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組織內病毒蛋白與核酸偵測之最佳化:鼠肝炎病毒與犬瘟熱病毒

Optimization of the *in situ* detection of viral protein and nucleic acid in tissue: mouse hepatitis virus and canine distemper virus

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Abstract

Mouse hepatitis virus (MHV) is the leading viral pathogen of laboratory mice in Taiwan. This study established a modified alkaline phosphatase-labelled avidin-biotin-complex (ABC-AP) method for detection of MHV in tissues. Mouse hepatitis virus antigen was clearly detected in samples of liver, stomach, caecal and colonic mucosa, and spleen. This method may prove useful in diagnosis when commercial antisera are unavailable or when immunosuppression prevents serological diagnosis. (**Chapter II**). Canine distemper virus (CDV) causes a highly contagious disease, which has been reported in Taiwan for many years; however the causative and its genes have never been identified and pathogenesis are poorly understood. The objectives of the dissertation were to set up a fast and easy diagnostic method of CDV infection, to isolate the field virus and do phylogenetic analysis of the viral H gene, to characterize the pathology of CD in Taiwan, and to assess the frequency of CNS demyelination and other pathological lesions in cases of CDV infection confirmed by immunohistochemistry (IHC) and/or reverse transcription polymerase chain reaction (RT-PCR). This study describes a modified non-biotin polymerized horseradish peroxidase (HRP) immunohistochemical method for the diagnosis of CDV infection from formalin-fixed, paraffin-embedded tissues. This method confirmed seven out of eight (87.5%) suspected cases. Labeled CDV antigen was observed in cerebrum, cerebellum, meninges, glial cells, neurons, vascular endothelium, periventricular areas and pericytes, and choroid plexus; grey and white matter and central canal of the spinal cord; renal pelvis and tubular epithelium, and urinary bladder epithelium; macrophages and lymphocytes in splenic white pulp and lymph nodes; skin epidermis; bronchiolar epithelium and alveolar macrophages; hepatic Kupffer cells, and gastric and intestinal mucosal epithelium; stratified squamous epithelium of the tongue and oesophagus.With the non-biotin HRP detection system, pretreatment by autoclaving followed by microwave heating gave better labeling results than did microwave pretreatment alone. No obvious difference was noted between the labeling results produced by the non-biotin HRP detection system and the Super Sensitive ™ Link-Label IHC detection system (**Chapter III, VI**). For the purpose of confirming the IHC labeling and improving the detection of CDV nucleoprotein RNA, *in situ* hybridization (ISH) was applied in paraffin-embedded tissues from selected dogs with spontaneous CDV infections. In addition to proteinase K, autoclaving in various solutions (Trilogy, TBS S3006, H-3301, and S1700) for pre-treatments were compared. The intensity was assessed by using the integrated optical density (IOD) and the integrity of the tissue morphology. The combination of proteinase K digestion and autoclaving in a Trilogy solution resulted in a 5- to 100-fold ISH signal enhancement of CDV RNA. This modified technique can be useful in the

retrospective viral studies across a broad range in the future (**Chapter IV**). For the purpose of CDV genotyping, during the period from 2003 to 2005, two CDV strains were isolated from 17 non-vaccinated puppies with suspected canine distemper by co-culture with peripheral blood mononuclear leucocytes and B95a cells. In addition, four cloned hemagglutinin (H) genes were obtained from 166 dogs infected with CDV. Indirect immunofluorescence assays and antigen tests confirmed that they were CDV. Analysis of the H genes of the six identified strains revealed that the deduced amino acid sequences contained nine potential sites for N-linked glycosylation, as had been found for the H proteins of Japanese isolates. The seventh site is characteristic to the Taiwan strains described in this report and to the recently reported Japanese strains. Furthermore, phylogenetic analysis of the H gene showed that the six isolates belong to the Asia-1 group and are closely related to the recently reported Japanese and Chinese strains (**Chapter V**). To realize the histopathological lesions of CDV in Taiwan, fifty two (IHC or RT-PCR positive) affected dogs were obtained from either animal clinics or dog shelters from 2000 to 2009, within which 32 were from clinics and 20 were from shelters. Postmortem and laboratory examination included, gross findings, histopathology, Luxol-fast blue cresyl echt violet (LFB-CEV) histochemistry, non-biotin HRP anti-CDV immunohistochemistry, and phosphoprotein gene RT-PCR. 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 crossbred. Totally, 79% (41/52) showed lymphoid depletion, 71% (37/52) had interstitial pneumonia, 65% (34/52) had CNS demyelination, 32% (17/52) had catarrhal enteritis. Younger dogs from clinic group frequently had lymphoid depletion (31/32, 96%), inclusion bodies (28/32, 87%), pneumonia 81% (26/32, 81%), and CNS demyelination (26/32, 81%), which were all statistically significantly different from those from shelter. 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. Twenty nine co-infections and other associated lesions were identified. However, no significant difference was seen in the frequency of occurrence between two groups with the exception of interstitial nephritis. In conclusion, lymphoid depletion, pneumonia, and CNS demyelination were the most common CDV-infected principal lesions, and the inclusion bodies had a high occurrence in lymph nodes, spleen as well as mucosa epithelium of lung, stomach, urinary bladder and kidney (**Chapter III, VI**). In the present study, it has been demonstrated that CDV in Taiwan has at least one clade of the phylogenetic tree showing more than 95% amino acid similarity (< 5%

amino acid variation) in the H gene. The modified non-biotin polymerlized horseradish peroxidase (HRP) IHC labeling and ISH method are easy, optimal and accurate for retrospective diagnosis of CDV and MHV. The occurrence of CDV-associated pathological lesions depend on the source of the animals, treatment history, age and tissue distribution.

Key word: mouse hepatitis virus; canine distemper virus; immunohistochemistry; reverse transcription polymerase chain reaction; *in situ* hybridization

中文摘要

鼠肝炎病毒（mouse hepatitis virus；MHV）是小鼠族群高度傳染性首要病原。本研究在缺乏特異性冠狀病毒初級抗體情況下，利用血清酵素免疫吸附法（ELISA）鼠肝炎病毒陽性檢體血清，結合卵白素(avidin)與生物素化(biotinylated)的二級抗體，在發病免疫缺陷鼠之福馬林固定組織，如肝、胃、脾臟、 盲腸、及結腸等偵測鼠肝炎病毒抗原存在(Chapter II)。犬瘟熱病毒（canine distemper virus, CDV）為一年代久遠的疾病，本病之發生，已遍及全世界。犬瘟熱病毒為一高傳染性疾病，且存在臺灣許多年。然而；犬瘟熱病毒分離，基因型卻不曾有報告。組織病變，免疫化學染色診斷，病理學分析及併發症等均缺乏全面性調查資料。本研究目的為建立快速及正確的犬瘟熱病毒感染診斷方法，分離犬瘟熱病毒株及分析其H基因之遺傳差異，及經免疫化學染色(IHC)或反轉錄聚合酶反應(RT-PCR)確診下之自發病例，分析其中樞神經脫髓鞘發生率及其它病理變化。研究發現經非生物素性聚合山葵過氧化酶（non-biotin HRP）免疫化學染色方法，對福馬林固定、石蠟包埋組織之犬瘟熱感染病例，其診斷率可達87.5%。犬瘟熱病毒特異性抗原可在下列組織發現：大腦皮層外錐狀細胞及樹狀突細胞質、小腦白質層星狀細胞及細胞質、軟腦膜、神經膠質細胞、神經元、血管內皮細胞、腦室周圍、室管膜細胞、脈胳叢；脊髓灰、白質及中央導水管；腎盂上皮及腎小管上皮細胞；膀胱黏膜上皮細胞；脾臟白髓及淋巴結巨噬細胞及淋巴球；皮膚海綿層細胞及細胞質；肺臟小支氣管黏膜上皮及肺泡巨噬細胞；肝臟巨噬細胞；胃腸黏膜上皮細胞；扁桃腺及食道複層扁平上皮細胞等。組織經高溫高壓滅菌鍋前處理的免疫化學染色方法，其抗原復舊程度遠遠超過傳統微波爐法，大大提高犬瘟熱病毒診斷率(Chapter III,VI)。為了要確認免疫化學染色訊號，本研究建立一個改良的原位雜交染色步驟，應用於上述病例。此方法除了傳統的蛋白水解酶K的雜交前處理外；另外比較了將切片置入於不同(Trilogy, TBS S3006, H3301, S1700)溶液中進行高溫高壓滅菌鍋前處理。同時原位雜交訊號使用平均光密度(IOD)及組織形態完整性進行半定量性效果分析。結果發現進行原位雜交前，切片如果配合蛋白質水解酶K先處理，再以Trilogy溶液高溫高壓處理後進行原位雜交，其訊號及組織完整性均較其它組強，此一方法建立，未來將廣泛應用於犬瘟熱病例或其它病毒性疾病之原位雜交診斷(Chapter IV)。為了解台灣地區犬瘟熱病毒基因型分析，2003-2005年間進行病毒分離，自17隻未經疫苗注射之發病幼犬，應用患犬血液單核球與 B95a 細胞株共同培養之技術，分離出兩株具誘發融合細胞病變之病毒，經免疫螢光染色及抗原測試確認為犬瘟熱病毒。將此兩株病毒之血球凝集素基因（H），與同期間自台灣大學動物醫院臨床送檢病例之另外4株犬瘟熱病毒之H基因進行核酸定序。經比對序列與樹狀圖分析發現，本土病毒株皆有9個N連結配醣位，其中第7個配醣位為日本或中國大陸流行之亞洲1型犬瘟熱病毒所特有。本研究顯示臺灣地區所流行之犬瘟熱病毒，其H基因經分析屬亞洲1型（Chapter V)。為了解台灣地區是否有犬瘟熱病毒感染之

病理變化差異，2000-2009年分析來自收容所或臨床動物醫院之犬瘟熱患犬計52隻(經IHC或RT-PCR確診)。診斷方法包括肉眼檢查、組織病理、脫髓鞘特殊染色(LFB-CEV)、IHC、RT-PCR等。結果發現32隻來自臨床動物醫院，20隻來自收容所。來自臨床動物醫院之犬，75%為6月齡或以下，但來自收容所之犬，85%為大於6月齡。共計11種品種，但67% 為混種犬。在病理學分析，淋巴減少(79%)、間質性肺炎(71%)、中樞神經脫髓鞘(65%)、卡他性腸炎(32%)為主要病變。臨床動物醫院組相對地在淋巴減少(96%)、病毒包涵體(87%)、間質性肺炎(81%)及中樞神經脫髓鞘(81%)發生率很高，且統計差異顯著。腸炎占二組別之30-34%，無統計差異顯著。犬瘟熱病毒病毒包涵体在膀胱、淋巴組織、肺臟及消化系統，二組別有顯著差異。在29種犬瘟熱病毒感染之併發症或協同病變分析中，除了間質性腎炎外，二組間皆無顯著性統計差異。總結而言，淋巴減少、肺炎、中樞神經脫髓鞘是主要病變，然而病毒包涵體出現却以淋巴組織及黏膜上皮如腎臟、膀胱、肺臟及消化系統較高(ChapterIII,VI)。綜合以上結果，本研究證明台灣犬瘟熱病毒至少存在一種以上H基因型(亞洲1型)，其彼此間的胺基酸差異小於5%。同時本研究所建立的免疫化學染色及原位雜交染色方法使回溯性犬瘟熱病毒及鼠肝炎病毒之研究及診斷更簡單及正確。犬瘟熱病毒分離之方法可應用於未來臺灣不同地區之病毒分離。犬瘟熱病毒感染之伴隨病變視動物來源、治療病史、年齡及組織分布而定。

關鍵字: 鼠肝炎病毒; 犬瘟熱病毒; 免疫化學染色; 反轉錄聚合酶反應; 原位雜交

Chapter I

General Introduction

1.1. Background

A critical factor for both *in situ* hybridization (ISH) and immunohistochemistry (IHC) detection of viral protein and nucleic acid is fixation. The optimal and best fixatives for tissue were either paraformaldehyde, low concentrationof formalin (5% of formalin) (Yan et al., 2010) or 10% NBF, zinc formalin and alcoholic formalin (Babic et al., 2010). In brief, all aspects of tissue processing, including time until tissue fixation, type of fixative, duration of fixation, post-fixation treatments, and sectioning of the sample, impact the staining results (Babic et al., 2010). However, the tissue fixative formaldehyde initiates DNA denaturation (interchain hydrogen bonds break and bases unstack) at the AT-rich regions of double-stranded DNA creating sites for chemical interaction (Srinivasan et al., 2002; Shi et al., 2001). In this case, some heat-induced antigen retrieval (HIAR) pretreatment of the histological slides can enhance the signal of IHC or optimize the detection effect of nucleic acid by ISH (Srinivasan et al., 2002; Shi et al., 2001).

Canine distemper (CD), which is caused by a morbillivirus genus, family Paramyxoviridae, produces systemic or central nervous system (CNS) infections of dogs and related species and it is associated with high mortality in Taiwan (Wu *et al*., 2000; Yu *et al*., 2001). It is a non-segmented, single–stranded negative RNA virus of

approximately 15,616 nucleotides. Within the genome are six genes that encode for one envelope-associated protein (M), two glycoproteins (the haemagglutinine/attachment protein H and the fusion protein F), two transcriptase-associated proteins (the phosphoprotein P and the large protein L), and the nucleo-capsid N that encapsulates the viral RNA (Sidhu et al.,1993).

Diagnostically, CDV is typically detected following either immunohistochemical labelling (Frisk et al., 1999; Engelhardt et al., 2005; Liang et al., 2007), reverse transcription-polymerase chain reaction(RT-PCR) (Frisk et al., 1999), a sample antigen test (Liang et al., 2007), histopathological detection of intranuclear and cytoplasmic inclusion bodies, demyelination of cerebellar and cerebral white matter (Koutinas et al., 2002; Vandevelde and Zurbriggen, 2005; Kubo et al., 2007; Sips et al., 2007), footpad hyperkeratosis (Engelhardt et al., 2005; Koutinas et al., 2004; Okita et al., 1997), immunofluorescence (Engelhardt et al., 2005), clinicopathological findings (Amude et al., 2007) or a combination (Frolich et al., 2000).

The characteristic CNS changes of CDV infection include polioencephalomacia, white matter demyelination, astrogliosis, eosinophilic intranuclear and cytoplasmic inclusion bodies, gemistocytes, and occasional multinucleated syncytial giant cells (Summers et al., 1984; Summers and Appel, 1985). However, syncytial giant cells were demonstrated in only 9 - 28 % of CDV-infected brains (Palmer et al., 1990; Wu

et al., 2000), and eosinophilic intranuclear or cytoplasmic inclusion bodies in only 17 % (Haines et al., 1999), 33% (Stanton et al., 2002), 38.1% (Wu et al., 2000) or 72% (Palmer et al., 1990). Thus, histological examination of lesions does not appear to be a reliable indicator of CDV infection.

1.2. Pathogenesis of canine distemper virus infection

The pathogenesis and clinical features have been widely reported (Appel, 1970; Beineke et al., 2009). Great variations in duration, severity and clinical presentation of distemper have been found in experimentally infected dogs as well as in animals suffering from this world-wide spontaneously occurring disease. The incubation period may vary from 1 to 4 weeks and depends on the viral strain, age of the animal at the time of infection, and immune status of the host. Disease manifestation ranges from virtually no clinical signs to severe disease with approximately 50% mortality. The virus is shed primarily by oro-nasal secretion. Tissue macrophages and monocytes located in or along the respiratory epithelium and tonsils represent the first cell type to pick up and propagate the virus. Following this local burst of virus replication, the pathogen is then disseminated by lymphatics and blood to distant hematopoietic tissues during the first viremic phase. However, its CNS pathogenesis is not completely clarified. In the early stage of the infection, demyelination is

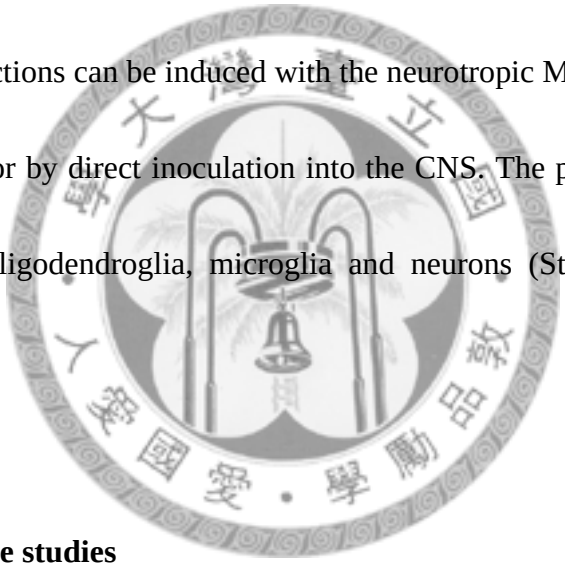
associated with viral replication in the white matter, in contrast, chronic inflammatory demyelination is due to a bystander mechanism resulting from interactions between macrophages and antiviral antibodies (Beineke et al., 2009; Vandevelde and Zurbriggen, 2005).

1.3. Pathogenesis of mouse hepatitis virus infection

Mouse hepatitis virus (MHV) which is caused by an enveloped positive stranded coronavirus genus, family Coronaviridae, causes a variety of diseases, such as enteritis, hepatitis, in susceptible rodents (Haring and Perlman, 2001). Enterotropic MHV strains, such as MHVY, initially replicate in epithelial cells of the gastrointestinal (GI) tract after infection of adult immunocompetent mice. Disease is acute and mild with minimal pathologic changes, and virus rarely disseminates to other organs. In contrast, morbidity and mortality are high in neonatal immunocompetent mice, and infection of immunocompromised mice can cause multisystemic, persistent infection with extended viral shedding and high mortality. Polytropic MHV strains, such as MHV-A59 or MHV-JHM (JHMV), initially replicate in the proximal respiratory tract epithelium after infection, then disseminate to many organs via viremia, lymphatic spread, or olfactory pathways from the nose to the brain. Infection of adult immunocompetent mice results in subclinical infection, hepatitis,

and/or encephalitis, whereas infection of immunocompromised micecan cause severe disseminated disease with high mortality (Compton et al., 1993; Liang et al.,1995).

The demyelinating JHM strain of MHV similar as CDV, infection of immunocompetent mice with the results in acute encephalitis, followed by the development of chronic demyelination in survivors (Haring and Perlman, 2001; Weiner et al., 1973). The pathogenesis of Neurotropic JHM strain is dependent upon viral dose, route of infection, the host's age and genetic background; however, prominent CNS infections can be induced with the neurotropic MHV strains either via the intranasal route or by direct inoculation into the CNS. The parental JHMV strain infects astrocytes, oligodendroglia, microglia and neurons (Stohlman and Hinton, 2001).



1.4. Objectives of the studies

The objectives of the studies were to set up an accurate and optimal IHC or ISH detection method of CDV and MHV infection, to isolate the field virus and do phylogenetic analysis of the viral H gene, to characterize the pathology of CD in Taiwan, and to assess the frequency of CNS demyelination and other associated lesions in cases of CDV infection confirmed by immunohistochemistry (IHC) and/or RT-PCR.

1.4.1. Immunohistochemical detection of mouse hepatitis virus infection

Mouse hepatitis virus (MHV) is the most common viral pathogen of mice, and seropositivity for MHV had been reported in 19–83% of animals in mouse colonies (Kraft and Meyer, 1986, 1990; Casebolt et al., 1988; National Research Council, 1991; Liang et al., 2009). In Taiwan, MHV is highly prevalent in mouse colonies, especially in immunosuppressed mice (Liang et al., 1995; Liang et al., 2004). Due to the inability of immunodeficient homozygous mice to produce antibody, immunohistochemistry may represent a useful replacement for serology in diagnosing MHV infection. In this study, high-titre serum from immunocompetent mice was used as primary antibody. The findings regarding the distribution of MHV antigen accorded with those of previous reports (Weir et al., 1987; Barthold et al., 1990), the antigen being demonstrated in the intestine, liver, spleen and stomach, consistent with multi-organ MHV infection (Compton et al., 1993).

1.4.2. Immunohistochemical detection of canine distemper virus infection

Immunohistochemical detection of CDV antigen in tissue sections was reported by Palmer et al. (1990), to be superior to the demonstration of inclusion bodies or syncytial cells for the diagnosis of canine distemper encephalitis. In the infected dogs,

immunohistochemical examination demonstrates CDV antigen in glial cells, neurons, ependymal cells, inflammatory macrophages, choroid plexus cells, and meningeal cells (Mitchell et al., 1991); in haired skin and footpad epithelium (Haines et al., 1999; Koutinas et al., 2004); and in lung, spleen, kidney, and lymph nodes (Iwatsuki et al., 1995).

