Canine Distemper in Taiwan from 2000 – 2009: Co-infections and the Use of RT-PCR and Immunohistochemistry to Detect Tissue Involvement in Two Groups of Dogs

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ABSTRACT

Canine distemper virus (CDV) causes a highly contagious disease, which has been reported in Taiwan for many years; however phylogenetic analysis of field CDV, histopathological lesions, and co-infection are poorly understood. The goals of this study were to characterize the pathology of CDV in dogs in Taiwan, and to assess the frequency of CNS demyelination in cases of CDV infection confirmed by immunohistochemistry (IHC) and/or reverse transcription polymerase chain reaction (RT-PCR). Fifty two (IHC or RT-PCR positive) affected dogs were obtained from either animal clinics or dog shelters from 2000 to 2009. Postmortem and laboratory examination included gross findings, histopathology, Luxol-fast blue cresyl echt violet (LFB-CEV) histochemistry, non-biotin HRP anti-CDV IHC, and phosphoprotein gene RT-PCR. Thirty two (32) dogs were submitted from clinic. Twenty (20) dogs were submitted from shel-

ter. Clinic cases had histories of treatment and or vaccination. Twenty four clinic cases (75%) were puppies less than 6 months old. Seventeen shelter cases (85%) were identified as 'adults' greater than 6 months old. There were 27 males and 25 females. Eleven dog breeds were represented, but most dogs (35/52, 67%) were mixed-breed. Totally, 79% (41/52) had lymphoid depletion, 71% (37/52) had interstitial pneumonia, 65% (34/52) had CNS demyelination, and 32% (17/52) had catarrhal enteritis. Younger clinic group frequently had lymphoid depletion (31/32, 96%), inclusion bodies (28/32, 87%), pneumonia (26/32, 81%), and CNS demyelination (26/32, 81%), also showed statistically significant difference compared to shelter group. Enteritis was identified in about one third of the animals in both groups. The distribution of inclusion bodies also showed significant difference in urinary bladder, lymphoid tissues, lung, and alimentary tract between the two groups. A variety of 29 co-infections and other associated lesions were identified. However, no significant difference in the frequency of occurrence between the two groups, the exception was interstitial nephritis. In conclusion, lymphoid depletion, pneumonia, and CNS demyelination were the most common CDV-infected principal lesions, and high occurrence of inclusion bodies were found in spleen, lymph node, and mucosa epithelium of urinary bladder, pelvis, bronchioles, and stomach.

INTRODUCTION

Canine distemper virus (CDV), which belongs to morbillivirus genus, family Paramyxoviridae, produces systemic or central nervous system(CNS) infections of dogs and related species, and often associated with high mortality in dogs in Taiwan.[14] Expected pathology findings in CDV infected dogs include lymphocyte depletion in lymphoid tissues, interstitial pneumonia, degenerative changes in epithelia of respiratory organs, urinary bladder and gastrointestinal tract, foot pad hyperkeratosis,[12] and intranuclear or intracytoplasmic eosinophilic inclusion bodies (INIB/ICIB) in epithelial cells of the urinary bladder or gastrointestinal tract.[1,4] Characteristic central nervous system CNS changes include polioencephalomacia,[15] white matter demyelination, astrogliosis, eosinophilic INIB/ICIB in astrocytes and neuron, gemistocytes and occasional multinucleated syncytial giant cell [3,19,24,25] and old dog encephalitis.[26]

We have noticed that in Taiwan, there have been changes in the suite of histopathological lesions seen in the prevalent infection of CDV since 2000;29 CNS signs are now marked and gastrointestinal involvement is rare.[14] Two distinct disease group of CDV infection, "enteritis and non-enteritis", have also been noted in Japan. The non-enteritis type of CD exhibited reduced epitheliotropism, might be the wild-type of CDV infection.[18] In other reports also, the frequency of CNS findings seemed lower than what we were experiencing in Taiwan eg. syncytial giant cells in 9 % of infected brains,[19] and eosinophilic intranuclear or cytoplasmic inclusion bodies in only 17-72 %.[8, 9, 23, 29]

In addition to the lesions of CDV itself, infected dogs may have a wide variety of concurrent infections, including canine adenovirus type 2 (CAV-2),[5,6] coccidiosis,[9] colibacillosis,[28] cryptosporidiosis,7 Parainfluenza viruses,[6] *Mycoplasma Cynos*,[5] toxoplasmosis,[17] Tyzzer's Disease,[9] documented in individual case reports. Based on the previous findings and lack of a large-scale of case analysis, it is interesting to know concurrent infections with CDV and characteristics of CDV-associated lesions in different environment for appropriate management. Thus, we conducted a retrospective study, for the 10 years from 2000 to 2009, to compare CDV histopathological lesions and complications in two groups of dogs in Taiwan, 32 that had been treated in clinics and 20 dogs from shelters.

