATTAAGTGCTCTACTCAGGCAAGCA 240
th_12-sm-uppl.nuc    181:..G.....G.....G.....A... 240
th_15-homo-uppl.nuc  181:..G.....G.....G..... 240
th_270BR-homo_uppl.nuc 181:.....G.....G..... 240
th_290BR-homo^uppl.nuc 181:.....G.....G.....A... 240
SI24C                181:..G.....G.....G.....T... 240
007Lm               181:..C.....G.....G..... 240
P01-2689cut         181:..G.....G.....T.G..... 240
P5804Gecut          181:..G.....G.....G.....A... 240
*****

Ondestepoort          241:GAAATGTAGGATTCAGAAAAGGGATGGGAAGACTCTGCAGTCCCACATAATCCCGAAG 300
th_12-sm-uppl.nuc    241:.....G.....A.....T.....C..... 300
th_15-homo-uppl.nuc  241:.....G.....A.....C..... 300
th_270BR-homo_uppl.nuc 241:.....A.....C..... 300
th_290BR-homo^uppl.nuc 241:.....A.....C..... 300
SI24C                241:.....G.....A.....T.....C..... 300
007Lm               241:.....G.....C..... 300
P01-2689cut         241:.....CA.....C.....T.A...A 300
P5804Gecut          241:.....A.....C.....C..... 300
*****

Ondestepoort          301:GTAAGACAAGGGATCCGGAGTGTGGATCCATTA AAAAGGGCACAGAAGAGAGGTCAGTCT 360
th_12-sm-uppl.nuc    301:.....GA.....G..C.G.....C... 360
th_15-homo-uppl.nuc  301:.....GA.....G.....C..... 360
th_270BR-homo_uppl.nuc 301:.....G.....G.....G.....C... 360
th_290BR-homo^uppl.nuc 301:.....G.....G.....G.....C... 360
SI24C                301:..C.....GA.....C.....G.....C... 360
007Lm               301:.....G.....G.....A.....C... 360
P01-2689cut         301:.....G.....C.....G.....C... 360
P5804Gecut          301:.....G.....G.....G.....C... 360
*****

Ondestepoort          361:CACATGGAATGGGGATAGTTGCTGGATCGA 390
th_12-sm-uppl.nuc    361:..... 390
th_15-homo-uppl.nuc  361:..C..... 390
th_270BR-homo_uppl.nuc 361:.....C..... 390
th_290BR-homo^uppl.nuc 361:..... 390
SI24C                361:..... 390
007Lm               361:..... 390
P01-2689cut         361:.....C..... 390
P5804Gecut          361:.....A..... 390
*****

```

Figure 15: Nucleotide sequence of 390 basepair P gene region; th-12-sm-uppl1.nuc: nucleotide sequence of sample 12, th-15-homo-uppl1.nuc: nucleotide sequence of sample 15, th\_270BR-homo\_uppl1.nuc: nucleotide sequence of sample 270Brain, th\_290BR^uppl1.nuc: nucleotide sequence of sample 290Brain. Dots (.) indicate identity.

```

Ondestepoort      1:AAGAGGTTAAGGGAATCGAAGATGCTGACAGTCTCGTGGTACCTGCAGGCACTGTCCGTA 60
007Lm             1:.....G..A..A... 60
S124C            1:.....G...A... 60
BR-gae           1:.....C.....G...AA.. 60
th_270BR-homo_upp1 1:.....C.....G...AA.. 60
LU-gae           1:.....C.....G...AA.. 60
th-270LU-homo-upp1 1:.....C.....G...AA.. 60
*****

Ondestepoort      61:ATCGAGGATTCGAGAGAGGAGAAGGAAGCCTTGATGATAGCACTGAGGATTCTGGCGAAG 120
007Lm            61:.....A.....A..... 120
S124C            61:.....C..... 120
BR-gae           61:G.....C...GA.....C..... 120
th_270BR-homo_upp1 61:G.....C...GA.....C..... 120
LU-gae           61:G.....C...GA.....C..... 120
th-270LU-homo-upp1 61:G.....C...GA.....C..... 120
*****

Ondestepoort      121:ATTATTCCGAAGGAAATGCTTCATCTAACTGGGGATATTCTTTCGGCCTTAAACCGGAC. 180
007Lm            121:.....A..... 180
S124C            121:.....G.....A..... 180
BR-gae           121:.....G.....A..... 180
th_270BR-homo_upp1 121:.....G.....A..... 180
LU-gae           121:.....G.....A..... 180
th-270LU-homo-upp1 121:.....G.....A..... 180
*****