The traditional avidin-biotin complex (ABC) immunohistochemical labelling method has several disadvantages. First, avidin has a high isoelectric point. At pH 7.4, it is positively charged and tends to bind to certain proteins (Haines and Chelack, 1991). The hydrophobic and electrostatic characteristics of the avidin conjugates appear to play a role in the non-specific binding (Masuoka et al., 2002). Second, kidney, pancreas, liver, lymphoid tissues, and nervous system tissue may contain lectin-like, negatively charged, endogenous, biotin-rich material which may adhere non-specifically to the ABC, resulting in false positive reactions (True, 1990; Ramos-Vara, 2005). Although the background can be greatly reduced by replacing avidin with streptavidin, background from endogenous biotin is still a problem with streptavidin methods (Ramos-Vara, 2005).

This report describes the development of a novel method of non-biotin chain polymer conjugate labeling combined with heat-induced epitope retrieval (Shi et al., 2001; Ramos-Vara, 2005; Liang et al., 2007) for CDV diagnosis.

1.4.3. *In situ* hybridization detection of canine distemper virus infection

In situ hybridization (ISH) can be used to detect CDV RNA in formalin-fixed, paraffin-embedded tissues (Muller et al., 1995; Gaedke et al., 1997; Hoyland et al., 2003; Engelhardt et al., 2005; Vandevelde and Zurbriggen, 2005; D'Intino, et al., 2006). However, in a retrospective study of the detection of viral RNA in formalin-fixed, paraffin-embedded tissues, the results of the ISH optimization following proteolytic enzyme digestion and heat-induced antigen retrieval (HIAR) pre-treatment were conflicting (Mcquaid et al., 1990; Gaedke et al., 1997; Kim and Chae, 2003; Shi et al., 2001). Additionally, few reports have focused on the optimization of the protocol for the detection of CDV RNA. While both proteinase K and saponin treatment preserved the morphology of tissues equally well, treatment with 0.1% saponin resulted in a more robust ISH signal than the proteinase K treatment (Yamawaki et al., 1993). Gaedke (1997) reported that the treatment with buffers made with DEPC water before hybridization, the storage time in paraffin wax, or the length of time the tissue was incubated in the fixative had no effect on the ISH-based detection of CDV RNA. Additionally, proteinase digestion, sodium borohydrite, or boiling in citrate buffer in the microwave had no effect.

However, other reports have demonstrated that proteinase K and protease VIII

treatment results in the maximal signal with minimal tissue degradation. With both enzymes, either increasing the concentration or increasing the length of the incubation produced no increase in the ISH signal. An incubation of 5 min at RT with 0.5 mg/ml protease VIII and microwaving resulted in the optimal signal for the detection of measles virus (Mcquaid et al., 1990). Similarly, antigen retrieval using a thermocycler combined with proteinase K digestion can enhance the ISH signal for the detection of porcine circovirus 2 (Kim and Chae, 2003). Because CDV are RNA virus which offer several unique limit regarding their *in situ* detection. The detection threshold of ISH which is estimated to be about 10 copies per cell. RNA viruses often produce lower copy numbers when they infect a cell and need *in situ* pretreatment before ISH (Amaro Filho and a Nicol, 2010).

The aim of this study was to optimize the ISH protocol and provided a combined HIAR pretreatment for the detection of CDV RNA in formalin-fixed, paraffin-embedded tissues in retrospective studies.

1.4.4. Viral isolation and phylogenetic analyses of field canine distemper virus

Dogs can be protected from the infection by live attenuated vaccine. Nevertheless, increasing incidences of canine distemper in pups in suburb areas and in urban kennel shops have recently been noticed in spite of vaccination. Apparent failure in

vaccination in Japan is considered to be due to the presence of maternal antibody which interferes with vaccine virus (Kai et al.,1993; Iwatsuki et al.,2000). Consequently, there are similar problems in Taiwan. As another possibility, antigenic changes in the currently prevalent CDV, which the current vaccine does not give complete protection, are also speculated (Kai et al., 1993; Lan et al., 2006). Characteristics of prevailing CDV in the field, however, have not been investigated in Taiwan due to difficulties in isolation of wild CDV from the field samples. The isolation of CDV was difficult (Imagawa et al., 1980; Metzler et al., 1984). Even using B95a cell, the viral isolation rate might be zero (0/32), However, that report used older dogs, from 3-month to 9-year-old as resource for viral isolation (Shin et al., 1995). CDV was rarely isolated from plain brain homogenates (Kimoto, 1986). However, using canine kidney tissue system (Ho et al., 1979; Evans et al., 1991), and canine lung macrophage cultures have been employed for the isolation of CDV without losing its virulence, but the cells are often contaminated with bacteria (Appel et al., 1967). Bovine proliferative cells and peripheral blood macrophages were also tried for CDV isolation (Metzler et al.,1981). The avirulent CDV may grow well in kidney cells but poorly in macrophages, whereas the virulent CDV grow well in macrophages but poorly in kidney cells (Evans et al., 1991). Vero cells have been widely used for the isolation of CDV as well as measles virus (Metzler et al.,1981,

1984; Kimoto ,1986; Evans et al.,1991), but this system is not practical since several passages are required for virus isolation (Ho et al.,1979). Vero.DogSLAMtag cells developed cytopathic effect (CPE) as early as 24 hours after inoculation, but it requires specific transfection techniques (Seki et al., 2003; Lan et al., 2006). Virulent CDV replicates well in mitogen-stimulated canine or ferret lymphocytes (Ho et al., 1981; Appel et al., 1992). However, CPE is not produced in these cells, so that the cultures have to be co-cultured with other susceptible cells such as Vero cells after confirming the presence of virus antigens by immunofluorescent technique (Appel et al., 1992). With such primary cells, their preparation and possibility of contamination with other canine viruses make them impractical.

Considering these drawbacks in the use of primary cells and long passage time of Vero cells, using B95a cells of marmoset B lymphoid cell line, which have been reported to be highly susceptible hosts for the isolation of wild measles virus from patient's materials (Kobune et al.,1989), and the canine distemper virus isolation (Kai et al., 1993; Mori et al., 1994; Imagawa et al.,1999). In this study, we succeeded in the isolation of CDV in Taiwan by using B95a cells and compared the H gene phylogenetic analyses with other reference isolates. Phylogenetic analyses place the field Taiwan CDV strains in the Asia-1 group (Liang et al., 2008). CDV isolates in Taiwan are all have 530 G, 549Y and 178A residues of Haemagglutinin(H) gene

(Liang et al., 2008). This is the first report of CDV viral isolation report in Taiwan.

1.4.5. Retrospective pathological analysis of canine distemper in Taiwan

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; CNS signs are now marked and gastrointestinal involvement is rare (Liang et al., 2007). Two distinct disease groups 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 (Okita et al., 1997). 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 the infected brains (Palmer et al., 1990), and eosinophilic intranuclear or cytoplasmic inclusion bodies in only 17-72 % (Haines et al., 1999; Palmer et al., 1990; Stanton et al., 2002; Wu et al., 2000).

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)(Chvala et al., 2007; Damian et al., 2005), coccidiosis (Headley et al., 2009), colibacillosis (Wada et al., 1996), cryptosporidiosis (Fukushima and Helman, 1984), parainfluenza viruses (Damian et al., 2005), *Mycoplasma cynos*(Chvala et al., 2007), toxoplasmosis (Moretti et al., 2006), Tyzzer's disease (Headley et al., 2009), 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 a retrospective study was conducted, for the 10 years from 2000 to 2009, to compare CDV histopathological lesions and complications in two groups of dogs in Taiwan, 32 dogs that had been treated in clinics and 20 dogs from shelters.

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Chapter II

Immunohistochemical diagnosis of mouse hepatitis virus and Mycoplasma pulmonis infection with murine antiserum

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Immunohistochemical Diagnosis of Mouse Hepatitis Virus and *Mycoplasma pulmonis* Infection with Murine Antiserum

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Summary

This study established a modified alkaline phosphatase-labelled avidin-biotin-complex (ABC-AP) method for diagnosis of mouse hepatitis virus (MHV) and *Mycoplasma pulmonis* infection from formalin-fixed, paraffin wax-embedded sections, murine antibody-positive serum being used as the primary reagent. With this method, MHV antigen in cAnNCrj.Cg-*Foxn1*^{nu}/*Foxn1*^{nu} mice and *M. pulmonis* antigen in Wistar rats were immunolabelled in tissue sections. MHV antigen was clearly detected in samples of liver, stomach, caecal and colonic mucosa, and spleen. *M. pulmonis* antigen was demonstrated on the luminal surface of bronchiolar epithelial cells. This method may prove useful in diagnosis when commercial antisera are unavailable or when immunosuppression prevents serological diagnosis.

Keywords: bacterial infection; mouse; mouse hepatitis virus; *Mycoplasma pulmonis*; viral infection

Introduction

Avidin-biotin-complex immunohistochemistry offers a sensitive, reliable method for the detection of pathogens in tissues. Mouse hepatitis virus (MHV) and *Mycoplasma pulmonis* are the most prevalent pathogens of laboratory mice and rats (National Research Council, 1991). Respiratory strains of MHV infect the nasal mucosa and then spread to the liver, lymphoid tissue, uterus, placenta, peritoneum, brain, vascular endothelium and bone marrow by the lymphatic or vascular route, or directly via olfactory pathways from the nose to the brain. Enterotropic strains of MHV usually infect the intestinal mucosal epithelium and nasal passages, with less involvement of other tissues (National Research Council, 1991; Compton *et al.*, 1993; Liang *et al.*, 1995).

M. pulmonis, an extracellular pathogen of mice and rats, preferentially colonizes the luminal surface of respiratory epithelium, the middle ear and endometrium (National Research Council, 1991; Percy and Barthold, 1993). Infection is usually diagnosed by microbial isolation, serological testing and histological examination. Microbial isolation requires multisite culture for reliable results, and serological methods are hampered by cross-reactivity between different species of mycoplasma (Cassell *et al.*, 1981). Serological testing is useful during the active and convalescent phases of disease (Feldman, 2001), but screening of immunodeficient mice is unreliable (Casebolt *et al.*, 1997). Immunohistochemistry is widely used to

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detect microbial antigen in tissue sections from naturally infected animals, except when specific primary antibodies are commercially unavailable or excessively expensive. In such cases, the use of positive antiserum from infected members of the homologous species is of potential valve. Although the use of homologous primary antiserum is not usually applicable in immunohistochemistry, a suitable method was recently described by Lu and Partridge (1998). In the present study, an avidin-biotin-complex and alkaline phosphatase (ABC-AP) method was developed in which ELISA-positive murine antiserum was used as the primary antibody for the diagnosis of MHV and *M. pulmonis* infection.

Materials and Methods

Animals

Experiment 1. Specific-pathogen-free cAnNCrj.Cg-*Foxn1*^{nu}/*Foxn1*^{nu} and *Foxn1*$^{nu/+}$ nude mice, originally obtained from Charles River Laboratories (Yokohama, Kanagawa, Japan), were maintained at the National Laboratory Animal Center (NLAC) in Taiwan. Thirty of these mice, consisting of 20 homozygous males aged 5 weeks and 10 homozygous females aged 9 weeks were sold, to a unit that maintains animals for use in research in Taipei City, on February 2 and March 8, 2000, respectively. Animals were housed in sterile microisolator cages (Laboratory Products, Maywood, NJ, USA), kept in animal cabinets (Nu-air; Plymouth, MN, USA), and placed in conventional rooms. Sterile water and commercial rodent chow (PMI Feeds, St Louis, MO, USA) was provided *ad libitum*. Bedding changes, water replenishment and supply of food were carried out in a class-II biological safety cabinet. The animal houses were maintained at 20 to 25 °C with a 12-h light/dark cycle.

At 42–49 days after arrival at the unit, the male nude mice had become emaciated, anorexic and dehydrated, with scaly skin, hunched posture, diarrhoea and ocular discharge, and 50% had died. The female nude mice showed similar but less severe signs. Four male and two female nude mice were humanely killed at the age of 15–16 weeks and samples were taken back to the NLAC for pathological examination.

Experiment 2. Sixty 6-week-old female Wistar rats from the NLAC were transferred to a clean conventional local area and housed in autoclaved microisolators. Bedding was changed twice a week in class II biohazard cabinets. Animals were maintained at a room temperature of 20–25 °C and humidity of 50–70%, with a 12-h light /dark cycle. Sterile water and commercial rodent chow (PMI Feeds) were provided *ad libitum*. Bedding (Beta-chip; Northeastern Inc., Warrensburg, NY, USA), polysulfone cages (Laboratory Products) and supplies were all autoclaved. A proportion (30%) of the animals showed sneezing 5–6 weeks after arrival. Thirteen of the affected animals, now aged 12–24 weeks, were killed for diagnostic evaluation. Necropsy revealed consolidation of the apical and cardiac lobes of the lungs. ELISA screening for pneumonia virus of mice (PVM), Sendai virus, lymphocytic choriomeningitis virus (LCMV), and sialodacryoadenitis virus (SDAV) was negative. Antibody titres of three rats for *M. pulmonis*, assessed by ELISA score, were 6.07 to 17.22. ELISA results were interpreted on the basis of the method described below.

Preparation of ELISA-positive Sera

The ELISA monitoring programme and diagnostic service of the NLAC were used to detect the following infectious agents: pneumonia virus of mice (PVM), reovirus (Reo-3), Sendai virus, lymphocytic choriomeningitis virus (LCMV), Theiler's murine encephalomyelitis virus (GD VII), minute virus of mice (MVM), mouse hepatitis virus (MHV), mouse adenovirus (Mad), ectromelia virus, Kilham's rat virus (KRV), sialodacryoadenitis virus (SDAV/RCV), *M. pulmonis*, hantavirus, K virus, and *Clostridium piliforme*. The procedure followed the Charles River ELISA scoring system (Serology Method Manual; Charles River Laboratories, Wilmington, MA, USA). Briefly, 50 µl of serum sample, diluted 1 in 60 in BLOTTO (Bovine Lacto Transfer Technique Optimizer; 5% non-fat dry milk in phosphate-buffered saline (PBS)) (Johnson *et al.*, 1984), were added to each appropriate antigen well and control well. The plate was covered and incubated for 40 min at 37 °C. After several washings, 50 µl of horseradish peroxidase-conjugated, affinity-purified horse anti-mouse or anti-rat IgG (Kirkegaard and Perry Laboratories, Maryland, USA), depending on species, were added to each well. After incubation for 40 min at 37 °C, the plate was washed again. One hundred µl of 0.4 mM ABTS-2.0 mM H_2O_2 chromogenic substrate were then added to each well and the plate was incubated at room temperature for 40 min. Absorbance was determined colorimetrically at 405 nm with an ELISA microplate reader (Thermo-max; Molecular Devices, Sunnyvale, CA, USA). Absorbance values were transmitted from the ELISA reader to a personal computer (PC) where they were converted to scores by dividing by 0.13.

The denominator of 0.13 divided net absorbance values of 0.13 to 1.3 into scores of 1 to 10. Integer scores were read and interpreted by comparison with the 3-decimal absorbance values. The PC was also used to compute net scores ($Score_{antigen}$ minus $Score_{tissue\ control}$). A result was considered non-specific and recorded as tissue control (TC) when both $Score_{antigen}$ and $Score_{tissue\ control}$ were 2 or above. Provided that the $Score_{tissue\ control}$ was lower than 2 (absorbance values lower than 0.26), net scores were interpreted as follows: 0–1, negative; 2–3, borderline; ≥3, positive. When the test serum was interpreted as "single agent (i.e., MHV)-positive", and the net score was ≥10, serum samples were collected and stored at −20 °C until used as primary antibody for immunohistochemistry.

Pathological Examination

The lungs, trachea, lymph nodes, heart, liver, spleen, small intestine, stomach, kidneys, urinary bladder, adrenal glands, skin and brain from affected animals in experiments 1 and 2 were fixed in 10% neutral buffered formalin. The tissue samples were processed by routine methods to paraffin wax-embedded blocks. Sections (6 μm) were stained with haematoxylin and eosin (HE).

Immunohistochemistry

The Mouse on Mouse kit (M.O.M.™; Vector Laboratories, Burlingame, CA, USA) was used for the immunohistochemistry study of MHV infection in experiment 1. The primary antibodies were ELISA-positive, mouse anti-MHV sera (ELISA score; 11.2–14.4). Infection with PVM, Reo-3, Sendai virus, LCMV, GD VII, MVM, Mad, ectromelia virus, K virus, *M. pulmonis* and polyoma virus were ruled out on the basis of ELISA results.

Tissue sections were dewaxed in xylene and rehydrated in a graded alcohol series. Antigen unmasking was performed by immersion of sections in Vector antigen unmasking solution 1% in PBS and boiling for 5 min in a 1450-W microwave oven (RE-C102; Sampo Co., Taiwan). The sections were then immersed in cool PBS for 10 min, rinsed in PBS, and incubated with trypsin (Sigma Chemical Co., St Louis, MO, USA) 0.1% in PBS for 5 min at 40 °C. Endogenous peroxidase activity was quenched with hydrogen peroxide 0.3% in methanol for 5 min at 40 °C. The sections were then rinsed in PBS, incubated for 30 min at 40 °C in mouse M.O.M.™ IgG blocking reagent, rinsed with PBS, and incubated in M.O.M.™ diluent for 5 min at 40 °C. Subsequently, they were incubated in ELISA-positive murine anti-MHV serum (diluted 1 in 60 in M.O.M.™ diluent) as the primary antibody for 24 h at 4 °C. Substitution of PBS or mouse serum (negative for any pathogen) for the primary antibody served as a negative control. The sections were then rinsed in PBS, incubated with M.O.M.™ biotinylated anti-mouse IgG reagent for 60 min at 40 °C, rinsed in PBS, incubated in Vectastain® ABC-AP reagent for 60 min at room temperature, rinsed in PBS, and incubated with alkaline phosphatase substrate solution (Vector® Red; Vector Laboratories) in 100 mM Tris HCl, pH 8.2–8.5, for 30 min at room temperature. Endogenous alkaline phosphatase activity of tissues was inhibited by adding one drop of levamisole (Vector Laboratories) to 5 ml of Tris HCl buffer before preparation of the substrate working solution. The sections were rinsed in distilled water, counterstained with Mayer's haematoxylin and examined microscopically.

Lung sections from experiment 2 were pretreated as described in experiment 1. They were then rinsed in PBS, incubated for 30 min at 40 °C in diluted (1 in 20) normal horse serum (Vector Laboratories), incubated with diluted (1 in 60) ELISA-positive murine anti-*M. pulmonis* serum (ELISA score:13.4–17.7) as primary antibody for 24 h at 4 °C, rinsed in PBS, and incubated with diluted biotinylated horse anti-mouse IgG secondary antibody (1 in 100, rat-absorbed) for 60 min at 40 °C. The subsequent procedures were the same as those in experiment 1.

Results

ELISA Monitoring Results

In the MHV test, 485 mouse serum samples were examined. Of these, 465 were negative (score <3), three were positive (score 3–10), and 17 had a high MHV score (>10). Seven of 17 samples with a high score were excluded because of simultaneous positivity for GDVII. Ten serum samples (ELISA score: 11.2–14.4) were collected for further diagnosis of MHV infection in experiment 1.

In the *M. pulmonis* test, 268 rat serum samples and 383 mouse serum samples were examined. Only six rat samples were positive, and 380 mouse samples were negative (score <3). Three mouse samples showed a high *M. pulmonis* score (>10), but one of these was excluded because of simultaneous positivity for MHV. The remaining two mouse serum samples (ELISA score: 13.4–17.7) were used for the diagnosis of *M. pulmonis* infection in Wistar rats in experiment 2. These Wistar rats

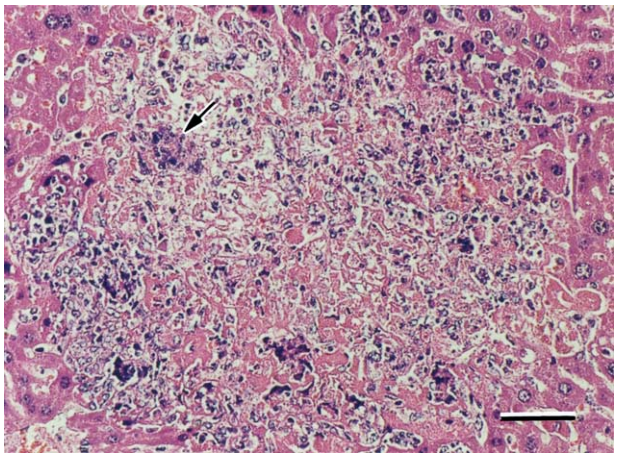

Fig. 1. MHV-infected mouse liver showing necrosis with multinucleated syncytial giant cells (arrow) at the periphery of the lesion. HE. Bar, 50 μm.

were confirmed as having *M. pulmonis* infection by testing 13 rat serum samples; three rats (23%) had an ELISA score of 6.07–17.22, and four (31%) had typical pulmonary lesions.