MATERIALS AND METHODS

Animals

This retrospective study was based on necropsy cases obtained between March

2000 and December 2009. The RT-PCR associated with the immunohistochemical labeling-confirmed CDV infection in 52 dogs, and the distribution of these cases over the 10-year-study-period was as follows: 2000 (26), 2001 (2), 2002 (4), 2003 (6), 2004 (2), 2005 (8) and 2009 (4). There were 27 males and 25 females. Most of the dogs were crossed-breed (35); the remainder were distributed among 11 breeds: Beagle (3), Shih tzu (3), Maltese (2), Labrador retriever (2), Chin (1), Chow chow (1), Dachshund (1), Lhasa apso (1), Miniature pinscher (1), Pomeranian (1), and Shiba (1).

Shelter submitted dogs, were submitted to our laboratory as 'puppies' considered to be less than 6 months old, or as 'adults', considered to be more than 6-month-old. Thus, for the purposes of comparison here age groups were classified as less than 6 months and more than 6 months. The dogs had died or were humanely euthanized by 80 mg/kg pentobarbital IV.

Immunohistochemistry (IHC)

CDV immunohistochemistry was reported previously,14 and was performed on deparaffinized tissue sections from paraffin blocks used for histology. Tissues examined by IHC included spleen, cerebrum, cerebellum, brain stem, urinary bladder, and lung for each dog. Briefly, the Super Sensitive TM Non-Biotin HRP Detection System (BioGenex Laboratories, San Ramon, CA, USA) was used. The primary antibody was mouse anti-CDV (MCA 1893, Clone DV2-12; Serotec, Kidlington, Oxford, UK). Substitution of TBS or negative mouse serum for the primary antibody on sections of CDV-infected cerebrum served as a non-specific negative control. Cerebral sections from dogs with no evidence of distemper infection served as a specific negative control. Positive control sections from a dog with numerous inclusion bodies were included in each IHC staining.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

This method was reported previously.[14] Briefly, DNA was extracted from serum, heparinized whole blood, cerebro-spinal fluid (CSF), nasal swab, ocular swab, and 10-fold dilution of vomitus or faeces from dogs with clinically suspected CD by the TRIzols reagent (Invitrogen, Carlsbad, CA, USA) phenol-chloroform extraction method. The primers specific to CDV AF378705 strain phosphoprotein (P) were used. Two groups of primer were used, one group was F2, R1 and R2 primer set. The target amplication sequence was a 200 base pair (bp) fragment. Another primer group was P2, P2b and P1. The target amplication sequence was a 78 base pair (bp) fragment (Table 1).

Pathology

All 52 dogs were examined post mortem (necropsy). Samples taken for postmortem examination consisted of 16 tissues: brain (including medulla oblongata, cerebrum, cerebellum, and brain stem), spinal cord, lymph nodes, spleen, heart, liver, stomach, small intestine, large intestine, kidneys, urinary bladder, adrenal glands, lungs, trachea, and skin. Fixation period was usually 36-48 h and did not exceed 7 days. Tissues were processed routinely and paraffin embedded, sectioned at 5μm transferred to glass slides, deparaffinized, stained with haematoxylin and eosin (H&E), and examined by the pathologist or examined immunohistochemically (Liang et al., 2007) to determine the type of lesion and the presence of inclusion bodies, syncytical cells, and CDV antigen.

For identification of fungal lesions, PAS stain and GMS stain, were employed. For identification of demyelinating lesions, Luxo-fast cresyl violet stain was employed. Histopathology assessment in each case included scoring for nine lesions associated with CDV infection. These were intranuclear or cytoplasmic inclusion bodies in epithelia or in CNS lesions, CNS demyelination (cerebrum, cerebellum, brain stem), lymphoid depletion (lymph nodes and /or spleen), catarral enteritis, interstitial pneumonia, foot pad hyperkeratosis, and syncytia in at least one of the lesions (Table 2). The presence of characterisitic intranuclear or intracytoplasmic inclusion bodies (INIB/ICIB) were recorded in nine tissues: urinary

Table 1. *RT-PCR primer sequence of the phosphoprotein (P) gene of CDV Onderstepoort, strain(AF378705)*