Ondestepoort      181:GAGCAGCTGATGTGAGCATGCTGATGGAAGAGGAATTAAGTCTCTACTCAGGACAAGCA 240
007Lm            181:.....C.....G.....G.....G.....T. 240
S124C            181:.....G.....G.....G.....A..... 240
BR-gae           181:.....G.....G..... 240
th_270BR-homo_upp1 181:.....G.....G..... 240
LU-gae           181:.....G.....G..... 240
th-270LU-homo-upp1 181:.....G.....G..... 240
*****

Ondestepoort      241:GAAATGTAGGGATTCAGAAAAGGGATGGGAAGACTCTGCAGTCCACATAATCCCGAAG 300
007Lm            241:.....G.....C.....C..... 300
S124C            241:.....G...A.....T.....C..... 300
BR-gae           241:.....A.....C.....C..... 300
th_270BR-homo_upp1 241:.....A.....C.....C..... 300
LU-gae           241:.....A.....C.....C..... 300
th-270LU-homo-upp1 241:.....A.....C.....C..... 300
*****

Ondestepoort      301:GTAAGACAAGGGATCCGGAGTGTGGATCCATTA AAAAGGCCACAGAAGAGAGGTCAGTCT 360
007Lm            301:.....G.....G.....A.....C... 360
S124C            301:.....C...GA...C.....G.....C... 360
BR-gae           301:.....G...G.....G.....C... 360
th_270BR-homo_upp1 301:.....G...G.....G.....C... 360
LU-gae           301:.....G...G.....G.....C... 360
th-270LU-homo-upp1 301:.....G...G.....G.....C... 360
*****