Histopathology and Immunohistochemistry

Experiment 1. Gross examination of the four affected male nude mice revealed that the liver was firm and pale with multiple, white, depressed foci, 2 to 3 mm in diameter. Microscopically, necrotic foci were seen to be scattered throughout the hepatic parenchyma, being well demarcated from the adjacent normal tissues (Fig. 1). Multinucleated syncytial giant cells with basophilic cytoplasmic granules (20 μm in diameter) were often present. Colonic and caecal mucosal ridges were attenuated and shortened, and contained syncytial cells (Fig. 2). Immunohistochemical results revealed

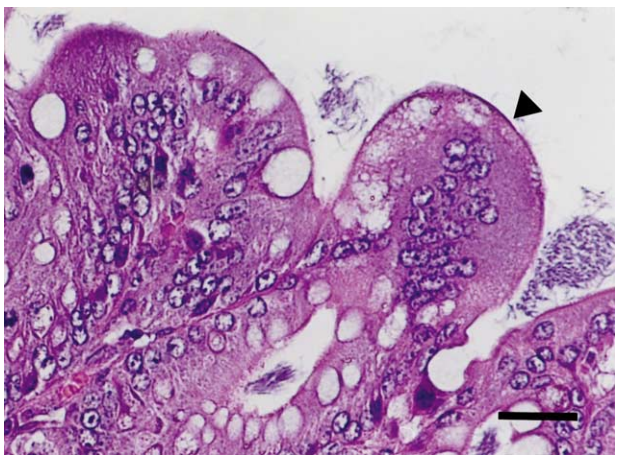

Fig. 2. Syncytial cells (arrowhead) in the surface epithelium of the colon of a MHV-infected mouse. HE. Bar, 25 μm.

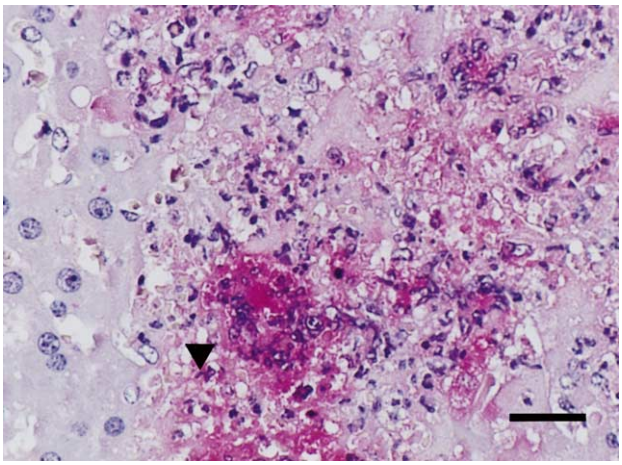

Fig. 3. MHV antigen within necrotic hepatocytes and syncytial cells (arrowhead) immunolabelled with ELISA-positive serum as primary antibody. ABC-AP with haematoxylin counterstain. Bar, 25 μm.

MHV antigen at the periphery of the necrotic foci in the liver (Fig. 3) and within the caecal and colonic multinucleated syncytial cells (Fig. 4), spleen, and jejunal and gastric mucosa.

Experiment 2. Microscopical examination of two affected Wistar rats showed extensive consolidation of the lung, with variable degrees of hyperplasia and metaplasia of bronchiolar epithelial cells, and mononuclear cell infiltration into the bronchioles and adjacent alveolar spaces. Loss of cilia and flattening of epithelial cells in the bronchioles were noted (Fig. 5). Aggregates of necrotic debris and neutrophils were present in the bronchiolar lumina. Peribronchial and perivascular cuffing by lymphocytes, macrophages and plasma cells was also observed. Sections of lung showed immunohistochemical labelling for *M. pulmonis*

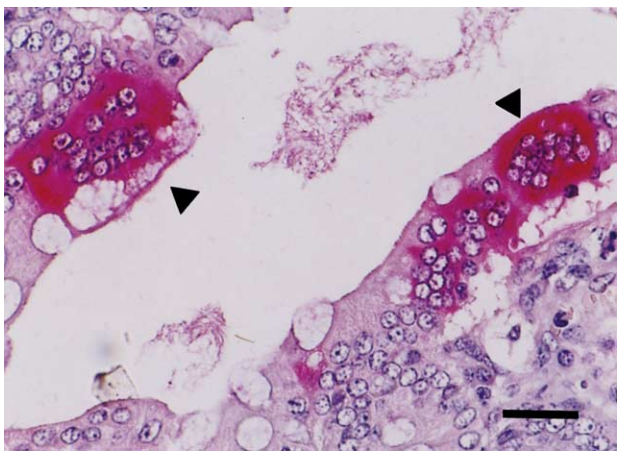

Fig. 4. Colonic epithelial and syncytial cells (arrowheads) immunolabelled for MHV with ELISA-positive sera. ABC-AP with haematoxylin counterstain. Bar, 25 μm.

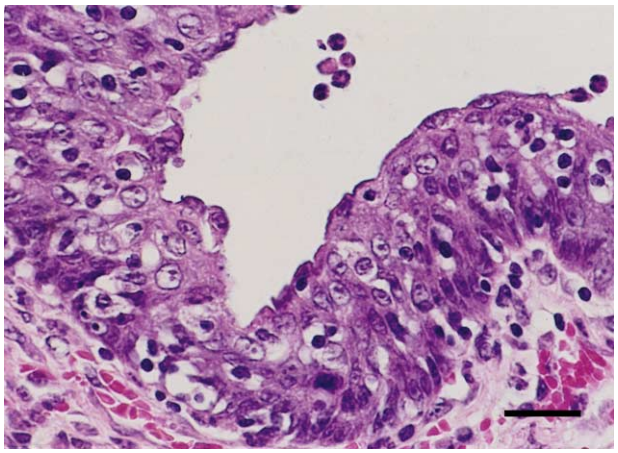

Fig. 5. Hyperplasia of bronchiolar epithelial cells of a rat infected with *M. pulmonis*. HE. Bar, 25 μm.

antigen over the luminal surface of hyperplastic bronchiolar epithelial cells (Fig. 6).

Discussion

Only 10 serum samples with a positive ELISA titre for MHV alone and two with a positive titre for *M. pulmonis* alone were used in this study. The results indicated the applicability of the technique to diagnosis in the absence of access to commercially produced antibodies. There have been few reports of the use of ELISA-positive murine sera as primary antibody for the immunohistochemical examination of rodent tissue. Polyclonal antiserum has been used for the diagnosis of MHV and *M. pulmonis* infection (Brownstein and Barthold, 1982; Brunnert *et al.*, 1994; Liang *et al.*, 1995). MHV is the most common viral pathogen of mice, and seropositivity for MHV had been reported in 19–83% of animals in mouse colonies (Kraft and Meyer, 1986, 1990; Casebolt *et al.*, 1988; National Research Council, 1991). In Taiwan, MHV is highly prevalent in mouse colonies, especially in immunosuppressed mice (Liang *et al.*, 1995). Due to the inability of immunodeficient homozygous mice to produce antibody, immunohistochemistry may represent a useful replacement for serology in diagnosing MHV infection. In this study, high-titre serum from immunocompetent mice was used as primary antibody. The findings regarding the distribution of MHV antigen in experiment 1 accorded with those of previous reports (Weir *et al.*, 1987; Barthold *et al.*, 1990), the antigen being demonstrated in the intestine, liver, spleen and stomach, consistent with multi-organ MHV infection (Compton *et al.*, 1993).

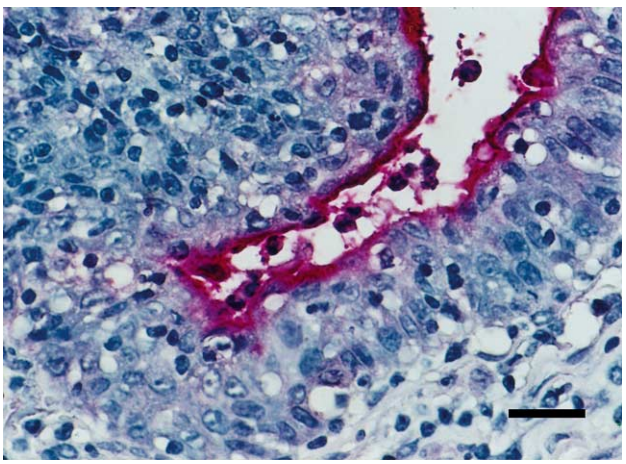

Fig. 6. *M. pulmonis* antigen is clearly demonstrated on the luminal surface of hyperplastic bronchiolar epithelial cells. ABC-AP with haematoxylin counterstain. Bar, 25 μm.

Serology and culture are widely used in the diagnosis of *M. pulmonis* infection, but discrepancies sometimes occur (Cassell *et al.*, 1981). ELISA has detected *M. pulmonis* infection in 8–78% of rat colonies and 35–91% of mouse colonies, depending on whether conventional or barrier-maintained facilities are used (Casebolt *et al.*, 1988; Kraft and Meyer, 1990). An advantage of ELISA is the low incidence of non-specific or false positive reactions as compared with haemagglutination inhibition (HI) (Kraft and Meyer, 1986). Discrepant results for *M. pulmonis* infection obtained by different serological tests may be due to reactive substances in the serum, such as lysozyme, antinuclear antibodies, protease and bacterial products (LaRegina *et al.*, 1987). Culture of *M. pulmonis* from tracheobronchial lavage fluid showed 89.6% positivity in rats and 36.5% positivity in mice in non-barrier-maintained facilities (Timenetsky and DeLuca, 1998). For routine monitoring of *M. pulmonis*, the preferred use of time-consuming culture procedures as opposed to serological testing is applicable only in acute or early infection. One-third of infected animals do not yield *M. pulmonis* in culture (Kraft *et al.*, 1982). Culture and histopathology may be misleading in evaluating a colony of rodents for mycoplasma infection, particularly when the prevalence is low (Cox *et al.*, 1988).

M. pulmonis infection in the chronic stage is readily detected histopathologically (Kraft *et al.*, 1982; Goto *et al.*, 1994), but in some instances MHV or mycoplasmal infection produces minimal or no lesions. In such instances, immunohistochemistry is valuable (Matthaei *et al.*, 1998). In experiment 2, labelling of *M. pulmonis* antigen was noted on

the luminal surface of bronchiolar epithelial cells, a site also affected by the cilia-associated respiratory (CAR) bacillus (Matsushita *et al.*, 1987). In a previous study (Brunnert *et al.*, 1994) the ABC method failed to detect *M. pulmonis* in formalin-fixed lung tissue but gave 27.4% (17/62) positive results with ethanol-fixed lung tissue; this compared with 96% (60/62) positive results given by the polymerase chain reaction (PCR). However, MHV antigens were demonstrated in formalin-fixed, paraffin wax-embedded blocks for as long as 2 years after preparation (Brownstein and Barthold, 1982). Immunohistochemistry, commonly used to detect rodent pathogens, takes much less time than that required for culturing mycoplasmas. In the present study, however, the incubation with primary antiserum was carried out overnight at 4 °C (Miller and van der Maaten, 1989), rather than for 30 min at room temperature or at 40 °C (Liu *et al.*, 1997; Liang *et al.*, 2000), the purpose being to increase the immunolabelling.

PCR is more sensitive than immunohistochemistry or microbial isolation but requires a high degree of technical expertise, and contamination leads to false positivity (Brunnert *et al.*, 1994). It offers an advantage over serological testing, however, in situations in which an antibody response is unlikely to be generated. In conclusion, the method described, in which ELISA-positive serum was used as primary antibody, may be useful in diagnosing infection in immunodeficient animals or when commercial immunohistochemical reagents are unavailable (Matthaei *et al.*, 1998).

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Chapter III

A non-biotin polymerized horseradish - peroxidase method for the immunohistochemical diagnosis of canine distemper

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A Non-biotin Polymerized Horseradish-peroxidase Method for the Immunohistochemical Diagnosis of Canine Distemper

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Summary

This report describes a modified non-biotin polymerized horseradish peroxidase (HRP) immunohistochemical method for the diagnosis of canine distemper virus (CDV) infection from formalin-fixed, paraffin wax-embedded tissues. This method confirmed infection in seven of eight (87.5%) suspected cases. Labelled CDV antigen was observed in the following sites: cerebrum, cerebellum, meninges, glial cells, neurons, vascular endothelium, periventricular areas and pericytes, and choroid plexus; grey and white matter and central canal of the spinal cord; renal pelvis and tubular epithelium, and urinary bladder epithelium; macrophages and lymphocytes in splenic white pulp and lymph nodes; skin epidermis; bronchiolar epithelium and alveolar macrophages; hepatic Kupffer cells, and gastric and intestinal mucosal epithelium; stratified squamous epithelium of the tongue and oesophagus. With the non-biotin HRP detection system, pretreatment by autoclaving followed by microwave heating gave better labelling results than did microwave pretreatment alone. No obvious difference was noted between the labelling results produced by the non-biotin HRP detection system and the Super Sensitive™ Link-Label IHC detection system.

Keywords: canine distemper; dog; non-biotin HRP; viral infection

Introduction

Canine distemper (CD), which is caused by a morbillivirus (genus *Morbillivirus*, family *Paramyxoviridae*), produces systemic or central nervous system (CNS) infections of dogs and related species and is associated with high mortality in Taiwan (Wu *et al.*, 2000; Yu *et al.*, 2001). The characteristic CNS changes include polioencephalomalacia, white matter demyelination, astrogliosis, eosinophilic intranuclear and cytoplasmic inclusion bodies, gemistocytes and occasional multinucleated syncytial giant cells (Summers *et al.*, 1984; Summers and Appel, 1985). However, syncytial giant cells were demonstrated in only 9–28% of canine distemper virus (CDV)- infected brains (Palmer *et al.*, 1990; Wu *et al.*, 2000), and eosinophilic intranuclear or cytoplasmic inclusion bodies in only 17% (Haines *et al.*, 1999), 33% (Stanton *et al.*, 2002), 38.1% (Wu *et al.*, 2000) or 72% (Palmer *et al.*, 1990). Thus, histological examination of lesions does not appear to be a reliable indicator of CDV infection. Immunohistochemical detection of CDV antigen in tissue sections was reported by Palmer *et al.* (1990) to be superior to the demonstration of inclusion bodies or syncytial cells for the diagnosis of canine distemper encephalitis. In infected dogs,

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immunohistochemical examination demonstrates CDV antigen in glial cells, neurons, ependymal cells, inflammatory macrophages, choroid plexus cells and meningeal cells (Mitchell *et al.*, 1991); in haired skin and footpad epithelium (Haines *et al.*, 1999; Koutinas *et al.*, 2004); and in lung, spleen, kidney and lymph nodes (Iwatsuki *et al.*, 1995). The traditional avidin–biotin complex (ABC) immunohistochemical labelling method has several disadvantages. First, avidin has a high isoelectric point. At pH 7.4, it is positively charged and tends to bind to certain proteins (Haines and Chelack, 1991). The hydrophobic and electrostatic characteristics of the avidin conjugates appear to play a role in the non-specific binding (Masuoka *et al.*, 2002). Second, kidney, pancreas, liver, lymphoid tissue and nervous system tissue may contain lectin-like, negatively charged, endogenous, biotin-rich material which may adhere non-specifically to the ABC, resulting in false positive reactions (True, 1990; Ramos-Vara, 2005). Although the background can be greatly reduced by replacing avidin with streptavidin, background from endogenous biotin is still a problem with streptavidin methods (Ramos-Vara, 2005).

This report describes the development of a novel method of non-biotin chain polymer conjugate labelling combined with heat-induced epitope retrieval (Shi *et al.*, 2001; Ramos-Vara, 2005) for CDV diagnosis.

Materials and Methods

Animals

Eight dogs (nos 1–8), from either an animal shelter or a private clinic, with clinical signs of CD were used in the study. All animals had at least one sign of each of the following: CNS infection (seizure, ataxia, circling and myoclonus); digestive tract infection (diarrhoea, vomiting, depression and anorexia); and respiratory tract infection (nasal and ocular discharge, cough, dyspnoea and sneezing). The animals were either humanely destroyed or died naturally.

Pathological Examination

Samples taken *post mortem* consisted of brain (including medulla oblongata, cerebrum and cerebellum), spinal cord, lung, trachea, lymph nodes, heart, liver, spleen, large and small intestine, stomach, kidneys, urinary bladder, adrenal gland, and skin. These samples were fixed in 10% neutral buffered formalin for at least 36–48 h and no more than 7 days. They were then processed by routine methods and embedded in paraffin wax. Sections (6 μm) were either stained with haematoxylin and eosin (HE) for histopathological examination or examined immunohistochemically, to determine the type of lesion and the presence of inclusion bodies, syncytial cells and CDV antigen.

Immunohistochemistry (IHC)

The Super Sensitive™ 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).

Tissue sections were dewaxed in xylene and rehydrated in a graded alcohol series. Antigen unmasking was performed by immersion of sections in Trilogy™ (Cell Marque, Hot Springs, Arkansas, USA) 5% in Q water (Milli-Q, UF Plus; Millipore Co. Billerica, MA, USA) and boiling for 15 min at 121 °C in an autoclave (SA-252F; Sturdy Industrial Co., Taipei, Taiwan). Sections were then immediately transferred to fresh hot Trilogy™ solution in a second staining dish, heated by a 1450-W microwave oven (RE-C102; Sampo Co., Taipei, Taiwan). This was left to stand for 10 min at 80 °C. The sections were then immersed in cool Tris-buffered saline (TBS; DakoCytomation, Carpinteria, CA, USA) for 10 min, and then rinsed in TBS. For evaluation of the antigen retrieval effect, the first autoclave treatment was omitted and the sections were placed directly in fresh hot Trilogy™ solution as above.

Endogenous peroxidase activity was quenched with hydrogen peroxide 3% in methanol (Merck, Darmstadt, Germany) for 10 min at room temperature. This was followed by rinsing in TBS and incubation in Power Block™ solution (BioGenex) for 10 min at room temperature. The primary antibody diluted 1 in 500 in antibody diluent (Ventana Medical System, Tucson, AZ, USA) was then applied for 24 h at 4 °C.

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. The sections were then rinsed in TBS, incubated with Super Enhancer™ reagent (BioGenex) for 60 min at room temperature, rinsed in TBS, and incubated in Polymer-HRP reagent (BioGenex) for 60 min at room temperature. To compare these results with conventional IHC, the Super Enhancer ™ reagent was replaced by Super Sensitive Multilink® reagent (BioGenex) and the Polymer-HRP reagent was replaced by streptavidin peroxidase reagent (Super Sensitive™ Link-Label IHC Detection System; BioGenex). The subsequent procedures were identical for each of the two methods compared. These subsequent procedures consisted of rinsing in TBS once more, incubation with AEC (3-amino, 9 ethyl-carbazole) or DAB (3, 3′- diaminobenzidine tetrahydrochloride) (BioGenex) chromogen

Table 1
Nucleotide sequences of primers

Primer	*Nucleotide sequence*	*Location*	*Direction*
F2	5′-TAAGGGAATCGAAGATGC-3’	2160–2177	Forward
R1	5′-CCATCAGCATGCTCACATC-3’	2341–2359	Reverse
R2	5′-GATCCCCCAGTTGACTTG-3’	2568–2585	Reverse

solution for 3–5 min at room temperature, counterstaining with Mayer's haematoxylin, and mounting with Super Mount® (BioGenex). The sections were then examined microscopically.

CDV Antigen Test

The RapiGen® Canine Distemper Antigen rapid test kit (RapiGen Inc., Keumjung, Gunpo, Kyonggi, Korea) was used, according to the manufacturer's instructions. Briefly, serum, plasma or ocular discharge from suspected cases was dispensed into the sample wells. The results were read after waiting for 5–10 min. One red or purple band in the control line (C) with no apparent band in the test line (T) indicated a negative result for CDV infection. One red or purple band in the control line (C) and one band in the test line (T) indicated a positive result.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RNA was extracted from serum, heparinized whole blood, cerebro-spinal fluid (CSF; without centrifugation), nasal swab in 1 ml of phosphate-buffered saline (PBS), ocular swab in 1 ml of PBS, and a 10-fold dilution of vomitus or faeces from dogs with clinically suspected CD. The RNA was extracted by the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) phenol-chloroform extraction method. Briefly, a 500 µl volume of homogenized tissue sample was placed in a 2-ml sterile microcentrifuge tube and 1 ml of TRIzol® reagent was added. The tube was left to stand for 5 min at room temperature. After vigorous shaking, followed by the addition of 200 µl of chloroform (Merck) and vortexing to ensure an even mix, the tube was left to stand for 2–3 min at room temperature before being centrifuged at 13 000 rpm (12000 **g**) for 10 min at 2–8 °C. The supernate was then transferred to another sterile microcentrifuge tube. An equal volume of isopropyl alcohol (Merck) was mixed with the supernate, which was then kept at −20 °C for 30 min before being centrifuged at 13 000 rpm (12 000 **g**) again for 15 min at 2–8 °C. The resultant supernate was discarded and the pellet mixed with 1 ml of 75% cold ethanol (Merck). Centrifugation was performed once again, as above for 10 min.The pellet was air dried in a lamina flow apparatus for 20 min. Finally, 20 µl of RNase free diethyl pyrocarbonate (DEPC) water were added to the RNA pellet. Primers specific to CDV AF378705 strain phosphoprotein (P) (Table 1) were used. cDNA synthesis was carried out in 20 µl of RT-PCR reaction cocktail mixture containing 1 µl of 10 µM primary F2 primer set mixture, 10 µl of RNA template, 5 X first strand buffer (including 250 mM Tris-HCl [pH 8.3], 375 mM KCl, 15 mM $MgCl_2$), 2 µl of 0.1 M dithiothreitol (DTT), 1 µl of 10 mM deoxynucleotide triphosphate mixture (dNTP), 1 µl of reverse transcriptase 200 IU/µl (SuperScript™ II Reverse Transcriptase) (Invitrogen). cDNA was synthesized with a thermocycler (GeneAmp PCR System 2400; Applied Biosystems, Foster City, CA, USA) at 42 °C for 1 h, and the sample was denatured by the same thermocycler at 70 °C for 15 min.