Primer	Nucleotide sequence (5'-3')	Nucleotide position	Target
Set 1			
F2	TAAGGGAATCGAAGATGC	2160-2177	-
R1	CCATCAGCATGCTCACATC	2359-2341	200
R2	GATCCCCCAGTTGACTTG	2585-2568	426
Set 2			
P2 (F)	ATGTTTATGATCACAGCGGT	2132-2151	429
P2b (F)	ATTAAAAAGGG(G/C)ACAGGAGAGAGATCAGCC	2482-2511	78
P1(R)	ATTGGGTTGCACCACTTGTC	2560-2540	-

Table 2. *Comparison of the occurrence of 9 diagnostic CDV lesions in shelter and clinic group of CDV infected dogs. Data is presented as number of positive cases, and percentage of positive cases.*

Diagnostic Lesions	Shelter N=20	Clinic N=32	chi-square test
Intranuclear inlcusion body	11 (55%)	28 (87%)	**
Demyelination, cerebrum	7 (35%)	25 (78%)	**
Demyelination, cerebellum	4 (20%)	23 (71%)	**
Demyelination, brain stem	6 (30%)	25 (78%)	**
Lymphoid depletion	10 (50%)	31 (96%)	**
Catarrhal enteritis	6 (30%)	11 (34%)	ns
Interstitial pneumonia	11 (55%)	26 (81%)	*
Hyperkeratosis	4 (20%)	9 (28%)	ns
Syncytium	6 (30%)	14 (43%)	ns

***, statistically significant, P<.01; * , statistically significant, P<.05; ns, not statistically significant*

bladder, lymphoid tissues, lung, cerebellum, cerebrum, brain stem, ependymal cells, alimentary tract and skin (Table 3, 4).

Additional 29 pathological findings including concurrent infections also were recorded. Concurrent infections included: Virus (1. adenovirus infection, liver, consistent with canine adenovirus I (infectious canine hepatitis); 2. adenovirus infection, lung, consistent with canine adenovirus II); Protozoa (3. coccidiosis, 4. toxoplasmosis, 5. babesiosis) ; Mycoplasma (6. hemobartonellosis); Fungi (7. aspergillosis); Parasite (8. cestodiasis, 9. ascaridiasis, 10. dirofilariasis, 11. acariasis (scabies).

Other findings included: Brain (12. brain microabscesses, 13. meningoencephalitis (without syncytia, inclusion bodies or demyelination), 14. polioencephalomalacia); Lung (15. suppurative bronchitis, 16 suppurative bronchopneumonia (without syncytia, or inclusion bodies),17. lung abscess) ; Heart (18. myocarditis;) ; Liver(19. hepatitis) ; Adrenal gland (20. nodular hyperplasia of adrenal gland; 21. hemorrhage and necrosis of adrenal gland) ; Spleen (22. extramedullary hematopoiesis (EMH)); Intestine (23. gastrointestinal (GI) crypt abscess) ; Testis (24. orchitis) ; Kidney(25. interstitial nephritis; 26. pyelonephritis) ; Bladder (27.

follicular cystitis; 28. suppurative cystitis); Skin (29. skin microabscess) (Table 6).

Data Analysis

Pearson's X2 test was used to compare : 1. age distribution of dogs in clinic and shelter groups; 2. pathological findings among the groups in two groups, (Table 2); 3. For each dog, we recorded the sites (organs) in which the inclusion bodies were seen (Tables 3 and 4). We then (i) compared the number of INIB/ICIB positive sites in the clinic and shelter groups, and (ii) for each of nine sites, compared the frequency of occurrence of INIB/ICIB between Clinic and Shelter groups by using the Pearson's X2 test (Table 5). 4. additional findings, including concurrent infections in two groups (Table 6); The two-tailed Student's t test was used to compare average number of occurrence of INIB/ICIB per dog and different co-infections and associated lesions in the clinic and shelter groups. For all statistical tests, a p value of < 0.05 was considered statistically significant.

RESULTS

Animals

From January 2000 to December 2009, RTPCR or IHC confirmed CDV infection of 52 dogs was submitted to our necropsy service. In the 32 dogs of clinic group, most were less than 6-month-old (24 cases, 75%); the remainders were older than 6-month-old (8cases, 25%). In contrast, in the 20 dogs of shelter group, most were older than 6 months (17 cases, 85%); the remainders were less than 6-month-old (3 cases, 15%).

IHC & RT-PCR

This study detected a total of 52 cases of CDV either tested by RT-PCR (73%, 38/52) only or IHC (63%, 33/52). The positive rate of RT-PCR diagnosis was 100% for the 38 cases for which specimens were available. The positive rate of immunohistochemical labeling was 97% (33 of 34) for 34 cases with specimens available for IHC. For cases combined tested both by IHC and RT-PCR, what was 95 % (19/20) agreement, only one case showed negative (Table 7).