Ondestepoort      361:CACATGGAATGGGGATAGTGTGGATCGA 390
007Lm            361:..... 390
S124C            361:..... 390
BR-gae           361:.....C..... 390
th_270BR-homo_upp1 361:.....C..... 390
LU-gae           361:.....C..... 390
th-270LU-homo-upp1 361:.....C..... 390
*****

```

Figure 16: The P gene region nucleotide sequences of 270Brain and 270Lung isolated samples compare with their homogenate samples. The th\_270BR-homo\_upp1 and th-270LU-homo-upp1 are 270Brain and 270Lung homogenate samples, BR-gae and LU-gae are the isolated samples. Dots (.) indicate identity.





Figure 17: The picture show the comparison of amino acid sequence of H gene region. Ond:  
Onderstepoort strain, BR-aa: the amino acid sequence of sample 270Brain, LU-aa: the  
amino acid sequence of sample 270Lung. Dots (.) indicate identity.



### 3.3.1 Phylogenetic analyses

The phylogenetic tree of P gene region showed that sample 1, 3, 5, 12, 14, 15 and 16 were in the same lineage and join the Asia1 lineage. The two strains from dog number 270 and 290 were in the same group are not related to the vaccine and other groups in Genbank database (Figure 18). The accession numbers of the reference strains were presented in the materials and method section.

Phylogenetic tree of H gene (Figure 19) showed that both new isolate strains were in the same lineage and distinguished from vaccine strains and other reference strains in Genbank database. The accession numbers of the reference strains were presented in the materials and methods section.

### 3.3.3 Nucleotide sequences accession numbers

The nucleotide sequences accession numbers in the Genbank database of P gene sequences of new isolates are: 3 IN (AB299191), 12 BR (AB299192), 14 BR (AB299193), 15 BR (AB299194), 16 BR (AB299195), 270 SP (AB299196), 270 LI (AB 299197), 270 BR (AB299198), 270 SP (AB299199), 290 SP (AB299200), 290 LN (AB299201), 290 BR (AB299202), 290 SP (AB299203), 290 BR (AB299204), 270 BR (AB301064) and 270 LU (AB301063).

The nucleotide sequences accession numbers in the Genbank database of H gene sequences of new isolates are: 270 BR (AB301065) and 270 LU (AB301066).

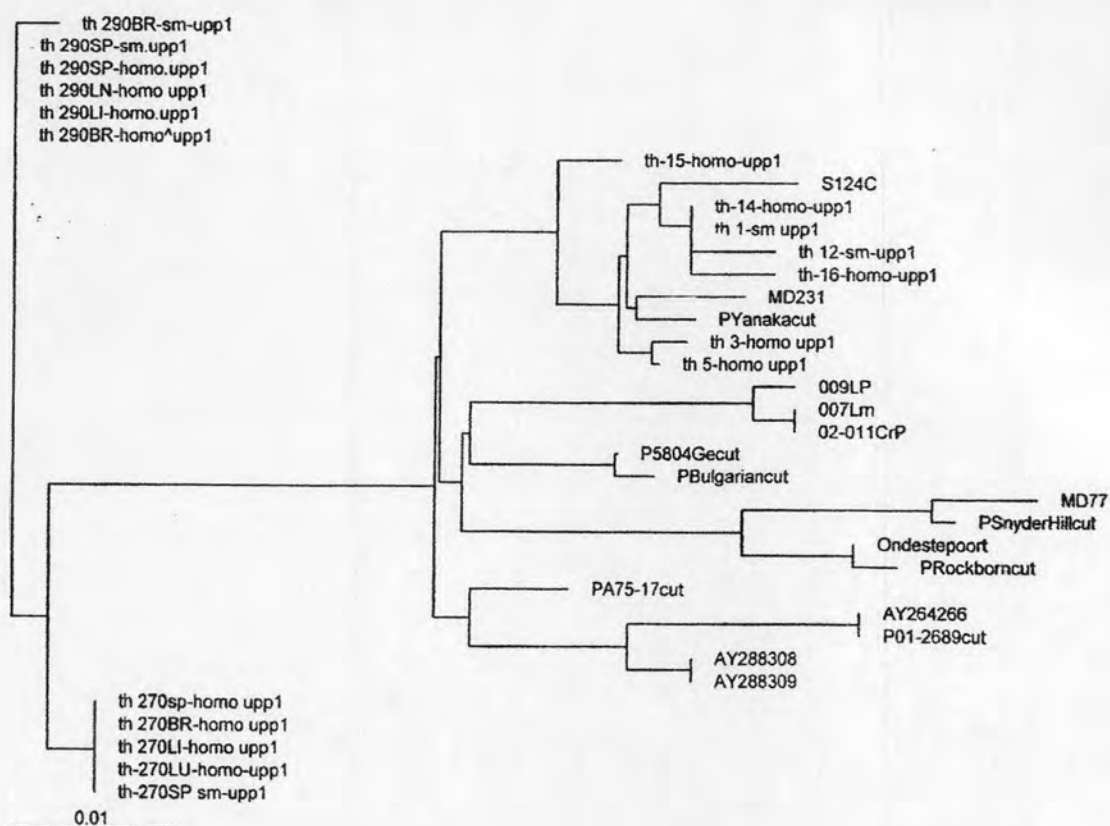
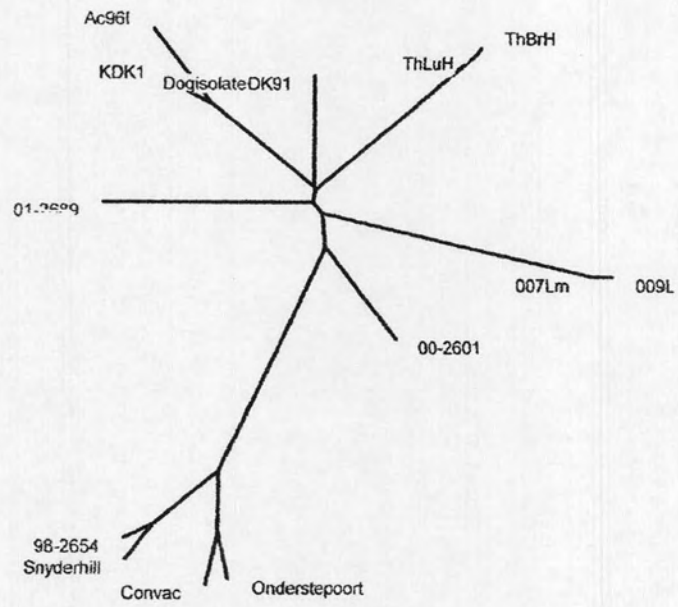


Figure 18: Phylogenetic analyses of CDV strains based on the nucleotide sequences of 390 bp gene fragments. The sample: th 290SP-homo.upp1, th 290LN-homo.upp1, th 290LI-homo.upp1 and th 290BR-homo.upp1 are the homogenated 290 samples from the spleen, lymph node, liver and brain, respectively. The sample: th 290SP-sm-upp1 and th 290BR-sm-upp1 are the supernatant of 290 spleen and lymph nodes that were inoculated in Vero-DST. The sample: th 270sp-homo.upp1, th 270BR-homo.upp1, th 270LI-homo.upp1 and th 270LU-homo.upp1 are the homogenated 270 sample from spleen, brain, liver and lungs, respectively. Sample th-270SP sm-upp1 is the supernatant of first time inoculated spleen of case 270 in Vero-DST. The sample: th 3-homo.upp1, th 5-homo.upp1, th 14.upp1, th 15-homo.upp1 and th 16-homo.upp1 are the homogenated sample number 3, 5, 14, 15 and 16, respectively. The sample: th 1-sm-upp1 and th 12-sm-upp1 are the supernatants of sample numbers 1 and 12 that were inoculated in Vero-DST.



0.01

Figure 19: Phylogenetic analyses of CDV strains based on the nucleotide sequences of H gene fragments. ThBrH is the 270BR strain and ThLuH is the 270LU strain.