The resulting cDNA product from each sample was used as a template in the following PCR procedure. The PCR reaction cocktail mixture (30 µl) contained 1 µl of F2 and R2 primer set (each 10 µM), 10 × PCR buffer (containing 200 mM Tris–HCl [pH 8.4], and 500 mM KCl), 1 µl of 50 mM $MgCl_2$, 1 µl of 10 mM dNTP mixture, and 0.5 µl of Taq DNA polymerase 5 IU/µl (Invitrogen). The primary PCR amplification was carried out with 35 cycles at 94 °C for 25 s, at 57 °C for 25 s, and at 72 °C for 35 s. The final extension was carried out at 72 °C for 5 min.

Primary PCR product (1 µl) was used as the subsequent PCR template and amplification was performed with nested F2 and R1 PCR primer set in a further 35 sequential cycles at 94 °C for 25 s, 57 °C for 25 s, and 72 °C for 25 s. The final extension consisted of one cycle at 72 °C for 5 min. The targeted amplification sequence was a 200 base pair (bp) fragment. The amplification products were analysed on 1.6% agarose gel and the electrophoresis results were observed under a UV illuminator after ethidium bromide staining.

Results

RT-PCR and Antigen Test

Positive results were obtained in four dogs (nos 1–4) with the RT-PCR and in three dogs (5, 6, 8) with the CDV antigen test (Table 2). Two of the eight dogs with CDV infection had not been vaccinated, but the vaccination status of the remainder was unknown.

Table 2
Details of the dogs with CDV infection

Dog no.	*Breed*	Age (*months*)	*Clinical signs*			*CDV antigen test*	*Positive RT-PCR*	*Vaccination record*
			CNS	*GI*	*Re*			
1	Labrador retriever	2	+	+		NA	Blood, CSF and urine	No
2	Chow chow	3	+		+	NA	Blood	NA
3	Shiba	3	+		+	NA	Nasal swab, ocular swab	NA
4	Dachshund	1		+	+	NA	Blood, stool, vomitus	No
5	Mongrel	3	+			+	NA	NA
6	Shih tzu	2	+		+	+	NA	NA
7	Pomeranian	60	+			NA	NA	NA
8	Miniature pinscher	60	+			+	NA	NA

CNS, central nervous system signs (seizure, ataxia, circling and myoclonus); GI, gastrointestinal signs (diarrhoea, vomiting, depression and anorexia); Re, respiratory signs including nasal and ocular discharge, cough, dyspnoea and sneezing; NA, not available.

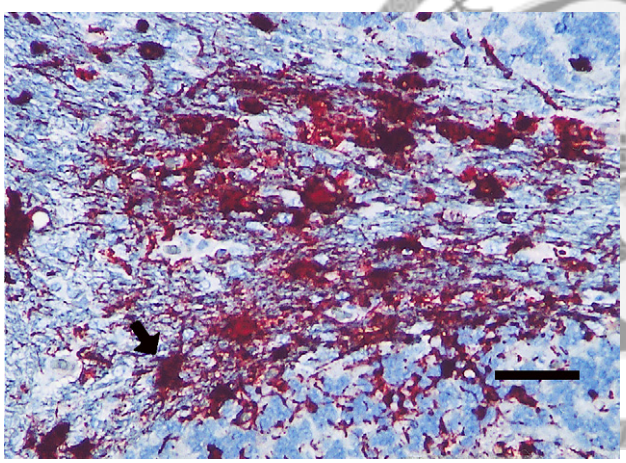

Fig. 1. Diffuse CDV immunolabelling in the cerebellar white matter is visible in the astrocytes (arrow). Non-biotin HRP (AEC) with haematoxylin counterstain; autoclave with microwave pretreatment. Bar, 50 µm.

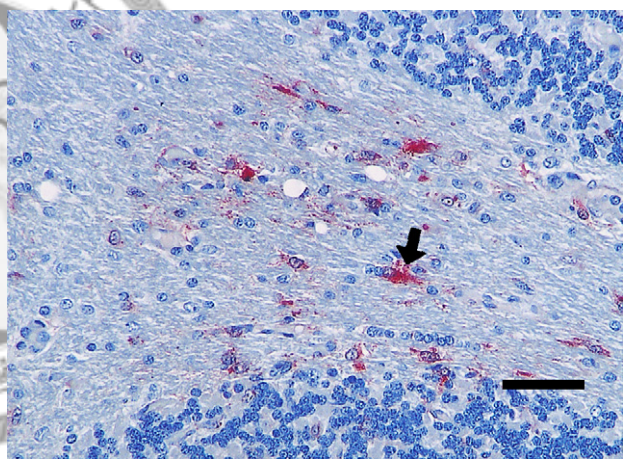

Fig. 2. Same area as in Fig. 1, showing CDV labelling in the cerebellar white matter, located focally in the astrocytes (arrow). Non- biotin HRP (AEC) with haematoxylin counterstain; microwave pretreatment only. Bar, 50 µm.

Histopathology and Immunohistochemistry

For antigen retrieval, autoclaving combined with microwave treatment yielded better labelling results (Fig. 1) than did microwave treatment only (Fig. 2). The non-biotin HRP detection system gave essentially similar labelling regardless of the substrate (AEC or DAB), but AEC labelling was preferable for small, fine antigen particles and for contrast. Immunolabelling produced by the non-biotin HRP detection system in CNS tissues did not differ significantly from that produced by the Super Sensitive™ Link-Label detection system.

All eight cases of suspected CD were confirmed by the combination of methods used (RT-PCR and CDV antigen test [Table 2], IHC [Table 3] and histopathology [including and the demonstration of intranuclear or cytoplasmic inclusion bodies]). Inclusion bodies were observed in seven cases (87.5%), albeit not readily, their prevalence varying in different tissues. The most common sites were the periphery of the splenic central arteries, macrophages and lymphocytes in the lymph nodes and urinary bladder epithelium, pulmonary macrophages and bronchiolar epithelium, ependymal cells lining the 4th ventricle, renal pelvis and cerebellar white matter astrocytes. Eosinophilic cytoplasmic inclusion bodies were occasionally seen in the renal pelvis and urinary bladder epithelium. Immunolabelling of the CDV antigen by

Table 3
Non-biotin HRP immunohistochemical labelling of CDV antigen in tissues of eight infected dogs

System	*Tissues*	*Results in dogs no.*								*Total*
		1	2	3	4	5	6	7	8	
Respiratory	Lung	+, ecs	+, ecs	+, macrophages	–	+, ecs and macrophages	+, ecs and septa	+, ecs and macrophages	+, ecs and septa	7/8
Digestive	Stomach	+	+	+	–	+	+	+	+	7/8
	Small intestine	+	+	+	–	+	+, focal	+	+	7/8
	Large intestine	+	+	+	–	+	+	+	+	7/8
Nervous	Cerebrum	+	+	+	–	+	+	+	+	7/8
	Cerebellum	+	+	+	–	+	+	+	+	7/8
	Brain stem	+	+	+	–	+	+	+	+	7/8
Lymphoid	Spleen	+	+	+	–	+	+	+	+	7/8
	Lymph node	+	+	NA	–	+	+	+	+	6/7
Urinary	Urinary bladder	+	+	NA	–	+	+, ecs	+, ecs	+, ecs	6/7
	Kidney	+	+	+	–	+	+, pelvis, ecs	+, pelvis, ecs	+, pelvis, ecs	7/8
Others	Skin	+	+	+	–	+	+	+	+	7/8
	Salivary gland	+	NA	+	–	+	+	+	+	6/7

NA, not available; ecs, epithelial cells.

the non-biotin HRP method was observed in seven cases (Table 3).

The brain lesions in the infected dogs consisted of demyelination and glial cell infiltration in the cerebellum, cerebellar peduncles and brain stem. CDV immunolabelling in the cerebral cortex appeared diffusely or focally in the dendrites of large pyramidal cells (Fig. 3). It was particularly striking at the periphery of the 4th ventricle (Fig. 4) and in ependymal cells, but was present in only a few Purkinje cells. In some cases with cerebellar demyelinating areas, only a few intranuclear inclusion bodies and neuropils were immunolabelled. In more severely affected cases, astrocytes in the demyelinating white matter and brain stem showed immunolabelling with a spidery and

Fig. 3. Diffuse CDV labelling in the cerebral cortex is seen in the dendrites of large pyramidal cells (arrow). Non-biotin HRP (AEC) with haematoxylin counterstain; autoclave with microwave pretreatment. Bar, 50 µm.

Fig. 4. Extensive immunoreactivity with CDV antigen is exhibited in the ependymal cells lining the 4th ventricle. Non-biotin HRP (AEC) with haematoxylin counterstain; autoclave with microwave pretreatment. Bar, 100 µm.

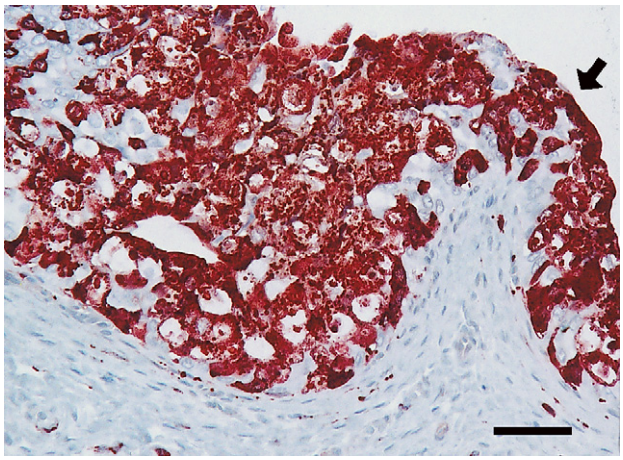

Fig. 5. Extensive CDV immunolabelling of the ballooning epithelial cells of the urinary bladder (arrow). Non-biotin HRP (AEC) with haematoxylin counterstain; autoclave with microwave pretreatment. Bar, 50 µm.

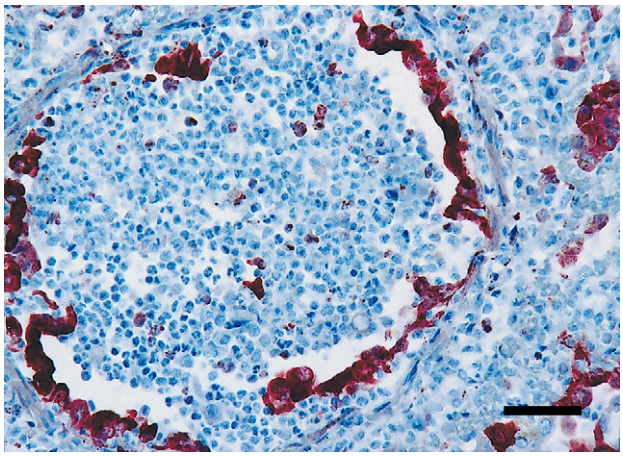

Fig. 7. Alveolar macrophages, bronchiolar epithelium and alveolar septa show extensive CDV immunolabelling. Non-biotin HRP (AEC) with haematoxylin counterstain; autoclave with microwave pretreatment. Bar, 50 µm.

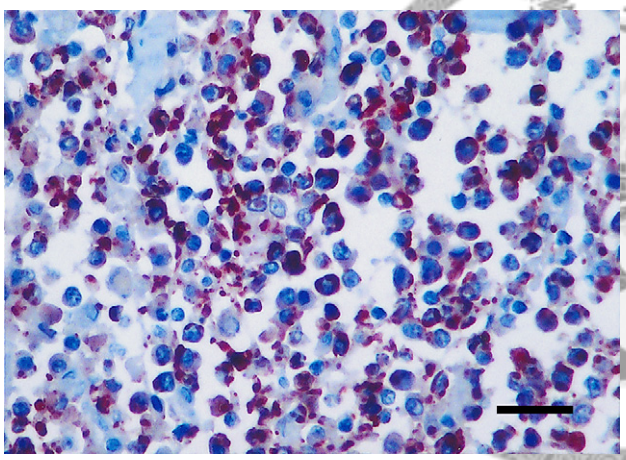

Fig. 6. Extensive CDV immunolabelling is seen in the cytoplasm of macrophages and sub-capsular lymphocytes of lymph nodes giving a beaded appearance. Non-biotin HRP (AEC) with haematoxylin counterstain; autoclave with microwave pretreatment. Bar, 25 µm.

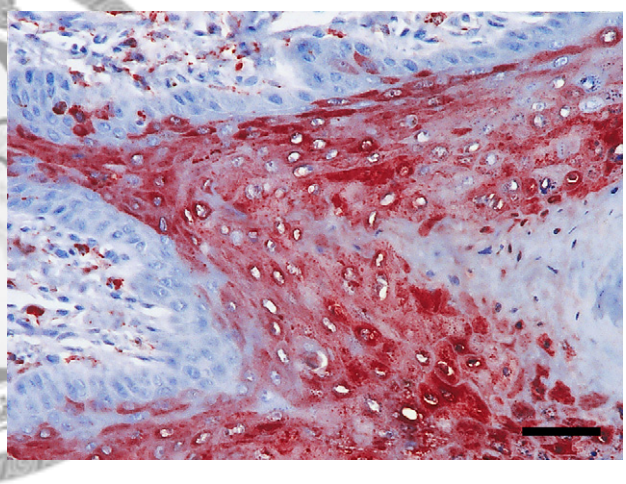

Fig. 8. Extensive CDV immunolabelling is seen in the cytoplasm of cells of stratum spinosum (rather than the stratum basale) of the skin. Non-biotin HRP (AEC) with haematoxylin counterstain; autoclave with microwave pretreatment. Bar, 50 µm.

beaded appearance. The CDV antigen was also present in the grey and white matter and central canal of the spinal cord. The urinary bladder epithelium showed ballooning degeneration, with small, granular, eosinophilic, cytoplasmic inclusions which were immunoreactive for CDV antigen (Fig. 5). Immunolabelling was also present in the renal pelvis epithelium, hepatic Kupffer cells, gastric and intestinal mucosal epithelia, and stratified squamous epithelium of the tongue and oesophagus. No significant inflammatory responses were noted at these sites.

The lesions in lymphoid tissues consisted of focal or diffuse tissue depletion and necrosis. Extensive immunolabelling was seen in the following sites: in the spleen—at the periphery of the central arteries and cytoplasm of lymphocytes or macrophages; in lymph nodes—in the cytoplasm of macrophages and subcapsular lymphocytes (Fig. 6); alveolar macrophages, alveolar septa and bronchiolar epithelium (Fig. 7); cytoplasm of salivary gland epithelial cells and ganglion cells; cytoplasm of the cells of the stratum spinosum of skin (Fig. 8).

Discussion

The use of non-biotin HRP for the immunohistochemical diagnosis of CDV infection has not previously been reported. In the present study, it produced improved

contrast against the background, making the detection of CDV antigen easier than the histological detection of intranuclear inclusion bodies. The cloned DV2-12 primary antibody used required pretreatment of formalin-fixed, wax-embedded sections by steaming, proteinase K pretreament having been shown by Ramos-Vara and Beissenherz (2000) to be without effect for this antibody.

The use of different fixatives, with or without microwave unmasking, may influence the immunolabelling intensity, depending on the type of antibody (Liang *et al.*, 2000). An alkaline phosphatase (AP) substrate detection system may be used in immunohistochemical diagnosis of infectious diseases (Liang *et al.*, 2004) but will not produce optimal labelling of fibres, processes or terminals in neural tissues. Unfortunately, the targeting of the CNS by CDV makes an AP system less suitable for use in its diagnosis. We tried to overcome these problems by using a microwave unmasking and AP substrate detection system in CDV diagnosis, but the results were unsatisfactory.

Seven of the eight cases examined were acute to subacute, with no significant associated inflammatory response. Only one case (no. 4) had no CNS lesions. In a previous study, the microscopical lesions of CDV encephalopathy were divided into four types, namely acute, acute with necrosis, subacute, and chronic (Headley *et al.*, 2001). Acute distemper white matter lesions were characterized by vacuolar changes, mild microgliosis with some reduction of myelin stain, and lack of perivascular mononuclear inflammation. Subacute changes were characterized by a "moth-eaten" appearance, moderate to severe demyelination, foci of malacia with Gitter cell infiltration, varying degrees of astrocytic hypertrophy and hyperplasia, a few multinucleated astrocytes and gemistocytes, and minimal to severe perivascular lymphoplasmacytic cuffs. Chronic lesions were characterized by loosely or densely arranged astrocytic proliferations and demyelination with or without perivascular lymphoplasmacytic cuffs. There were no hypertrophic astrocytes or macrophages in such lesions (Baumgartner *et al.*, 1989). The predominance of early distemper encephalitis over late lesions in the present study was similar to that reported previously (Frisk *et al.*, 1999; Headley *et al.*, 2001).

A higher percentage of cases had eosinophilic intranuclear or cytoplasmic inclusion bodies in this study (87.5%) than in previous studies (17–72%) (Palmer *et al.*, 1990; Haines *et al.*, 1999; Wu *et al.*, 2000; Stanton *et al.*, 2002), but this discrepancy may be attributable to the small sample size. An explanation for the discrepancy between the detection of inclusion bodies and the immunohistochemical findings may be that the formation of intranuclear inclusion bodies should be viewed as paradoxical for an RNA virus, which replicates entirely in the cytoplasm (Summers and Appel, 1994). Bellini *et al.* (1994) presumed that intranuclear inclusions were composed of RNA surrounded only by the viral nucleocapsid (N) protein and appeared to be devoid of any other viral antigen. However, intranuclear inclusion bodies in the present study were more common than cytoplasmic inclusion bodies. The eosinophilic inclusion bodies were found in gastric (dogs 1, 2 and 8; data not shown) or urinary epithelial cells in this study, despite the absence of obvious histopathological changes. However, a previous study revealed gastrointestinal lesions and inclusion bodies in the epithelial cells of the intestines or urinary epithelial cells only in cases with enteritis (Okita *et al.*, 1997). In the present study, only two cases had digestive symptoms. In seven out of the eight cases CDV antigen was identified in the digestive tract mucosa.

In previous studies, CDV infection was confirmed immunohistochemically in 58–76% of suspected cases (Frisk *et al.*, 1999; Stanton *et al.*, 2002). In these earlier studies, CDV RNA was detected by RT-PCR in 86% of serum samples and 88% of whole-blood and CSF samples from dogs with immunohistochemically confirmed distemper. There was no evidence of inhibition of *Taq* DNA polymerase by haemoglobin (Frisk *et al.*, 1999). However, with the aid of RT-PCR, CD was diagnosed in 17 cases (81%). The positive results given by different organs varied from 71.4% (cerebellum), to 33.3% (whole-blood). The specific amplicons consisted of phosphoprotein (P) gene, which is unable to identify false-positive results due to vaccination, and no immunohistochemical comparisons were made (Wu *et al.*, 2000).

Two dogs (1 and 4) in this study had not been vaccinated but still gave positive RT-PCR results in various tissues. In addition, Kim *et al.* (2001) reported that CDV antigen, as detected by nested PCR, disappeared 14 days after vaccination. Dog 4 gave positive RT-PCR results in blood, faeces and vomitus but was inclusion body-negative and IHC-negative. Dog 4, aged only 1 month, had peracute broncho-interstitial pneumonia and splenic lymphoid depletion (data not shown), giving no time for the accumulation of CDV antigen. Stanton *et al.* (2002), who studied 12 dogs with CD, found discrepancies between the positive results given by RT-PCR (83%), inclusion bodies detection (33%), immunofluorescent labelling (50%) and immunohistochemical labelling (58%). Frisk *et al.* (1999) reported that in the chronic stage of infection, immunolabelling of CDV antigen was found mainly in the CNS but rarely elsewhere. The immunohistochemical method described provides a more sensitive method of diagnosing CD encephalitis than methods based on demonstration of inclusion bodies or syncytial cells. Disadvantages include the relatively high cost of

non-biotin kits and the limited life span of slides; moreover, AEC, if used, is soluble in organic solvents, necessitating the use of a water-based mounting medium (Haines and Chelack, 1991).