Of the 52 cases, 65% (34/52) had CNS demyelination confirmed by H&E or LFB-CEV. Of these demyelination cases, 68% (23/34) were RT-PCR positive for CDV, and 82% (28/34) were IHC positive for CDV. Of the demyelination cases, 97% (33/34) had inclusion bodies. The IHC labeling (Fig. 1B, D). was more easy interpreted for diagnosis of CDV than was routine H&E staining (Fig. 1A,C). In general, the cerebrum (Fig. 1B), cerebellar white matter, 4th ventricle, lung(Fig. 1D), urinary bladder, and spleen were likely to be IHC-positive in CDV-infected dogs, and characteristic of IHC labelling as reported previously14 and had at least six of pathology findings consistent with canine distemper (Table. 2).

Pathology

All cases were examined by histology. Totally, the most common CDV diagnostic lesions of the dogs were lymphoid depletion, INIB/ICIB, interstitial pneumonia, and CNS demyelination in decreasing order. The positive rates were 79% (41/52), 75% (39/52), 71% (37/52), and 65% (34/52), respectively. However, the distribution of the nine CDV lesions varied considerably between the two groups of dogs. In the clinic dogs, the three most common lesions were lymphoid depletion (96% of the dogs), intranuclear inclusion bodies (87%), and interstitial pneumonia (81%). In the shelter dogs, these three lesions were also the most common, although the percentage of affected dogs was lower in each case, 50%, 55%, and 55%, respectively. In both groups of dogs, the least common lesion was foot pad hyperkeratosis, which affected 28% of the clinic dogs and 20% of the shelter dogs (Table 2).

We recorded eosinophilic INIB/ICIB in different organs of each dog of two groups (Table 3, 4). Two types of inclusion bodies were noted. One type was characterized as one, large, round or ovoid, distinct, homogenous, intensely eosinophilic INIB. The most common sites were cerebral astrocytes (Figure 2A), cerebellar white matter astrocytes, ependymal cells lining the 4th ventricle, the periphery of the splenic central arteries (Figure 2B), macrophages

***Table 3**. Sites of inclusion body in shelter group of CDV infected dogs.*

Lot No.	Urinary bladder	Lymphoid tissues	Lung	Cerebellum	Cerebrum	Brain stem	Ependymal cells	Alimentary tract	Skin
NTU00-171	-	-	-	-	-	-	-	-	-
NTU00-172	-	-	-	-	-	-	-	-	-
NTU00-233	+	+	+	+	+	+	+	+	+
NTU00-276	-	+	+	+	+	+	-	-	-
NTU00-350	-	+	-	-	-	-	+	-	-
NTU2000-384	-	-	-	-	-	-	-	-	-
NTU00-385	-	-	-	-	-	-	-	-	-
NTU00-403	+	-	-	-	-	-	-	-	-
NTU00-412	-	-	-	-	-	-	-	-	-
NTU00-433	-	-	-	-	-	-	-	-	-
NTU00-434	-	-	-	-	-	-	-	-	-
NTU00-457	-	-	-	-	-	-	-	-	-
NTU00-458	+	-	-	-	-	-	-	-	-
NP007	+	+	+	+	+	+	+	-	+
NP24	NA	+	-	+	+	+	+	-	-
NP49	-	-	-	+	-	+	+	-	-
NP106	-	-	-	+	-	-	-	-	-
NP107	-	-	-	+	-	-	-	-	-
NP110	+	+	-	-	-	-	-	+	-
NP159	-	+	-	-	-	+	-	-	-
No. detected	5	7	3	7	4	6	5	2	2
% positive	26	35	15	35	20	30	25	10	10

[a]: + : positive; -: negative; NA: not available

and lymphocytes in the lymph nodes, tonsil lymphoid tissues, pulmonary macrophages and bronchiolar epithelium, renal pelvis, urinary bladder epithelium (Figure 2C) and gastric glandular cells (Figure 2D). Another type was one or several smaller eosinophilic ICIB, seen chiefly in the renal pelvis, urinary bladder epithelium (Figure 2C) and skin epidermis.

The 29 co-infections and associated lesions with CDV infection are recorded and compared in two groups (Table 6). Most common co-infections were Cestodiasis, and Dirofilariasis. However, the co-infections included the basophilic adenovirus inclusion bodies (type II) (Figure 3A), adenovirus inclusion bodies (type I), coccidiosis, babesiosis, hemobartonellosis, aspergillosis and suppurative bronchitis were found in clinic group only. The parasitic infestation was more common in the older shelter group. Other common associated lesions included: 13. meningoencephalitis, 16 suppurative bronchopneumonia 22. extramedullary hematopoiesis (EMH) and 25. interstitial nephritis showed high incidence in shelter group (Table 6).

The microfilariae of *Dirofilaria* immitus were very common in both groups. They were found in pulmonary capillaries but were also noted in the interstitium with mixed leukocytic aggregates and erythrocytes (Figure 3B). Some rarely found infections included adenovirus infection in he-

***Table 4**. Distribution of inclusion bodies among 9 sites in clinic group of CDV-infected dogs.*

Source	Lot No.	Urinary bladder	Lymphoid tissues	Lung	Cerebellum	Cerebrum	Brain stem	Ependymal cells	Alimentary tract	Skin
C	NTU00-175	-	-	-	+	+	+	+	-	-
C	NTU00-396	-	-	-	-	-	-	-	-	-
C	NTU00-448	+	+	+	+	+	+	+	-	-
CV	NTU00-491	+	+	+	+	-	-	-	+	-
C	NTU00-504	+	+	+	+	+	+	+	-	-
C	NTU00-536	-	-	-	-	-	-	-	-	-
CV	NTU00-539	+	+	+	-	-	-	-	+	-
C	NP001	+	+	+	-	-	-	-	+	+
C	NP002	+	+	+	+	+	+	+	-	+
C	NP003	+	+	+	-	-	-	-	+	+
C	NP008	-	+	-	-	-	-	-	+	-
C	NP012	+	+	+	-	-	-	-	-	-
C	NP140	-	-	-	-	-	-	-	-	-
C	NP138	+	+	-	-	-	-	-	-	-
C	NP109	NA	+	+	+	+	+	+	-	-
C	NP126	+	-		+	+	-	-	-	
C	NP188	+	+	+	+	+		+	+	-
C	NP190	+	+	-	-	-	-	-	-	+
C	NP160	-	+	+	-	-	-	-	+	-
C	NP161	-	-	-	+	+	-	-	+	-
C	NP29	NA	+	+	+	-	-	+	+	NA
C	NP128	-	+	+	-	-	-	-	+	-
CV	NP135	-	-	-	+	+	-	-	+	-
CV	NP158	+	+	+	+	-	+	+	-	-
CV	NP189	+	-	+	-	-	-	-	+	-
CV	NP200	+	+	+	+	-	-	-	+	-
C	NP108	-	-	-	-	-	-	-	-	-
CV	NP151	NA	+	+	+	-	+	+	-	-
C	98-5102	NA	+	-	-	+	-	-	-	NA
C	dog1	NA	-	-	+	+	+	+	-	NA
C	NTU09-997	NA	-	-	+	+	+	+	-	-
C	NTU09-887	NA	-	+	+	+	-	+	+	NA
	No. detected	17	21	20	18	15	13	14	14	7
	% positive	68	65	62	56	46	40	43	43	25

C:clinic ; CV: clinic treatment with CDV vaccination history; +: positive; -: negative; NA: not available; r

Table 5. *Comparison of the frequency of intra-nuclear and cytoplasmic inclusion bodies (INIB/CIB) in 9 sites in shelter and clinic CDV-infected dogs. Data presented are number of INIB/CIB positive dogs, total number of dogs examined, and the percentage of dogs INIB/CIB positive.*

Sites of INIB/CIB	Shelter group		Clinic group		chi-square test
	nos	%	nos	%	
Urinary bladder	5/19	26	17/25	68	**
Lymphoid tissues	7/20	35	21/32	65	*
Lung	3/20	15	20/32	62	**
Cerebellum	7/20	35	18/32	56	ns
Cerebrum	4/20	20	15/32	46	ns
Brain stem	6/20	30	13/32	40	ns
Ependymal cells	5/20	25	14/32	43	ns
Alimentary tract	2/20	10	14/32	43	*
Skin	2/20	10	7/28	25	ns

*+ positive; - negative; statistically significant * $P < 0.05$, ** $P < 0.01$; ns, not significant; nos, number of samples*

patic cells (type I) (Figure 3C), babesiosis as well as hemobartonellosis were also found in this study. One case showed disseminated pyogranulomatous pneumonia with numerous, intralesional banana-shaped protozoal tachyzoites strongly suggested toxoplasma-like protozoa (Figure 3D) infection. One case showed disseminated pyogranulomatous lesions in the cerebrum, kidney, liver and serosa of gastrointestinal tract with intralesional PAS-positive thin septae hyphae, and Y-shaped branching, strongly suggested Aspergillosis infection.