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Chapter IV

Improving detection of canine distemper virus in formalin-fixed, paraffin-embedded, tissues: using in situ hybridization with integrated optical density to give a semi-quantitative assessment

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Abstract

Improving enhancement for the detection of canine distemper viral (CDV) nucleoprotein RNA was applied in paraffin-embedded tissues from dogs with spontaneous CDV infections. In addition to proteinase K, autoclaving in various solutions (Trilogy, TBS S3006, H-3301, and S1700) for pre-treatments were compared. The *in situ* hybridization (ISH) intensity was assessed by using the integrated optical density (IOD) and the integrity of the tissue morphology. A combination of proteinase K digestion and autoclaving in a Trilogy solution resulted in an optimal ISH signal enhancement of CDV RNA. This modified technique can be useful in the retrospective viral studies across a broad range.

Introduction

Canine distemper virus (CDV) is a non-segmented, single-stranded negative RNA virus that belongs to the genus *Morbillivirus* within the family *Paramyxoviridae*. CDV is the most common viral pathogen of canine encephalitis (Appel 1970). The CDV is typically detected by immunohistochemistry (IHC), immunofluorescence labelling, reverse transcription-polymerase chain reaction, clinicopathological findings, or a combination of these approaches (Beineke et al., 2009).

Additionally, CDV RNA and viral transcription can also be detected in acute demyelinating lesions in formalin-fixed, paraffin-embedded tissues, by using ISH (Muller et al., 1995; Gaedke et al., 1997; Hoyland et al., 2003; Engelhardt et al., 2005; D'Intino, et al., 2006; Zurbriggen et al., 1998; Vandevelde and Zurbriggen 2005) or in antigens that are not well demonstrated by IHC in chronic infection (Mitchell et al., 1991). For ISH, there are a variety of commonly used pre-treatments for the formalin-fixed paraffin-embedded tissue sections, including saponin (Yamawaki et al., 1993), diluted acids, detergents, alcohols, proteases (such as proteinase K, pronase, and pepsin) (Mcnicol and Farquharson 1997), sodium borohydrite (Gaedke et al., 1997), and boiling sections in citrate buffer in a microwave (Gaedke et al., 1997; Lan et al., 1996). Among the pre-treatments for ISH, the most common is the application of proteases.

However, problems with ISH persist, because different types of tissues require different digestion conditions in order to achieve optimal results. Few reports have focused on optimising the CDV RNA detection protocol in formalin-fixed, paraffin-embedded tissue sections. Furthermore, there are conflicting results from ISH optimisation following proteolytic enzyme digestion and heat-induced antigen retrieval (HIAR) pre-treatments (Mcquaid et al., 1990; Gaedke et al., 1997; Kim and Chae 2003).

Thus, the intent of our study was to improve the ISH protocol, and combine it with integrated optical density measurement (IOD), to provide a semi-quantitative and simple method for the detection of CDV RNA in formalin-fixed, paraffin-embedded tissues in retrospective studies.

Materials and Methods

Animals

The spontaneous CDV infections in eight dogs were previously confirmed and described by either RT-PCR and histopathology or by immunohistochemical labelling (Liang et al. 2007). The tissue samples obtained were fixed in 10% neutral buffered formalin for between 36 h and 7 days. The samples were then processed and embedded in paraffin, using a routine process.

Probe preparation

The total RNA was obtained from field CDV-infected B95a cells (Liang et al. 2008), and isolated using an RNeasy Mini Kit (Qiagen). The cDNA probe was prepared by using primers that amplify the CDV nucleoprotein (Frisk et al. 1999). The RT-PCR product (287 bp) was cloned using the PCR 2.1-TOPO TA cloning kit (Invitrogen). Digoxigenin (Roche)-labelled, double-stranded DNA probes were generated by PCR using Platinum Taq DNA polymerase (Invitrogen). The reactions were performed under the following conditions: 95℃ for 10 min, 35 cycles at 95℃ for 30 sec, 59.5℃ for 30 sec, and 72℃ for 30 sec, with a final extension for 10 min at 72℃. The resultant 287 bp DIG-dUTP-labelled dsDNA probe was then purified using a QIAquick® PCR purification kit (Qiagen), and analysed on a 2 % agarose gel.

Pre-treatment before pre-hybridization

From each of the formalin-fixed, paraffin-embedded tissues, we cut 6- μm thick serial sections. The sections were then deparaffinised, rehydrated, and de-proteinised, and then treated with one of the following pre-treatments, either (a) digested at 37℃ for 15 min with proteinase K (20 μg/ml, Roche) in PBS (DEPC), each washed with 0.2% glycine and 2× SSC for 10 min, and pre-hybridised and hybridised; or (b) digested at 37℃ for 15 min with proteinase K (20 μg/ml, Roche) in PBS(DEPC), washed with 0.2% glycine, immersed in 5% concentrated Trilogy stock (Cell Marque) in Q water (Milli-Q), and boiled for 15 min at 121℃ in an autoclave (SA-252F, Sturdy Industrial, Taipei, Taiwan).

Next, the autoclaved sections were immediately transferred to a fresh 5% Trilogy solution in a second staining dish that had been pre-heated to 80℃ in a 1450-W microwave oven (RE-C102; Sampo, Taipei, Taiwan), and were left to stand for 10 min at 80℃. The sections were then immersed in 10% concentrated stock Tris-buffered saline (TBS S3006; Dako Cytomation) in Q water at room temperature for 10 min, washed with 2× SSC, and pre-hybridised and hybridised.

The pre-treatment c, d, and e samples were processed as previously described; however, the solutions used in both the autoclaving and microwaving were changed to (c) 10% stock Tris-buffered saline (TBS S3006) in Q water, (d) a 1% vector

citrate-based stock antigen unmasking solution (H-3301, Vector Laboratories) in Q water, or (e) a target-retrieval solution (S1700; Dako Cytomation). After the different autoclaving and microwaving pre-treatments (b, c, d, and e), the sections were immersed in a 10% concentrated stock TBS S3006 solution in Q water at room temperature, and each rinsed in 2x SSC for 10 min before being pre-hybridised and hybridised.

***In situ* hybridization (ISH)**

We used the pre-hybridisation and hybridisation protocol as previously described by Chueh et al. (1999) and also in the Appendix of this study.

Immunohistochemistry (IHC)

We used the IHC protocol of Liang et al. (2007), which used the Super Sensitive™ Non-Biotin HRP Detection System (BioGenex Laboratories, San Ramon, CA, USA). The primary antibody was mouse anti-CDV (MCA 1893, Clone DV2-12; Serotec, Kidlington, Oxford, UK).

Postive area evalution

The ISH intensities of the five pre-treatments were assessed by estimating the area of the objects, and the medium pixel intensity per object, as the integrated optical

density (IOD) of the positively stained areas, by using the image analysis program Image-Pro® Plus 6.0 (Media Cybernetic, NY, USA). For each tissue section, we calculated the IOD, which was equal to area × average optical density of positive staining. The average IOD scores were calculated from duplicate valves from each section.

Tissue morphologies were graded on a semi-quantitative four-point scale: -, <10% to no damage; +, 10-25% mild damage; ++, 25-50% moderate damage; and +++, >50% severe damage.

Results

The pre-treatment process was conducted on a mock control (other dog sections with no CDV infection) and a reagent control (serial sections of infected cases without the addition of the probe), wherein no positive labelling was found, which directly contrasted the aforementioned observations made in the cases of infected sections where probes were used.

Consecutive serial tissue sections of the major CDV-infected organs, including the cerebellum, cerebrum, lung, urinary bladder, and spleen, were tested in parallel with the addition of different HIAR regimens. The results are summarised in Table 1 and Fig. 1, wherein it can be observed that the proteinase K pre-treatment in combination with autoclaving and microwaving enabled the detection of strong signals with very low non-specific backgrounds in spleen and urinary bladder tissues. The results corresponding to bronchiolar mucosa, cerebellar white matter, 4th ventricle, as well as cerebral cortex tissues were similar (data not shown); however, sections that were subjected to a combination of proteinase K pre-treatment and autoclaving in a Trilogy solution exhibited a stronger hybridization signal(Fig. 1B, 1G).

The ISH intensities of the five pre-treatments in the spleen tissue were assessed by average IOD, wherein the corresponding average IOD scores were 0, 19913, 10097, 0, and 2575 (Fig. 1A-1E). Negative to strong hybridization signals, which were

blue/purple to black in colour, were observed in the splenic periarteriolar white pulp areas. The retrospective study, splenic lymphoid depletion and necrosis with intranuclear inclusion bodies and other lesions, which are characteristics of CDV infection, were observed.

The ISH intensities of the five pre-treatment samples of urinary bladder were also assessed by IOD, wherein the average IOD scores were 30, 5624, 5150, 0, and 4123 (Fig. 1F-1J). Hyperplasia and ballooning changes with intranuclear and cytoplasmic inclusion bodies were also noted in the bladder mucosa epithelial cells (data not shown).

In terms of tissue morphology, tissues sections that had only been subjected to proteinase K pre-treatment exhibited intact and clear tissue outlines; however, the hybridization signal was rarely detected in tissues that did not undergo an autoclaving pre-treatment (Fig. 1A and 1F). The sections that were subjected to proteinase K pre-treatment in combination with autoclaving in Trilogy solutions exhibited mild losses in tissue architecture (Fig.1B and 1G).The sections that were subjected to proteinase K pre-treatment in combination with autoclaving in TBS solutions also exhibited mild losses in tissue architecture (Fig.1C and 1H). However, the sections that were subjected to proteinase K pre-treatment in combination with autoclaving in a H3301 solution exhibited severe losses in tissue architecture without any ISH signal

(Fig. 1D and 1I). The sections that were subjected to proteinase K / autoclaving pre-treatment in S1700 solution exhibited moderate losses in tissue architecture but had distinct signals (Fig. 1E and 1J).

Discussion

Heat-induced antigen retrieval (HIAR)

In this study, through a combination of proteinase K digestion and autoclaving in a Trilogy solution, we found a relatively simple method that demonstrates an optimal ISH signal enhancement of CDV RNA.

The formaldehyde as a 10% neutral buffered formalin is the most widely used universal fixative because it preserves a wide range of tissues and tissue components. However, attempts to extract usable DNA from formalin-fixed tissues for molecular biological studies have been variably successful (Srinivasan et al., 2002). The formaldehyde fixative initiates DNA denaturation (interchain hydrogen bonds break and bases unstack) at the AT-rich regions of double-stranded DNA creating sites for chemical interaction. There are four interactions of formaldehyde with DNA: 1) The first is an addition reaction. Formaldehyde is added to the nucleic acid base to form a hydroxymethyl (methylol) group (-CH2 OH). 2) The second is a slower electrophilic attack of *N*-methylol on an amino base to form a methylene bridge between two amino groups. 3) Formaldehyde treatment can generate AP (apurinic and apyrimidinic) sites via hydrolysis of the N-glycosylic bonds, leaving free pyrimidine and purine residues. AP sites have a highly unstable cyclic carboxonium ion that hydrolyzes rapidly to yield 2-deoxy-D-ribose. 4) Formaldehyde may also cause slow hydrolysis

of the phosphodiester bonds leading to short chains of polydeoxyribose with intact pyrimidines. When compared to the DNA isolated from frozen tissues, formalin-fixed tissues exhibit a high frequency of nonreproducible sequence alteration (Srinivasan et al., 2002; Shi et al., 2001).

Previous attempts have used thermocycling (Kim and Chae, 2003), microwaving (Lan et al. 1996; Gaedke et al. 1997) or autoclaving (Relf et al. 2002) to enhance the ISH signal. Recent employment of the HIAR has been shown to enhance the extraction of nucleic acid or increase the efficiency of subsequent ISH detection of a target sequence. The process of crosslinking makes probe access to the target sequence difficult. Therefore, tissues must be digested to improve probe access to the specific mRNA while minimizing loss of mRNA and tissue morphology. Many such digestion strategies have been employed to permeabilize fixed cells or tissues using acids, detergents, alcohols, and enzymes such **as** proteinase K, pronase, and pepsin. However, this step remains problematic in that each tissue type requires a different set of digestion conditions (Mcquaid et al., 1990; Lan et al. 1996; Kim and Chae, 2003; Shi et al., 2001; Weise et al., 2005; Yamashita 2007). Heating cleaves inter- and intra-crosslinks in proteins and nucleic acids, and since a gel-like structure formed by the crosslinks is destroyed and the macromolecules are partially extracted, antibodies can easily penetrate into tissue sections and the immunoreaction is greatly intensified

(Yamashita, 2007).

The HIAR Trilogy solution is a novel product that has been shown to have good antigen retrieval effects for the immunohistochemical labelling of RNA viruses (Faoláin et al., 2005; Ward et al., 2006; Liang et al., 2007); however, due to the advantage of combining deparaffinisation, rehydration, and the retrieval of antigens during pressure cooking, this solution has never been used in an ISH protocol. In this study, Trilogy solution was found to be the idealist for retrieval of RNA in comparison to other solutions. The fact that the HIAR effect could be used as an approach to enhance the extraction of nucleic acids or to increase the efficiency of subsequent detection of a target sequence was not emphasized until recently (Shi et al., 2001; Kim and Chae, 2003). Trilogy solution is a novel product that combines deparaffinization, rehydration, and unmasking of antigens during pressure cooking (Faoláin et al., 2005). In recent reports, Trilogy solution has been shown to have good antigen retrieval effects for the immunohistochemical labelling of RNA viruses (Ward et al., 2006; Liang et al., 2007) or cancer diagnosis (Kuo et al., 2006) but never been tried in ISH protocol, which has lead to the development of novel ISH labelling protocols that are especially important for retrospective studies. The HIAR effect (Mcquaid et al., 1990; Kim and Chae, 2003) can greatly enhance the ISH signal and provides a simple detection method in formalin-fixed, paraffin -embedded tissues.

However, the tissue damage caused by the retrieval solution needs to be taken into consideration. The pH valve of the antigen retrieval solution is another important co-factor (Shi et al., 2001; Ramos-Vara 2005). Some antigens will be retrieved only with high pH solution, others with a wide range of pH. Most antigens, HIAR with 0.01M sodium citrate buffer (pH 6.0) will give satisfactory results and good cell morphology (Ramos-Vara 2005). However, the pH values of the Trilogy (7.69), TBS (8.22), H3301 (9.14), and Dako S1700 retrieval working solutions (9.22) were from neutral to weak basic pH valve in the present study. S1700 and H3301 retrieval solution had very similar pH value but gave totally different results. The citrate-based Vector H3301 retrieval solution combined with autoclave pre-treatment showed the most severe destruction of the tissue morphology.

The results demonstrated that a simple and modified combined pre-treatment of HIAR autoclaving the tissue sections in antigen retrieval buffer and proteinase K digestion resulted in stronger hybridization signals than proteinase K digestion alone. Importantly, autoclaving in either Trilogy solution or TBS resulted in strong hybridization signals of major organs, including spleen, urinary bladder, and lung, infected by CDV with mild to no tissue morphological damage. Similar retrieval effects of autoclaving in Trilogy solution for ISH were also noted in cerebellar sections (data not shown). Since formalin is the most commonly used fixative in

routine tissue fixation, our modified methods can be useful in retrospective and pathogenesis study of CDV infection. However, most RNA viruses are low-copy infections; and thus, RT *in situ* PCR is often the best method available to detect the virus *in situ* (Nuovo, 1995) and will be tried in the future to compare the results in this study.

Proteinase K effect

In the beginning of this study, using proteinase K treatment only, the CDV RNA ISH signals were observed in the immunohistochemistral labelling positive sites of the seven cases. However, the intensities and distribution of ISH signals were not as distinct and strong as those obtained following non-biotin, horseradish peroxidise-based IHC labelling (Liang et al., 2007). Therefore, we investigated if the CDV RNA decayed in formalin-fixed, paraffin-embedded tissues. The specificity of the ISH probe used in this study was validated by detecting CDV RNA in B95a cells infected with field Taiwan CDV strain. In this study, 20 μg/ml proteinase K was used for formalin-fixed, paraffin-embedded tissues. In contrast, the final concentration of proteinase-K used in the detection of CDV-infected B95a cells was 1 μg/ml. To facilitate probe access in ISH, sections are frequently treated with diluted acid, proteolytic enzymes and/or non-ionic detergents. While proteinase K is the most commonly used enzyme for ISH pre-treatment (Lewis and Wells,1992; Mcnicol and

Farquharson, 1997), the final working concentration used is variable, with previous studies using 1 μg/ml (Muller et al., 1995; Gaedke et al., 1997), 5 μg/ml (D'Intino, et al., 2006), 20 μg/ml in the present study, 300 μg/ml (Kim and Chae, 2003) and 1–4 mg/ml (Mcquaid et al., 1990). The concentration of proteinase K used is critical, however, if morphology is to be preserved, and is tissue dependent. The optimal proteinase K concentration of brain and lung is 0.25 mg/ml; 0.5 mg/ml in kidney, liver, intestines, cervical, anal, and laryngeal section; However, it is not critical for protease digestion time for ISH, unless it is too concentrated and too long, and destroys the tissue, to be a factor in background with formalin-fixed tissue (Nuovo, 1995).

Probe effect

The dsDNA probes for ISH have the disadvantage that they do not allow to distinguish between positive and negative stranded RNA. However, the digoxigenin PCR labeling method still can be used in detection of new RNA virus (Sritunyalucksana et al., 2006). The DNA probes are still the most frequently used type of probes. Firstly, they are relatively easy to make in large quantities. Secondly, they are the best characterized. Thirdly, the advent of nucleic acid amplification techniques such as polymerase chain reaction (PCR) has increased their availability greatly. Finally, DNA probes can come in all sizes (Harvey and Schonau, 2006).

In this study, 50% formamide was added to the prehybridization and hybridization

buffer, as higher concentrations did not improve the staining results and the omission resulted in negative staining (Gaedke et al., 1997). The CDV RNA-specific ISH signals in infected B95a cells were stronger compared to the formalin-fixed tissues using proteinase K treatment only. This observation may partially be explained by the fixation of the B95a cells in 4% paraformaldehyde. In the present study, 400 ng/ml of a dsDNA probe (287 bp) was used for formalin-fixed and paraffin -embedded tissues. In contrast, the final concentration of probe and proteinase K used in the detection of CDV-infected B95a cells was 100 ng/ml. However, the concentration of the dsDNA probe for the detection of CDV is variable, with previous reports using 170 or 500 ng/ml, depending on the sizes of probes 126 and 287 bp, respectively (Gaedke et al. 1997). The final working concentration of a CDV-specific RNA probe is also variable, with previous studies using 2 ng/ml (Engelhardt et al., 2005), 100 ng/ml (Gaedke et al., 1997), 1 μg/100 μl (D'Intino, et al., 2006) or 10 μg/100 μl (Muller et al., 1995). as well as 1 mg/ml in muscle, heart, placenta and spermatozoa section (Lewis and Wells, 1992). The same probe sequence (DNA-1) in our study still resulted in a 66% positive rate compared to either shorter DNA-2 probe, an RNA probe or immunohistochemistry shown in a previous study (Gaedke et al., 1997). CDV RNA was still detected in 60% of the samples by ISH compared to 100% of the samples by *in situ* RT-PCR (Hoyland et al., 2003). In order to reduce this false negative rate in

formalin-fixed, paraffin-embedded tissues, we combined HIAR antigen retrieval techniques with proteinase K pre-treatment in this study.

IOD evalution

The IOD, which has previously been successfully applied to analyze IHC staining (Xu et al., 2008), was used in the present study. This method provides a more objective and semi-quantitative evaluation of the viral ISH labeling.

In summary, in order to simplify the tedious procedures that use enzyme digestion for ISH pre-treatment, which requires adjustments in time, temperature, and concentration for different tissues, the present study has established a modified HIAR autoclaving technique. In addition to a fixed concentration of proteinase K digestion used in different tissues, most importantly this technique employed autoclaving in a Trilogy solution. Strong hybridization signals were resulted in CDV-infected major organs, including the spleen and urinary bladder, with mild to no tissue morphological damage and a weak non-specific background. Similar retrieval effects of autoclaving in a Trilogy solution for ISH were also observed in cerebellar and lung sections (Table 1). This modified ISH method could be useful in the retrospective studies of CDV and other viral infections.

Conflict of interest statment

Neither of the authors of this paper has a financial or personal relationship with

other people or organisations that could inappropriately influence or bias the content of the paper.