Data Analysis

The clinic group showed significantly difference of younger age than the shelter group. There were significant differences in lesion frequencies, or disease pattern, between the clinic and shelter groups. The occurrence of 6 of the 9 diagnostic CDV lesions was significantly higher in the clinic dogs than in the shelter dogs: intranuclear inclusion bodies; demyelination in the cerebrum, cerebellum and brain stem; lymphoid depletion, and interstitial pneumonia (Table 2). For the remaining three lesions, catarral enteritis, hyperkeratosis, and syncytium, there was no significant difference in frequency of occurrence between the two groups of dogs.

In the clinic group (n = 32), inclusion bodies were most common in urinary bladder (68%), lymph node or spleen (65%), lung (62%) and cerebellum (56%), with 50% positive rate of detection overall in this group. In the shelter dogs, the most common sites for the inclusion bodies were the lymphoid tissues and cerebellum (both 35%), followed by the brain stem (30%); the least common sites were the alimentary tract and the skin (both 10%). The inclusion bodies of shelter group were identified in the same tissues but were significantly less common, with overall 23% detection rate (Table 5).

The occurrence of the INIB/ICIB in four of the nine sites was significantly higher in the clinic dogs than in the shelter group, namely in the urinary bladder, lymphoid tissues, lung, cerebellum, and alimentary tract. For the remaining tissues, there were no significant differences in occurrence between the two groups of dogs. Furthermore, the mean number of inclusion body positive tissues per dog in the clinic group (4.6 ± 0.6), was significantly higher than that

Table 6. *Twenty-nine complication and associated lesions distribution in shelter and clinical group of CDV infected dogs (Percentage of different lesions and co-infections /total animal counted*

Co-infections /Organ	Co-infections and associated lesions	3Shelter group, (N=20) (%)	Clinic group (N=32) (%)
Co-infections			
Virus	Adenovirus infection, liver, type I	0	3
	Adenovirus infection, lung, type II	0	16
Potozoa	Coccidiosis	0	6
	Toxoplasmosis	5	0
	Babesiosis	0	3
Mycoplasma	Hemobartonellosis	0	3
Fungi	Aspergillosis	0	3
Parasite	Cestodiasis	30	9
	Ascaridiasis	10	3
	Dirofilariasis	25	9
	Scabies	5	0
Associated lesion			
Brain	Brain microabscesses	0	3
	Meningoencephalitis	25	6
	Polioencephalomalacia	5	6
Lung	Suppurative bronchitis	0	16
	Suppurative bronchopneumonia	25	16
	Lung abscess	0	3
Heart	Myocarditis	5	3
Liver	Hepatitis	5	6
Adrenal gland	Nodular hyperplasia of adrenal gland	10	6
	Hemorrhage and necrosis of adrenal gland	5	0
Spleen	Extramedullary hematopoiesis(EMH)	30	19
Intestines	GI crypt abscess	5	3
Testis	Orchitis	0	3
Kidney	Interstitial nephritis	15	0
	Pyelonephritis	0	3
Bladder	Follicular cystitis	5	0
	Suppurative cystitis	5	0
Skin	Skin microabscess	5	0

One dog can have more than one co-infections or associated lesions; In 28 of the 29 co-infections and associated lesions, there were no significant differences in frequency of occurrence between shelter and clinic dogs (X2 chi-square test); the exception was interstitial nephritis, which was significantly more common in the shelter dogs than in the clinic dogs.

Figure 1. *Comparison between H & E and IHC labelling in two CDV infected dogs. A, B. Case 58, brain. A. H & E, showing multifocal spongy form of the mid-brain thalamus neuropil B, non-biotin HRP (AEC) with haematoxylin counterstain, showing diffuse immunolabelling of thalamus neuropil. C, D. Case 57, lung. A. H & E, showing interstitial pneumonia with exfoliated pulmonary macrophages and necrotic debris in the alveolar lumen. D, non-biotin HRP (AEC) with haematoxylin counterstain showing intracytoplasmic immunoreactivity in the bronchiolar epithelium and macrophages. All scale bars = 40 µm.*

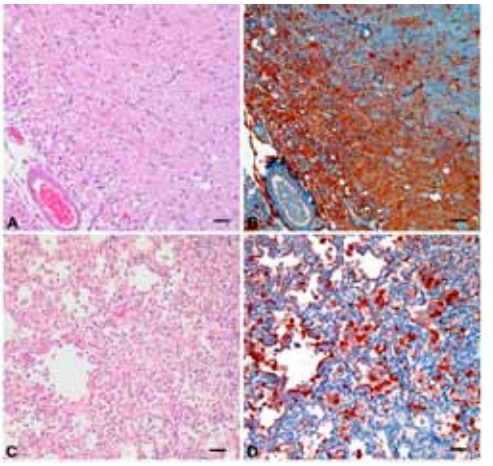

Figure 2. *. Eosinophilic intranuclear and intracytoplasmic inclusion bodies in four dogs with CDV. H & E. A, multifocal spongy form of the mid-brain thalamus neuropil with eosinophilic intranuclear inclusion bodies in the sub-ependymal astrocytes (arrowhead), case 58. B, splenic lymphoid depletion and necrosis with eosinophilic intranuclear inclusion bodies (arrowhead) in the white pulp, case 49. C, eosinophilic intranuclear and intracytoplasmic inclusion bodies (arrowhead) in the bladder ballooning mucosa epithelium, case 51. D, eosinophilic intranuclear inclusion bodies (arrowhead) in the gastric mucosa gland, case 50. All scale bars = 10 µm.*