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Table 1. Comparisons on the five pre-treatments of the major organs of the enhancement for the ISH detection of CDV RNA

Pretreatment methods	Spleen		Urinary bladder		Lung		Cerebellum	
	Hybridization signal[a]	Tissue morphology[b]	Hybridization signal[a]	Tissue morphology[b]	Hybridization signal[a]	Tissue morphology[b]	Hybridization signal[a]	Tissue morphology[b]
1.Proteinase K only	-,0[c]	-	+, 30	-	+	-	-	-
2. Proteinase K with Trilogy (Cell Margue)	+++, 19913	+	+++, 5624	++	++	+	++	+
3. Proteinase K with TBS (Dako, S3006)	++, 10097	+	++, 5150	++	++	+	++	+
4. Proteinase K with citrate-based solution (Vector, H3301)	-, 0	+++	-, 0	+++	-	+++	-	+
5. Proteinase K with AR solution (Dako, S1700)	++, 2575	++	++, 4123	++	++	++	+	+

a Hybridization signal: -, negative; +, weak; ++, moderate; +++ strong

b Tissue morphology: -, no damage; +, mild damage; ++, moderate damage; +++, severe damage.

c: IOD score

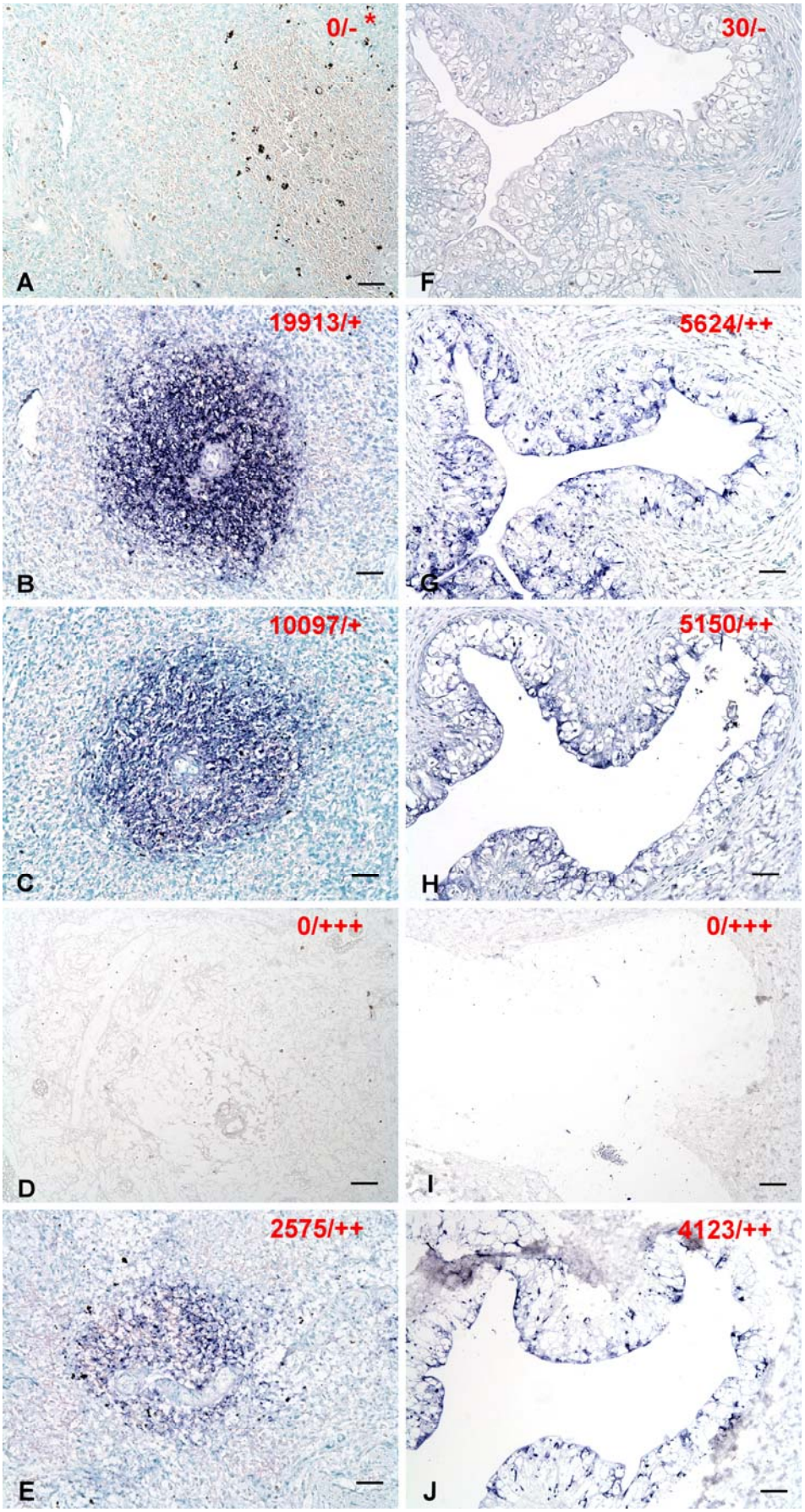
0/- *
A
30/-
F
19913/+
B
5624/++
G
10097/+
C
5150/++
H
0/+++
D
0/+++
I
2575/++
E
4123/++
J

Fig. 1. The effect of five pre-treatments on consecutive serial sections of the CDV infected spleen (A-E) and urinary bladder (F-J) were compared by hybridization signal and tissue morphological damage. The asterisk indicates “hybridization signal IOD score /tissue morphology evaluation”. A negative hybridization signal with no tissue morphological damage was observed in spleen (A) and bladder (F) subjected to only proteinase K pre-treatment. Sections showed strongest hybridization signals which were blue/purple to black in colour, with mild tissue morphological damage, were observed in the splenic periarteriolar white pulp (B) and cytoplasm of bladder mucosal epithelial cells (G). The proteinase K pre-treatment was combined with autoclaving in a Trilogy solution. Moderate hybridization signals with mild tissue morphological damage were noted in spleen(C) and bladder (H). The proteinase K pre-treatment was combined with autoclaving in a TBS solution. A negative hybridization signal was present in spleen (D) and bladder (I), in addition to a severe loss of splenic architecture. The proteinase K pre-treatment was combined with autoclaving in a H3301 solution. Moderate hybridization signals and tissue damage were observed in in spleen (E) and bladder (J). The proteinase K pre-treatment was combined with autoclaving in a S1700 solution. NBT/BCIP, counterstained with methyl green. Bar= 40 μm.

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Chapter V

Phylogenetic analysis and isolation of canine distemper viruses in Taiwan

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Phylogenetic Analysis and Isolation of Canine Distemper Viruses in Taiwan

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ABSTRACT During the period from 2003 to 2005, two canine distemper virus (CDV) strains were isolated from 17 non-vaccinated puppies with suspected canine distemper by co-culture of peripheral blood mononuclear leucocytes and B95a cells. In addition, four cloned hemagglutinin (H) genes were obtained from 166 dogs infected with CDV. Indirect immunofluorescence assays and antigen tests confirmed that they were CDV. Analysis of the H genes of the six identified strains revealed that the deduced amino acid sequences contained nine potential sites for N-linked glycosylation, as had been found for H proteins of Japanese isolates. The seventh site is characteristic of the Taiwan strains described in this report and of recently reported Japanese strains. Furthermore, phylogenetic analysis of the H gene showed that the six isolates belong to the Asia-1 group and are closely related to the recently reported Japanese and Chinese strains. [Liang CT, Chueh LL, Lee KH, Huang HS, Uema M, Watanabe A, Miura R, Kai C, Liang SC, Yu CK, and *Liu CH. Phylogenetic Analysis and Isolation of Canine Distemper Viruses in Taiwan. Taiwan Vet J 34 (4): 198-210, 2008. *Correspondence author TEL/FAX: 886-02-23633289, E-mail: chhsuliu@ntu.edu.tw]

Key words: canine distemper virus, hemagglutinin gene, Taiwan

INTRODUCTION

Canine distemper (CD) was first described in Spain in 1791, and the agent (canine distemper virus, CDV) was first isolated by Carre in 1905. The pathogenesis and clinical features of this infection have been widely reported [1]. Over a period of 200 years after the initial recognition of the disease, it has been the most common viral infection in dogs, although CDV can also infect wild carnivores and large cats [2,7,24]. CDV, which belongs to the genus Morbillivirus, family Paramyxoviridae, is a non-segmented, single-stranded negative RNA virus of approximately 15,690 nucleotides. Within the genome are six genes that en-

code for one envelope-associated protein (M), two glycoproteins (the hemagglutinin/attachment protein H and the fusion protein F), two transcriptase-associated proteins (the phosphoprotein P and the large protein L), and the nucleocapsid N that encapsulates the viral RNA [28].

Dogs can be protected from the infection by immunisation with live attenuated vaccine. However, due to the presence of maternal antibodies that interfere with the vaccine virus, increasing incidences of canine distemper in pups in suburban areas and urban kennel shops have been noted in spite of vaccination in Japan [14,15]. Antigenic changes have been speculated as the cause for the currently prevalent CDV, against which the current vaccine fails to give complete protection [14,17]. Although similar problems have also been reported in Taiwan [22], the characteristics of prevalent CDV in the field have not been investigated due to difficulties in the isolation of wild type CDV from field materials [12,16,25]. The most antigenic variation occurs in the H protein [3] and it has been suggested that analyses of CDV genetic variation should focus on this protein [9].

Many cell lines have been tested for their ability to grow wild type CDV and maintain its virulence and homogeneity. Among them, cells from the marmoset B lymphoid line B95a have been reported to be highly susceptible hosts [15,27]. The aim of this study, we successfully obtained two CDV field isolates in Taiwan by co-culturing peripheral blood leukocytes (PBML) with B95a cells, four cloned H genes from dogs infected with CDV, and totally six strains compared their nucleic acid sequences of the H genes with those of other reference isolates.

MATERIALS AND METHODS

Animals Seventeen non-vaccinated puppies, less than 3-4 months old, from an animal shelter and 440 dogs clinically suspected of CDV infection from the diagnostic services at National Taiwan University were included in this study. In the 440 clinical cases, a positive diagnosis of CDV infection was confirmed for 166 (37.7 %) by P gene RT-PCR. From these positive cases, samples were selected quarterly for further H gene analysis from October, 2003 to December, 2005. All 166 dogs showed at least one of the signs of CDV infection, such as central nervous system signs including seizure, ataxia, circling and myoclonus; digestive signs including diarrhea, vomiting, depression and anorexia; and respiratory signs including nasal and ocular discharge, cough, dyspnea and sneezing.

Cell culture and virus isolation The B95a cells were propagated in RPMI medium 1640 (Gibco, Grand Island, NY, USA) supplemented with 2 or 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 2 mM glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM Hepes, and 1 mM sodium pyruvate under a 5% CO_2 atmosphere. Virus isolation from peripheral blood leukocytes (PBML) separated on Ficoll Hypaque solution (Histopaque-1077, Sigma-Aldrich) was performed using a modification of the method reported by Blixenkrone-Moller [3]. Briefly, three to five mL heparin-stabilised whole blood of the 17 puppies with suspected CDV infection were diluted 1:1 in RPMI 1640 supplemented with 2% heat-inactivated FBS. PBML were isolated by centrifugation over a Ficoll density gradient of 1.077 g/mL at 400 g for 45 min at room temperature. The interface cells were harvested and washed two times in RPMI 1640 with 2% FBS by centrifugation at 400 g for 3 min. The 50 - 60% confluent B95a cells cultured in 25 cm^2 flasks were overlaid with PBML for observation of cytopathic effect (CPE) within 2-3 days. When B95a cells became 100% confluent and no CPE was detected, a second blind passage was carried out by removing the adherent B95a cells with cell scrapers (Cell scraper 353085, Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA). One mL of scraped adherent cells was suspended at fresh 50% confluent B95a cells cultured in 25 cm^2 flasks (Corning, NY, USA) for the next passage and examination.

CDV antigen test When the infected B95a cells showed 40-50% CPE, the cells were scraped and four drops of the suspension were dispensed into the Bioindist International Technical (BIT) rapid color CDV sample wells. The BIT rapid color CDV kit (Donghwa-Ri, Bongdam-Eup, Hwaseong, KyungGi-Do, Korea) was used to test for the presence of CDV anti-

gen. Briefly, whole blood (serum or plasma), ocular or nasal discharge solution from suspected cases was dispensed into the sample wells. The results were read by naked eye after waiting for 5 to 10 min. One red or purple band in the control line (C) with no apparent band in the test line (T) indicated a negative result for CDV infection. One red or purple band in the control line (C) and one band in the test line (T) indicated a positive result.

Indirect immunofluorescence assay (IFA) When infected B95a cells in 25 cm^2 flasks showed 40-50% CPE, the cells were harvested and spread on 10-well immunofluorescence assay (IFA) slides (Assistant-Präzisions, Glaswarenfabrik, Karl Hecht KG, Sondheim, Germany). The slides were air dried and fixed by acetone/methanol (1:1) at 4℃ for 15 min. The virus antigens in the fixed cells were examined microscopically by IFA (Olympus BX51, Shinjku-Ku, Tokyo, Japan). The primary antibody, a mouse anti-CDV Ab(MCA 1893, Clone DV2-12; Serotec, Kidlington, Oxford, UK) was diluted 1 in 150 in antibody diluent (Ventana Medical System, Tucson, AZ, USA), and then applied to the cells for 60 min at 37℃. The cells were then immersed in phosphate buffered saline (PBS; DakoCytomation, Carpinteria, CA, USA) for 10 min, followed by incubation with goat anti-mouse IgG FITC-labelled secondary antibody (Chemicon 5008, Single Oak Drive Temecula, CA, USA) for 60 min at 37℃. Then the cells were rinsed in PBS, counterstained with 0.02% Evans Blue dye and mounted with mounting fluid (Chemicon 5013, CA, USA). When the results were positive, the IFA test results were reconfirmed by an eight-well Lab-Tek II Chamber slide system (Nalge Nunc, Naperville, IL, USA). For this assay, 4×10^4 fresh B95a cells in 0.5 mL RPMI medium 1640 were seeded on each well of the slide and incubated for one day, and then inoculated with 100 mL of field virus. Two to three days after inoculation, once CPE was observed, the slides were fixed and examined by IFA as described above.

Titration of CDV B95a cells infected with CDV were frozen and thawed three times, and the titer of the released virus was determined by endpoint dilution assays to calculate the tissue culture infective dose 50% ($TCID_{50}$). The assays were carried out in 96 well plates (Costar 3599, Corning, NY, USA) by a limiting dilution method. In each well, 0.1 mL of 3×10^4/mL B95a cells was inoculated with 0.1 mL of sequential 10-fold viral dilutions. During the next 7- 10 days, each well was examined daily to detect the appearance of CPE, and then viral titers were calculated.

RT-PCR of H gene Four positive results for the H gene from the 166 CDV-infected clinical cases, NTU 1-2004, NTU 4-2003, NTU 3-2004, and NTU 2004, and two CDV isolates from the virus-infected B95a cells were analysed. Total RNA was extracted by using the RNeasy Mini Kit (Qiagen). First-strand cDNA synthesis was performed at 55 ℃ for one hour with a specific reverse primer (CDVHR2, nucleotides 8928 to 8905, 5'-CAATTGARATGTGTATCATCATAC-3'), by using SuperScript™ III reverse transcriptase (Invitrogen Inc., Carlsbad, CA, USA). H genes were amplified by using Platinum® Pfx DNA polymerase (Invitrogen) with the primer set, CDHR2 and forward primer (CDHF1, nucleotides 7063 to 7082, 5'-CTCAGGTAGTCCARCAATGC-3') yield an amplicon of 1,866 base pairs, in the following conditions: 94℃ for 2 min, then 35 cycles of 94℃ for 15 sec, 53℃ for 30 sec and 68℃ for 2 min, with a DNA thermal cycler (Applied Biosystems, CA, USA). The primers nucleotide position based on the CDV strain 5804 (AY386315). The amplified PCR products were purified from agarose gels with the QIA quick PCR purification kit (Qiagen, Inc., CA, USA).

Sequencing and accession numbers of H genes from isolated CDVs Most purified PCR products were directly sequencing using the primers used for amplication. All direct sequencing reactions were verified independently at least three times. Alternatively, some purified PCR products were 3'-end-adenylated by incubation with dNTP and Platinum® Taq DNA polymerase, and then cloned into the pCR® 2.1-TOPO® TA cloning vector (Invitrogen, Inc., Carlsbad, CA, USA). The direct and cloned H genes were sequenced using the ABI PRISM Big Dye™ Terminator Cycle Sequencing Ready reaction kit and an ABI 3730 XL DNA analyzer (Applied Biosystems, CA, USA). Sequence analysis was performed with ABI PRISM

DNA sequencing analysis software V 5.2. The six CDV strains identified in this study have been submitted to GenBank. The code and GenBank accession number for each of these strains are as follows: NTU 1-2004 (DQ191175), NTU 4-2003 (DQ191767), NTU 3-2004 (DQ191766), NTU 2004 (DQ191765), NTU 2005-1 (DQ887547), and NTU 2005-2 (DQ887548).

Phylogenetic analysis Phylogenetic analysis of the nucleotide sequences of H genes was performed with the LASERGENE Biocomputing (DNASTAR, 1998, Wisconsin, USA) software package. The tree was inferred using the neighbor-joining (NJ) method and the Kimura 2-parameter model, constructed with the MEGA4 software package [29]. The codes, accession numbers and provenience for the sequences are as follows (Table 1): Taiwan isolates, Dog/NTU 1-2004 (DQ191175, Taiwan), Dog/NTU 4-2003(DQ191767, Taiwan), Dog/NTU 3-2004 (DQ191766, Taiwan), Dog/NTU 2004 (DQ191765, Taiwan), Dog/ NTU 2005-1 (DQ887547, Taiwan), Dog/NTU 2005-2 (DQ887548, Taiwan), and Dog/Taichung 2003 (AY378091, Taiwan); China isolates, Dog/TN-China 2003 (AY390347, China), Giant panda/China 1999 (AF178038, China), and Lesser panda/China 1999 (AF178039, China); Japanese isolates, Dog/KDK1 (AB025271, Japan), Dog/Hamamatsu (D85754, Japan), Raccoon dog/Tanu 96 (AB016776, Japan), Dog/Ueno (D85753, Japan), Dog/Yanaka (D85755, Japan), Dog/HM-3(AB040767, Japan), Dog/HM-6(AB040768, Japan), Dog/26D (AB040766, Japan), and Dog/98-002 (AB025270, Japan); European and USA isolates, Dog/404 (Z77671, Germany), Dog/2544 (Z77672, Germany), Dog 324/03 (DQ494317, Italy 06), Mink/DK86 (Z47759, Denmark), Dog/DK91, B+C (Z47761, Denmark), Dog/US89 (Z47762, USA), Black leopard/US91 (Z47763, USA), Javelina/US89 (Z47764, USA), Raccoon/US89 (Z47765, USA); Old CDVs and vaccine isolates, Onderstepoort (AF378705), Wyeth-Lederle (AF014953), Convac vaccine (Z35493), Snyder Hill (AF259552, USA), and A75-17(AF164967); the Arctic isolates, Dog 179/04 (DQ226087, Italy 06) and Dog/GR88 (Z47760, Northern Greenland).

RESULTS

Isolation of field CDV strains Two CDV strains, NTU 2005-1 and NTU 2005-2, were successfully isolated from 17 dogs with suspected CDV infection between 2003 and 2005 in Taiwan. The appearance of CPE was recognized in the fifth passage (12th day) of B95a cells co-cultured with PBL of the NTU 2005-1 strain and in the second passage (6th day) of the NTU 2005-2 strain. The CPE was observed as a polygonal to stellate or large round shape. Multinucleated giant cells with more than 50 nuclei were observed (Fig. 1A). Mock controls of B95a cells showed normal appearances (Fig. 1B).

Indirect Immunofluorescence assay (IFA) The IFA results for infected B95a cells showed positive labelling in the cytoplasm of altered cells. The positive green-color labelling was more extensive and distinct in the cytoplasm of syncytial cells with red-color background (Fig. 2A). The same field counterstaining with 0.02% Evans Blue also showed the positive FITC labeling cells in Fig. 2A was multinucleated syncytial giant cells (Fig. 2B).

Titration of CDV The titers of the stock solution of NTU 2005-1 and NTU 2005-2 strains were 3.1×10^3 $TCID_{50}$/mL and 2.14×10^4 $TCID_{50}$/mL, respectively.