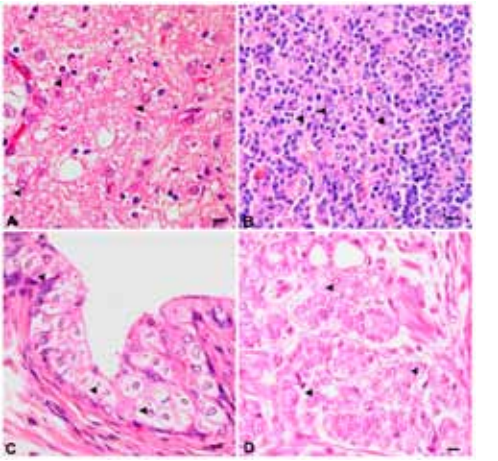

Figure 3. *Co-infections and associated lesion in four dogs with CDV. H & E. A, purulent bronchointerstitial pneumonia with smudge 8-10µm adenovirus, basophilic intranuclear inclusion bodies in the pulmonary bronchiolar epithelium (arrow), case 58. B, 10-40µm (body length) microfilaria of Dirofilaria immitis (arrows)deposited in the pulmonary alveolar septa, and capillaries with nodular aggregates of pyogranulomatous epithelioid macrophages, eosinophilic intranuclear inclusion bodies in the macrophages(arrowhead), case 49. C, hepatic cell necrosis with 5-8µm basophilic adenovirus, intranuclear inclusion bodies (arrows) in the hepatocytes, case 51. D, hemorrhagic to necrotizing pneumonia with numerous, 4-8 x 2-4 um, intra-lesional, curvilinear tachyzoites (arrow) free within areas of pulmonary necrotic parenchyma, case 50. All scale bars =10 µm.*

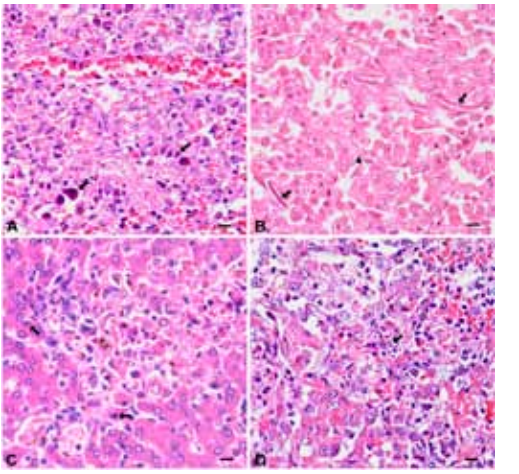

Table 7. *Diagnostic Criteria of CDV cases included in this study, N=52*

Single test	RT-PCR	IHC	Demyelination	INIB/ICIB
positive	38(73%)	33(63%)		
negative	0	1		
not available	14(27%); S 2; C12	18(35%); S 13;C5	34 (65%)	39 (75%)
Combined tests	20			
positive	19			
negative	1			

C: clinic; INIB/ICIB: intranuclear or intracytoplasmic inclusion bodies; IHC: immunohistochemistry; RT-PCR: Reverse Transcription Polymerase Chain Reaction, S: shelter.

in the shelter group (1.8 ±0.6) by two-tailed Student's t test.

However, there was no significant difference in the mean number of co-infections and associated lesions per dog between the two groups (2.2 in the shelter group, and 1.5 in the clinic group). Furthermore, the occurrence of 28 of the 29 co-infections and associated lesions was not significantly different between the two groups by two-tailed Student's t test; the exception was interstitial nephritis, which was significantly more common in the shelter dogs than in the clinic dogs.