Sequencing and phylogenetic analysis of H gene The H gene of the six isolated CDV strains was 1824 nucleotides long and the deduced amino acid sequence was 607 amino acids long. Nucleic acid sequences of the H genes of the NTU 2005-1 and NTU 2005-2 strains showed 98.6% identity, and their deduced amino acid sequences showed 98.8% identity (Table 2). At the amino acid level, the NTU 2005-1 and NTU 2005-2 strains showed more than 98.2% identity with Asia-1 isolates [10,13,26] (Hamamatsu, KDK-1 and dog/TN-China 2003), 93.1% to 93.4% identity with the Asia-2 isolate (98-002) [10,26], but only 90.5% identity with the Onderstepoort vaccine strain. At the nucleotide level, similar results were also noted (Table 2). In the deduced H protein of the six new isolates, nine asparagines that are potential sites for N-linked glycosylation (N-X-S/T) were found at

Table 1. Nucleotide sequence accession numbers of the H gene of the strains mentioned in this study

Strains	Origin	Accession	Reference
NTU 1-2004	Dog, Taiwan	DQ191175	Present study
NTU 4-2003	Dog, Taiwan	DQ191767	Present study
NTU 3-2004	Dog, Taiwan	DQ191766	Present study
NTU 2004	Dog, Taiwan	DQ191765	Present study
NTU 2005-1	Dog, Taiwan	DQ887547	Present study
NTU 2005-2	Dog, Taiwan	DQ887548	Present study
Taichung 2003	Dog, Taiwan	AY378091	Hsieh et al., 2003, unpublished
TN-China 2003	Dog, China	AY390347	Meng et al.,2003, unpublished
China 1999	Giant panda, China	AF178038	Li et al., 1999, unpublished
China 1999	Lesser panda, China	AF178039	He et al., 1999, unpublished
KDK1	Dog, Japan	AB025271	[26]
Hamamatsu	Dog, Japan	D85754	[13]
Tanu 96	Raccoon dog, Japan	AB016776	Kai and Ohashi,1998, unpublished
Ueno	Dog, Japan	D85753	[13]
Yanaka	Dog, Japan	D85755	[13]
HM-3	Dog, Japan	AB040767	[10]
HM-6	Dog, Japan	AB040768	[10]
26D	Dog, Japan	AB040766	[10]
98-002	Dog, Japan	AB025270	[26]
404	Dog, Germany	Z77671	[9]
2544	Dog, Germany	Z77672	[9]
324/03(Italy 2006)	Dog, Italy	DQ494317	[23]
DK86	Mink, Denmark	Z47759	[4]
DK91, B+C	Dog, Denmark	Z47761	[4]
US89	Dog, USA	Z47762	[4]
US91	Black leopard, USA	Z47763	[4]
US89	Javelina, USA	Z47764,	[4]
US89	Raccoon, USA	Z47765	[4]
GR88	Dog,Northern Greenland	Z47760	[4]
Onderstepoort	vaccine	AF378705	[31]
Wyeth-Lederle	vaccine	AF014953	[28]
Convac	vaccine	Z35493	[17]
Snyder Hill	vaccine	AF259552	[9]
A75-17	vaccine	AF164967	Wiederkehr et al.,1999 unpublished
179/04 (Italy 2006)	Dog, Italy	DQ226087	[23]

amino acid positions 19-21, 149-151, 309-311, 391-393, 422-424, 456-458, 584-586, 587-589 and 603-605. Of these nine sites, the seventh (584-586) that had been found in recent Japanese isolates [10,11,13,18,19] was also identified in the Taiwan strains. Within the H protein sequences of the six isolates, 12 cysteine residues known to be important for the secondary structure of the protein were conserved

Table 2. Nucleotide and amino acid sequence identities between the CDV H genes (%). Nucleotide identities are present in the upper half of the matrix and amino acid identities in the lower half

	Virus	Nucleotide (%)								
		NTU2005-1	NTU2005-2	NTU2004	Dog/Taichung 2003	TN-China 2003	Dog Hamamatsu	Dog KDK-1	Dog 98-002	Onderstepoort
Amino Acid (%)	NTU2005-1	*	98.6	99.2	99.2	98.7	99.2	98	92.7	90.8
	NTU2005-2	98.8	*	98.4	98.4	98.3	98.7	97.7	92.1	90.3
	NTU2004	99.2	98.2	*	99.5	98.6	99	97.8	92.3	90.8
	Dog/Taichung 2003	99.3	98.4	99.3	*	98.6	99	97.8	92.3	91.1
	TN-China 2003	99	98.3	98.7	98.8	*	98.8	97.7	92.2	91.2
	Dog Hamamatsu	99.2	98.5	98.5	98.7	98.7	*	98.4	92.7	91.1
	Dog KDK-1	98.8	98.2	98.2	98.4	98.3	98.5	*	93.1	91.2
	Dog 98-002	93.4	93.1	93.1	93.3	93.2	93.1	93.4	*	91
	Onderstepoort	90.5	90.5	90.5	91	90.6	90.1	90.6	90.1	*

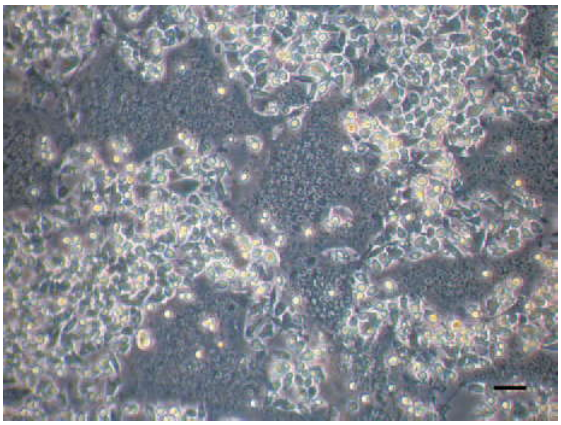
* = 100 %

at positions 139, 154, 188, 283, 296, 377, 382, 390, 490, 566, 575 and 602 (Fig. 3). Phylogenetic analysis of the H gene showed that all of the six CDV field strains isolated in this study belong to the Asia-1 group that is distinct from the Asia-2 group (Fig. 4).

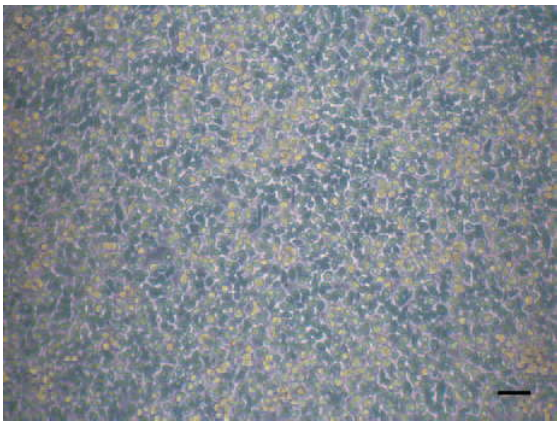
DISCUSSION

This study of CDV isolation was first carried out in tissue homogenates of adult CDV-infected dogs diagnosed by RT-PCR or histopathology, but where this failed to show CPE in either B95a or MDCK cell lines as previously reported [9,21]. Thus, we also employed a co-culture of PBML from 17 unvaccinated puppies with suspected CDV infection with B95a cell lines. The combination of IFA and CDV antigen tests successfully identified the virus and found CPE in different passages of these co-cultures. Virus isolation has been reported to be easier from young puppies than adult dogs. Uema et al. [30] reported that two to three month-old infected dogs with or without vaccination history can be used for CDV isolation. Co-cultivation of mononuclear cells with B95a cells also yielded higher CDV isolation rates [15,27]. False negative IFA results were occasionally noted when compared with antigen tests, especially when CPE was present in 100 % of the cells or when scraped infected B95a cells were used. The chamber slide method [13] for IFA showed better detection of CDV positive results than the scraped IFA slide method in this study. The titers of the two stock strains were 3.1×10^3 and 2.14×10^4 $TCID_{50}$/mL, respectively. The titers were a little lower than those of CDV isolated from lymph nodes, large intestines, tonsil or spleen [19].

Recent extensive acquisition of molecular data on the H gene of CDVs has allowed the identification of six distinct phylogenetic clades with regard to their geographic origin (Asia-1 [10,13,26], Asia-2 [10,26], Europe [4,5,6,9,23,30], USA [4,20,30], Arctic [6,23], and Old CDVs [9,17,28,31]. In a given geographical region, two to three genotypes of CDVs can be present [4,5,6,8,10,20,23,26,30]. Based on the phylogenetic analysis of the H gene, six Taiwan strains were reported in this study, including NTU 1-2004, NTU 4-2003, NTU 3-2004, NTU 2004, NTU 2005-1, and NTU 2005-2. These strains belong to the Asia-1 group

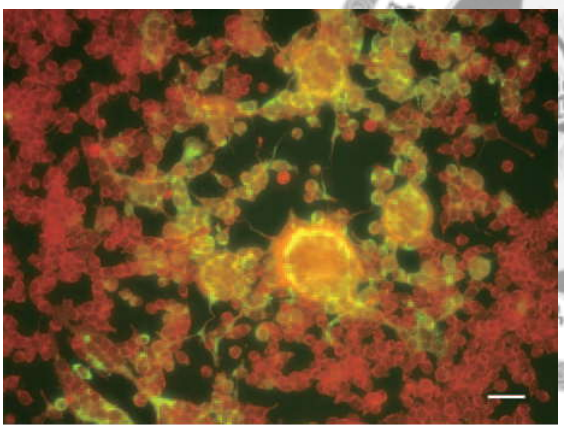


1A

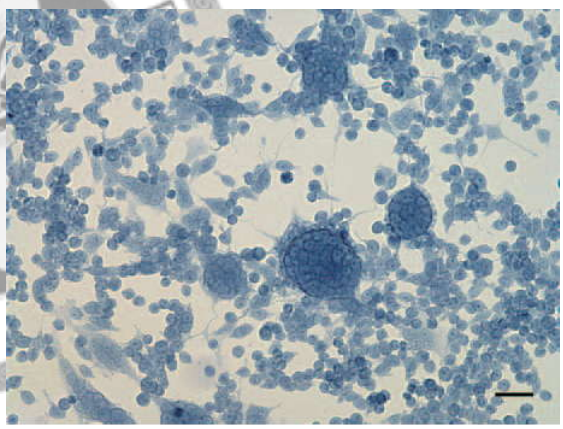


1B

Fig. 1 Syncytium type of CPE in infected B95a cells (A) B95a cells infected with NTU 2005-2 CDV strain. Multinucleated syncytial giant cells with more than 50 nuclei were observed. (B) Mock control of the B95a cells showed normal appearance. Bar, 50 μm.



2A

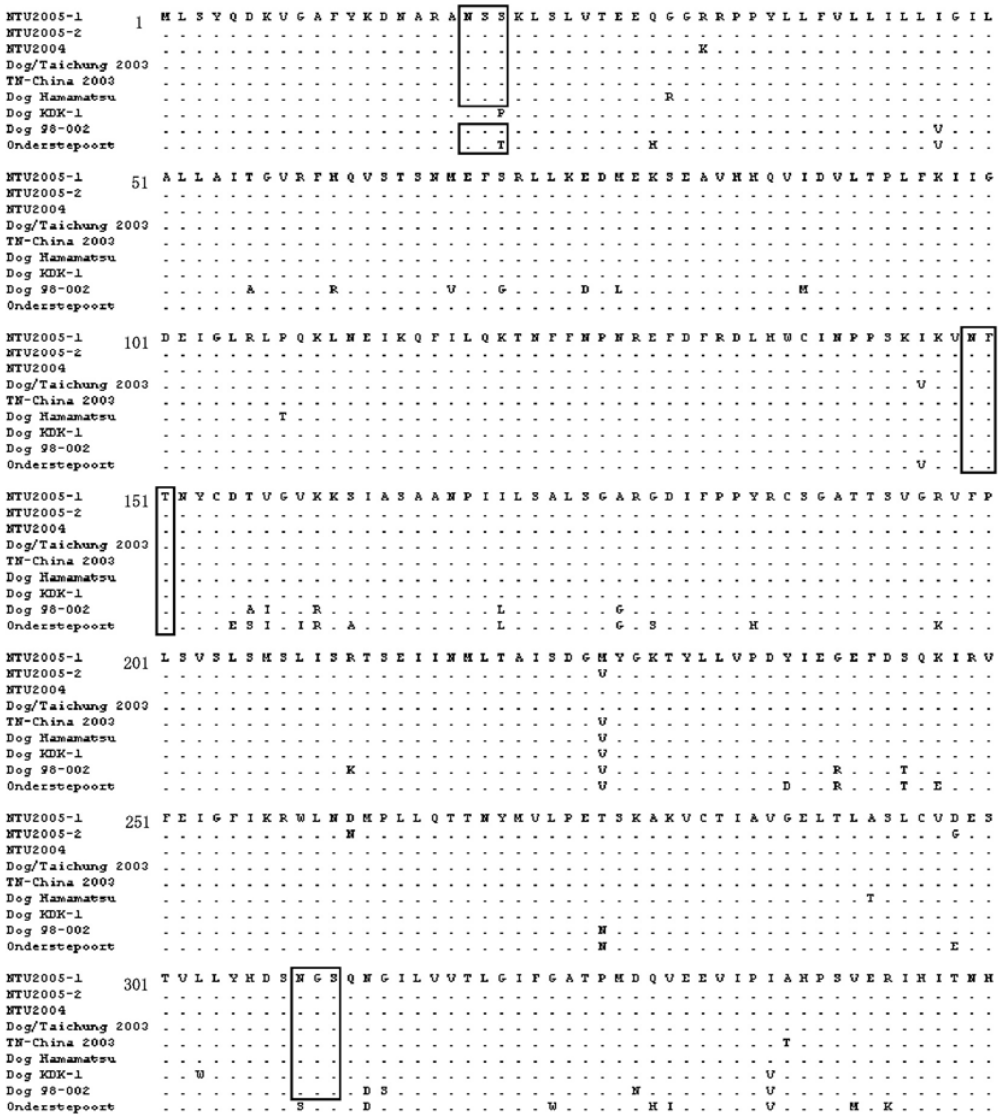


2B

Fig. 2 Detection of virus antigen in B95a cells infected by a NTU 2005-1 strain (A) The positive green-color FITC labelling was distinct in the cytoplasm of syncytial cells with red-color background. (B) Same field as in 2A, counterstaining of infected B95a cells showed multinucleated syncytial CPE appearance. Bar, 40 μm.

[10,13,26], which is distinct from the old CDVs and Asia-2 groups [10,26].

The six CDV strains identified in this study were tightly related to each other (< 2% amino acid variation). This might be due to all the samples coming from northern Taiwan during a limited period (2003-2005). However, strains in the same clade of the phylogenetic tree showing more than 95% amino acid similarity (< 5 % amino acid variation) in the H gene may be considered to belong to the same genotype [26]. The 8137th nucleotide on the viral genome (the 1059th one on the H gene), a thymine (T) in the vaccine strains, is substituted to cytosine (C) in the wild-type strains [6]. This point mutation was also found in all six Taiwanese strains in this study. The H gene RFLP analysis using EcoRV and SspI [30] may be useful for the detection of Taiwanese field strains. The field CDV strains in this study possessed nine asparagines that were


NTU2005-1
NTU2005-2
NTU2004
Dog/Taichung 2003
TN-China 2003
Dog Hamamatsu
Dog KDK-1
Dog 98-002
Onderstepoort

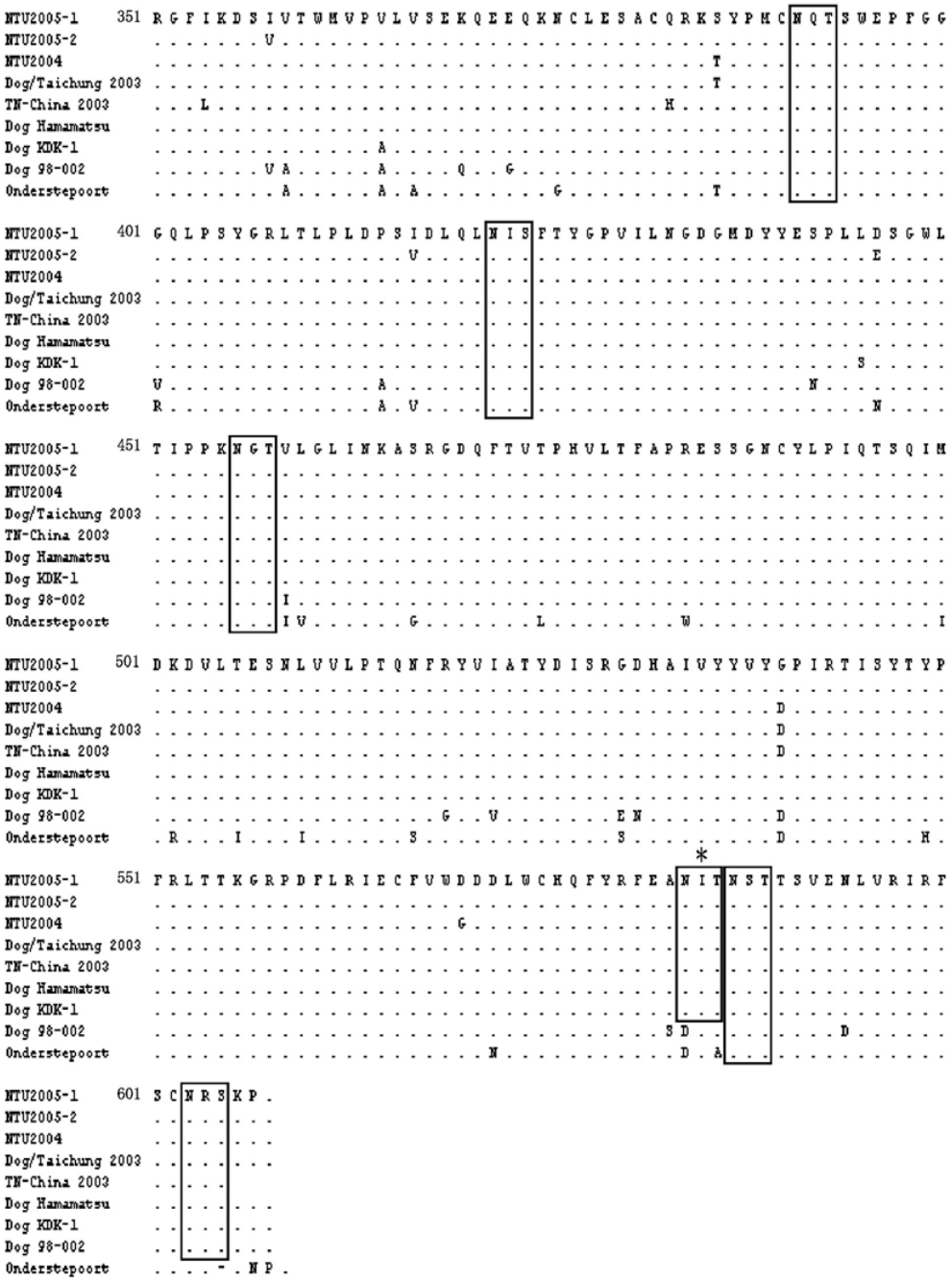


Fig. 3 Multiple alignment of deduced amino acid sequences of the H gene of the Taiwan CDV strains. CDV strains are referenced in Table 1. Identical residues are shown by dots. The potential N-linked glycosylation sites are boxed. An asterisk indicates the seventh potential N-linked glycosylation site.

potential sites for N-linked glycosylation in their deduced H protein. The same nine potential sites were also reported in Japanese strains [10,11,13,18,19]. Among them, the seventh (584-586) site is especially characteristic of the Taiwanese strains in this report and for recently reported Japanese strains.

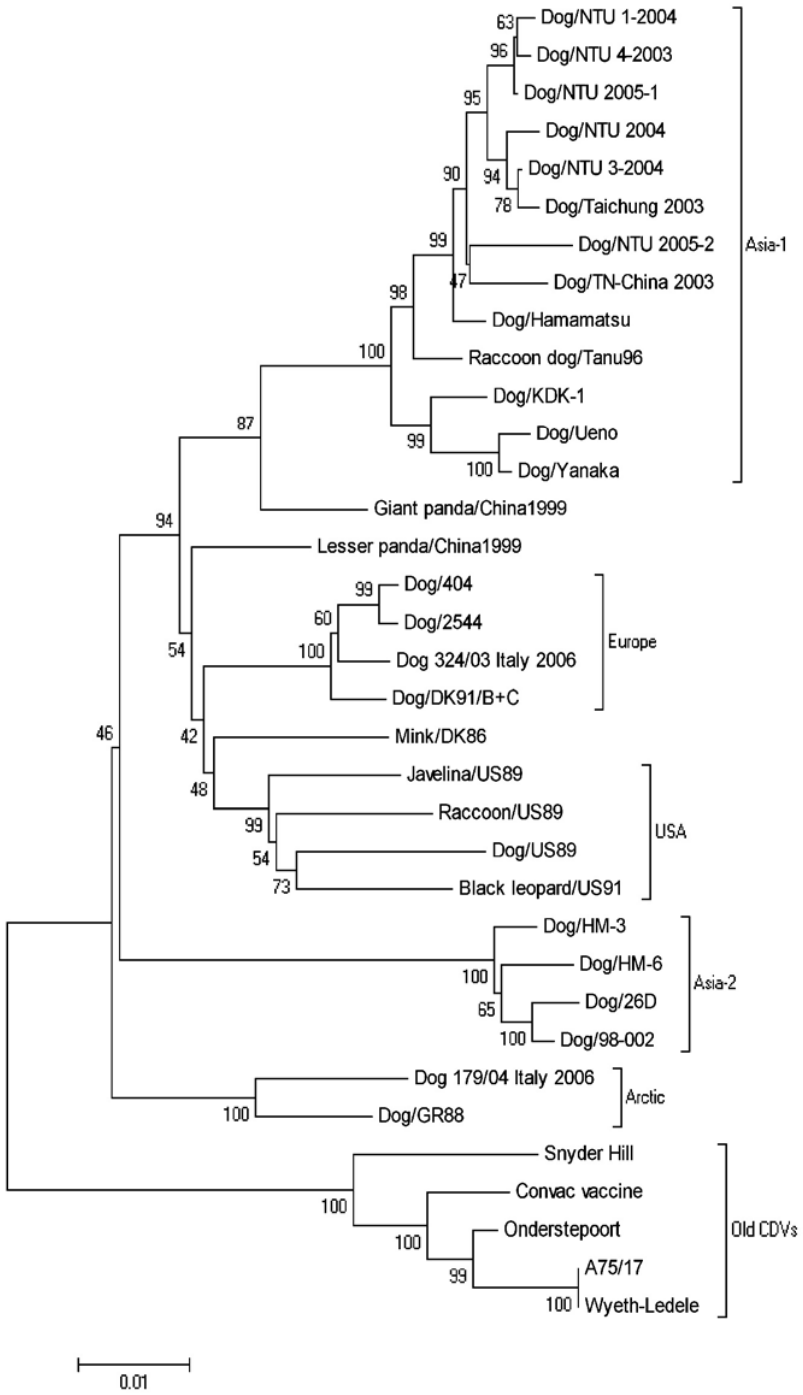


Fig. 4 Neighbor-Joining tree of CDV strains analysed in this study based on the nucleotide alignment of the H protein. CDV strains are referenced in Table1.The optimal tree with the sum of branch length = 0.39956060 is shown. The bootstrap values indicate the number of times that each branching was found in 1000 bootstrap analyses. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 1814 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 [29].