DISCUSSION

To the best of our knowledge, this study is a first comprehensive, retrospective study of the pathology of CDV infection, that includes statistical analysis of the distribution of lesion, inclusion bodies, and co-infections in two contrasting groups of dogs. The principal findings were: (i) The CDV diagnostic lesions including lymphoid depletion, frequency of INIB/ICIB, pneumonia and demyelination, were significant different between younger clinic and older shelter group, (ii) The high occurrence of INIB/ICIB of CDV in Taiwan was noted in spleen, lymph nodes, lung and gastric mucosa epithelium. However, its positive rates were high in younger clinic group. In this study, most of the cases were "non-enteritis group" which showed pneumonia (55 to 81%) and degenerative demyelinating lesions in the CNS (20-78%) depending on sheler or clinic group (Table 2). These results were quite similar as "non-enteritis group" of canine distemper in Japan. Those cases were suspected different antigenically from the vaccine strain (Okita et al.,1997). However, the high occurrence of INIB/ICIB in urinary bladder (26-68%), spleen and lymph nodes (35-65%), lung (15-62%) and gastric mucosa epithelium(10-43%) in this study (Table 5) were similar to "enteritis group"of CDV in Japan (Okita et al.,1997). (iii) The positive rates of RT-PCR, IHC labeling, CNS demyelination and INIB/ICIB were 73%, 63%, 65%, 75%, respectively. The positive rates of either IHC labeling only 63% of total cases or RT-PCR diagnosis only 73% could be higher, because some case blocks are damage (35% not available, n =18), so we did not test IHC and few cases did not had RT-PCR results (27% not available, n=14) (Table 7).

When all 52 dogs were considered together, the positive rates of distemper inclusion bodies was 75%, which was slightly higher than previously reported; previous studies have ranged from 17 to 72%.8,19,23,29 However, intranuclear inclusion bodies were chiefly finding in this study rather than intracytoplasmic inclusion bodies in neuron.25 The occurrence of inclusion bodies in 4 of 9 tissues was significantly higher in the clinic dogs than in the shelter dogs between our two groups of dogs (Table 5). This may reflect the fact that the clinic group was acute stage of CDV infection, and younger, contrasting with the chronic stage and older shelter group.[3,27]

However, 75% of these dogs in clinic group are under 6-month-old were noted in the present Taiwanese study. The antigenic genetic variation of field CDV in Taiwan may account for these cases. Because the H gene of field isolated CDV in Taiwan had 10% amino acid variation from the vaccine Onderstepoort strain.[13] The present study showed that the immunohistochemical detection of CDV antigen in tissue sections was superior to the demonstration of inclusion bodies or syncytial cells for the diagnosis of canine distemper. This finding is consistent with previous reports.[6,14,19,20]

Among our findings of co-infections and associated lesions of particular note, was the high occurrence of pulmonary adenovirus type 2, which affected 15% of the clinic dogs (Table 6). A similar high value was also reported.5,6 The primary CDV infection, which might causes immunosuppression, and have predisposed the dog to secondary Tyzzer's disease and intestinal coccidiosis;[9] other viruses or with *Bordetella bronchiseptica* and Mycoplasma spp. infection,[6] or concurrent toxoplasmosis.[17]

There was a single case of simultaneous disseminated *Aspergillus* infection, with the kidney, spleen, liver, gastrointestinal serosa and brain involvement being similar to that described previously.[11] However, the etiology of mycotic meningoencephalitis also should consider *Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Cladophialophora bantiana*, species of *Aspergillus* and *Fusarium*, and *Sporobolomyces roseus*.[22]

We also noted a high incidence of *Dirofilaria* microfilariae (25% of shelter dogs, and 9% of clinic dogs) (Table 6); we are unable to determine the species, because we did not undertake the PCR test needed to discriminate between different species of canine microfilaria.[21] Since pathogenic mycoplasmas are rarely fulminant in a healthy host,16 our finding suggests possibile immunosuppression induced by CDV.

In addition to the co-infections documented in Table 6, which were determined by examination of lesions in histological sections, it is likely that additional infections were also present, however these were not detected because we did not do cultures or PCR. Examples of such infections are Parainfluenza viruses,[6] *Mycoplasma cynos*,[5] Colibacillosis,[28] cryptosporidiosis ,[7] and Tyzzer's Disease.[9] Two lesions previously reported to be associated with CDV, namely associated metaphyseal bone lesions [2] and myocardial degeneration,[10] were not found in the present study.

In conclusion, the systemic and demyelinating CDV lesions in two groups of dogs in Taiwan were identified using RT-PCR along with immunohistochemical labeling. The combination of these two techniques provides the accurate CD diagnosis rather than depending on examination of inclusion bodies, and provides a more specific, and sensitive method to confirm CDV infection. However, the incidence of histopathological lesions of distemper including inclusion bodies, varied from 20 to 96%, depending on organs, animal age, source, and treatment history. This comprehensive pathological study of CDV can serve as a comparative database for emerging lesions of CDV infection in the future.

CONFLICT OF INTEREST STATEMENT

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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