In conclusion, the genetic variability among these sites of the CDV isolates is an important consideration for elucidating the immune response [9,13,19]. Furthermore, phylogenetic analysis of the H gene showed that the six isolates in this study were closely related to Chinese and Japanese isolates. These similarities between Taiwanese, Chinese and Japanese isolates might reflect the common prevalence of CD in Taiwan, China and Japan.

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臺灣地區犬瘟熱病毒分離及血球凝集素基因分析

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摘要 於 2003-2005 年間自 17 隻未經疫苗注射之發病幼犬，應用患犬血液單核球與 B 95a 細胞株共同培養之技術，分離出兩株具誘發融合細胞病變之病毒，經免疫螢光染色及抗原測試確認為犬瘟熱病毒。將此兩株病毒之血球凝集素基因(H)，與同期間自台灣大學動物醫院臨床送檢病例之另外 4 株犬瘟熱病毒之 H 基因進行核酸定序。經比對序列與樹狀圖分析發現，本土病毒株皆有 9 個 N 連結配醣位，其中第 7 個配醣位為日本或中國大陸流行之亞洲 1 型犬瘟熱病毒所特有。本研究顯示臺灣地區所流行之犬瘟熱病毒，經其 H 基因分析屬亞洲 1 型。[梁鍾鼎、闕玲玲、李洧泓、黃宣憲、Masashi UEMA、Akira WATANABE、Ryuichi MIURA、Chieko KAI、梁善居、余俊強、劉振軒。臺灣地區犬瘟熱病毒分離及血球凝集素基因分析。台灣獸醫誌 34 (4): 198-210，2008。*聯絡人TEL/FAX: 886-02-2363 3289，E-mail: chhsuliu@ntu.edu.tw]

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Chapter VI

Canine distemper in Taiwan from 2000 – 2009: co-infections, and 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 shelter. 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 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 X^2 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 X^2 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 previously[14] 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 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 hepatic 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 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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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 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 inclusion 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

Table 3. Sites of inclusion bodies in shelter group of CDV infected dogs(N=20)

Lot No./organ	Urinary bladder	Lymphoid tissues	Lung	Cerebellum	Cerebrum	Brain stem	Ependymal cells	Alimentary tract	Skin
NTU00-171	-	-	-	-	-	-	-	-	-
NTU00-172	-	-	-	-	-	-	-	-	-
NTU00-233	+	+	+	+	+	+	+	+	+
NTU00-276	-	+	+	+	+	+	-	-	-
NTU00-350	-	+	-	-	-	-	+	-	-
NTU00-384	-	-	-	-	-	-	-	-	-
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

Table 4. Sites of inclusion body in clinic group of CDV-infected dogs(N=32)

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	09-997	NA	-	-	+	+	+	+	-	-
C	09-887	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 intranuclear and intracytoplasmic inclusion bodies (INIB/ICIB) in 9 sites in shelter and clinic CDV-infected dogs. Data presented are number of INIB/ICIB positive dogs, total number of dogs examined, and the percentage of dogs INIB/ICIB positive.

Sites of INIB/ICIB	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

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	Shelter 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 (X^2 chi-square test); the exception was interstitial nephritis, which was significantly more common in the shelter dogs than in the clinic dogs.

Table7. Diagnostic Criteria of CDV cases included in this study, N=52

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

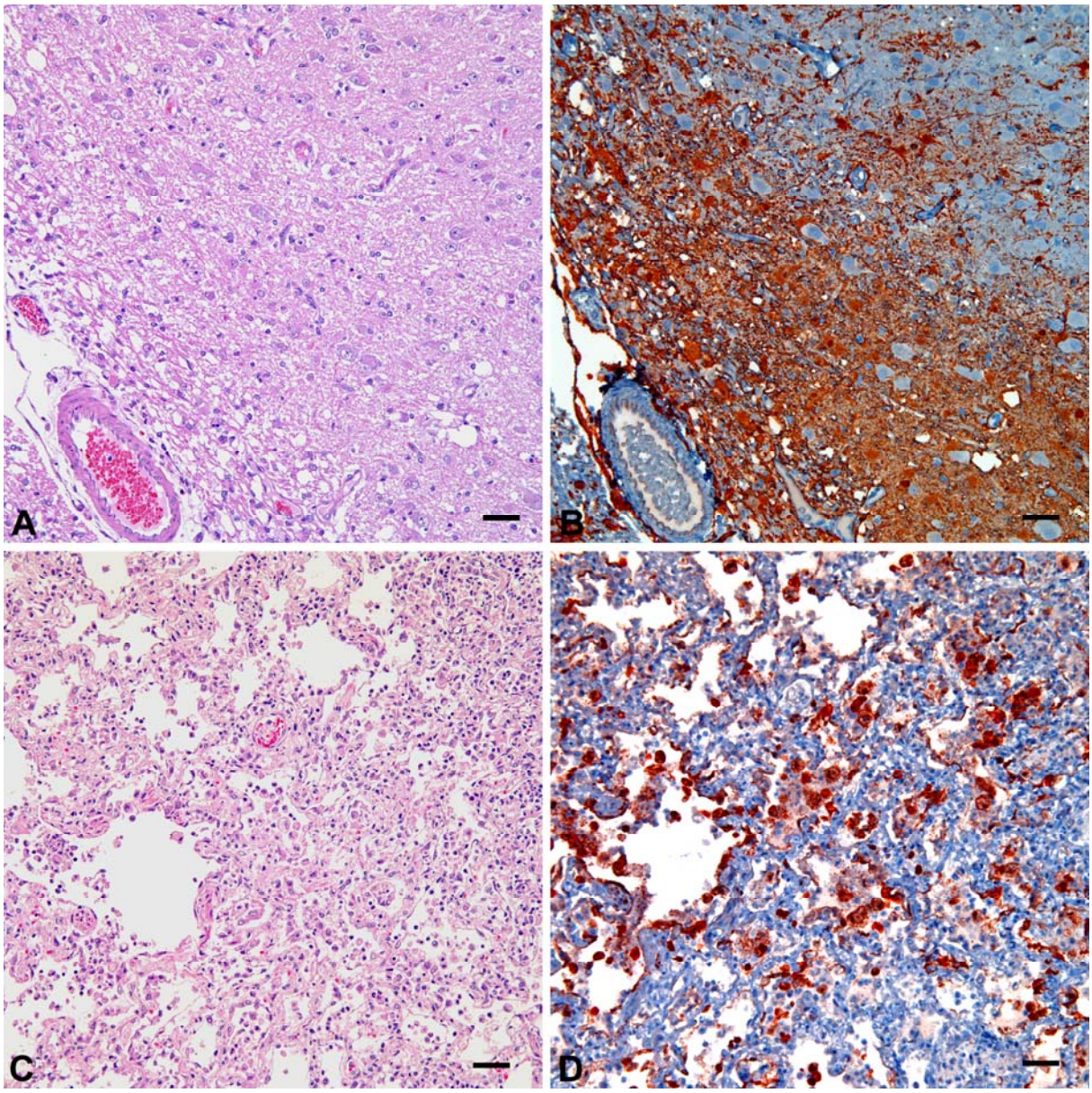

Figure 1. Comparison between H & E and IHC labelling in two CDV infected dogs. A, B. Case 58, brain. A. H & E, showing multifocal spongy form of the mid-brain thalamus neuropil B, non-biotin HRP (AEC) with haematoxylin counterstain, showing diffuse immunolabelling of thalamus neuropil. C, D. Case 57, 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.

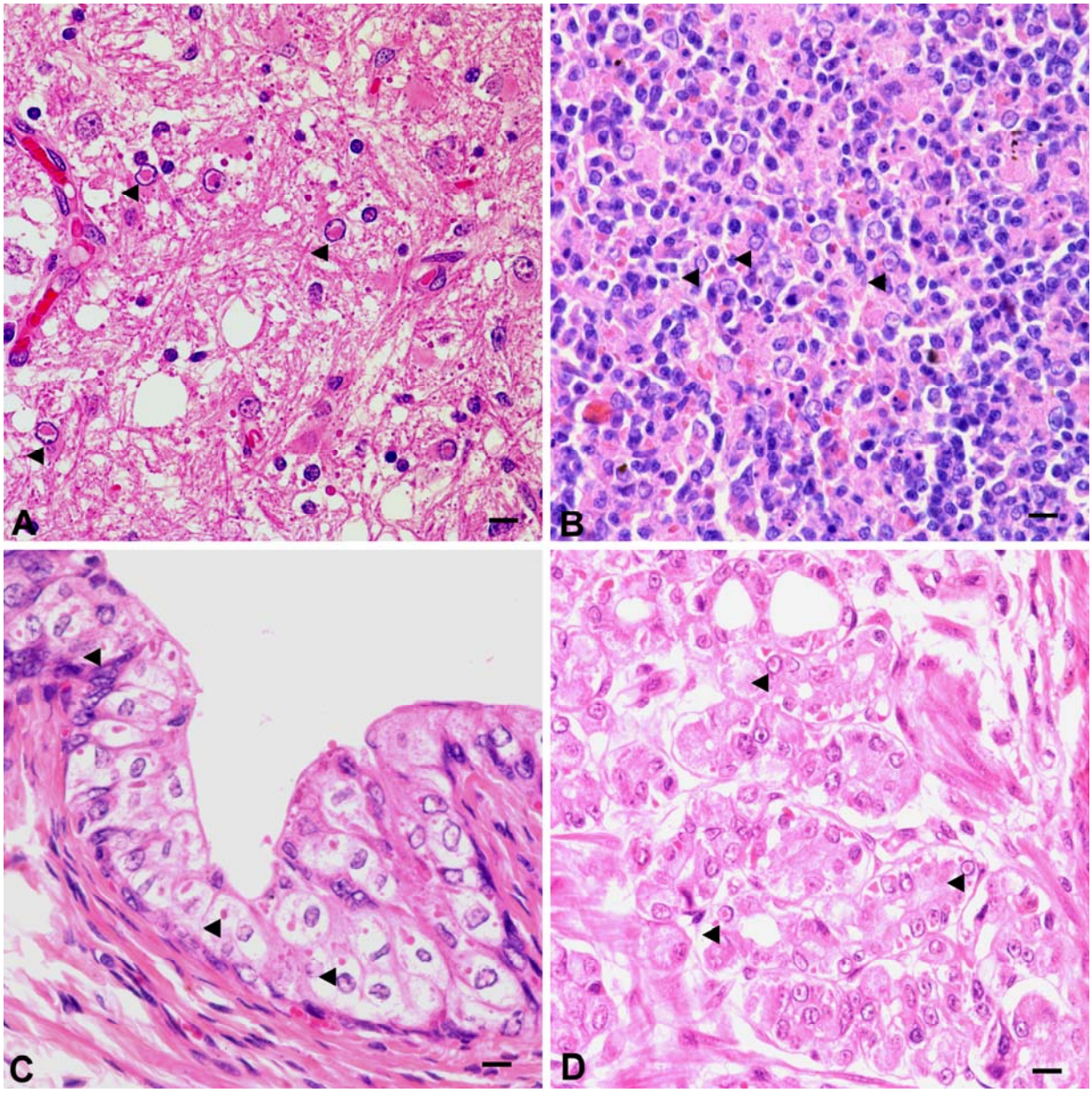

Figure 2. Eosinophilic intranuclear and intracytoplasmic inclusion bodies in four dogs with CDV. H & E. **A**, multifocal spongy form of the mid-brain thalamus neuropil with eosinophilic intranuclear inclusion bodies in the sub-ependymal astrocytes (arrowhead), case 58. **B**, splenic lymphoid depletion 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.

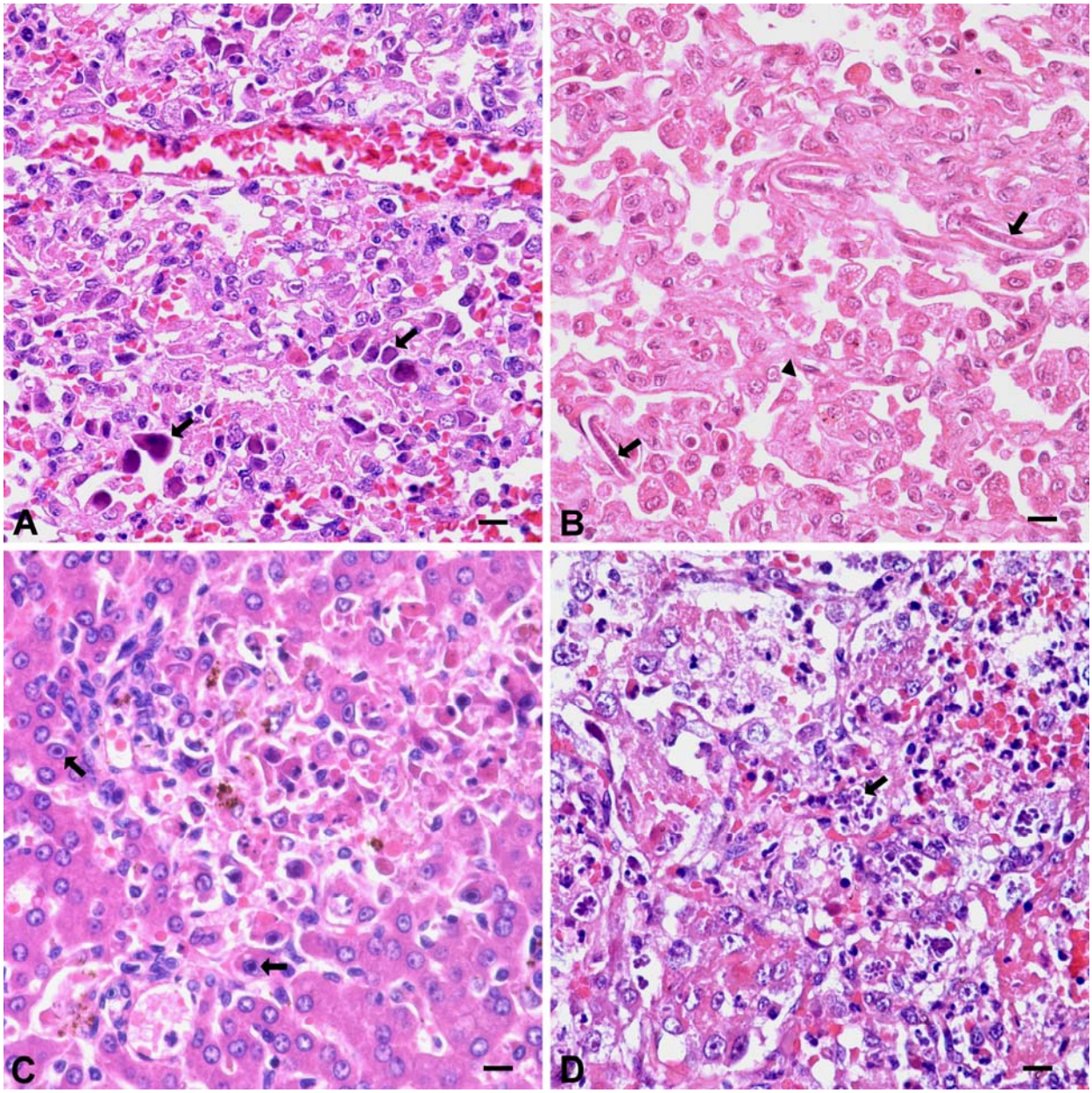

Figure 3. Co-infections and associated lesion in four dogs with CDV. H & E. **A**, purulent bronchointerstitial pneumonia with smudge 8-10μm adenovirus, basophilic intranuclear inclusion bodies in the pulmonary bronchiolar epithelium (arrow), case 58. **B**, 10-40μm (body length) microfilaria of *Dirofilaria 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 5-8 μ m 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.

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Chapter VII

General Discussion

Immunohistochemical detection of CDV and MHV infection

1. An alkaline phosphatase (AP) substrate IHC detection system may be used in immunohistochemical diagnosis of infectious diseases (Chapter II) but will produce variable labeling of fibres, processes or terminals in neural tissues. Unfortunately, the targeting of the CNS by CDV makes an AP system less suitable for use in its diagnosis.

2. With the non-biotin HRP detection system, pretreatment by autoclaving following by microwave heating gave better immunohistochemical labeling of CDV infection than did microwave pretreatment alone.

The use of non-biotin HRP for the immunohistochemical diagnosis of CDV infection has not previously been reported (Chapter III,VI). In the present study, it produced improved contrast against the background, making the detection of CDV antigen easier than the histological detection of intranuclear inclusion bodies. The cloned DV2-12 primary antibody used required pretreatment of formalin-fixed, wax-embedded sections by steaming, proteinase K pretreament having been showen by Ramos–Vara and Beissenherz (2000) to be without effect for this antibody. In this study, the positive areas of IHC labeling quite often exhibited neither histopathological lesions nor occurrences of inclusion bodies. These characteristics are quite similar as “non-enteritis” group in Japan. These “non-enteritis” group were

considered antigenically different from vaccine strain (Okita et al.,1997). However, another cases in this study had numerous INIB/ICIB in pelvis, urinary bladder, lung and stomach. These group are similar as “enteritis” group in Japan (Okita et al.,1997).

The use of different fixatives, with or without microwave unmasking, may influence the immunolabelling intensity, depending on the type of antibody (Liang et al., 2000). The immunohistochemical method described provides a more sensitive of diagnosing CD encephalitis than demonstration of inclusion bodies or syncytial cells.

3. A combination of proteinase K digestion and autoclaving in a Trilogy solution resulted in optimal ISH signal enhancement of CDV RNA.

As seen in the study (Chapter IV), this modified technique can be useful in the retrospective viral studies across a broad range. The HIAR effect (Mcquaid et al., 1990; Kim and Chae, 2003) can greatly enhance the ISH signal and provides a simple detection method in formalin-fixed, paraffin -embedded tissues. However, the tissue damage caused by the retrieval solution needs to be taken into consideration.

The IOD evalution method was used in this study. This method provided a more objective and semiquantitative evaluation of the viral ISH labeling.

CDV viral isolation and H gene phylogenetic analysis

1. According to the phylogenetic analysis of these H gene amino acid sequences, six

Taiwan strains, Dog/NTU 1-04, Dog/NTU 4-03, Dog/NTU 3-04, Dog/NTU 04, Dog/ NTU 05-1, Dog/ NTU 05-2 reported in this thesis were grouped in a similar cluster with CDV detected in dogs from Asia-1 strains, but quite distinct from the Onderstepoort old vaccine strain and Asia-2 strain.

2. Co-cultivation of mononuclear cells with B95a cells will give higher CDV isolation rates (Chapter V)(Kai et al., 1993; Mori et al., 1994). The higest antigenic variation was found in the H protein of CDVs, whereas the F and P proteins were affected to a lesser extent (Blixenkrone-Moller et al., 1992). The nucleocapsid (NP) protein does not reflect the variation in pathogenicity of clinical groups. In the recent years, extensive acquisition of molecular data on the H gene of CDVs has allowed the identification of distinct phylogenetic clades with regard to their geographic origin (Asia-1, Asia-2, Europe, USA, Arctic), and the year of isolation (Hashimoto et al., 2001; Martella et al., 2002; Hirama et al., 2004). In a given geographical region, two to three genotype of CDVs can be present (Haas et al., 1999; Hashimoto et al., 2001; Lednicky et al.,2004; Martella et al., 2006; Calderon et al.,2007; Dremer et al.,2007). These six strains in this study (Chapter V), were 98.4 to 99.6 % identical in nucleotide. Same nine potential sites for asparagines N-linked glycosylation in the H gene of the CDV isolates were also reported in Japan (Iwatsuki et al., 1997; Hirama et al., 2004). However, eight glycosylation sites had also been reported in Asia-2 group (Haas et

al.,1997; Mochizuki et al.,1999; Hashimoto et al., 2001; Lan et al.,2007) or Arctic group (Demeter et al.,2007). Four glycosylation sites (positions 19-21, 149-151, 422-424, 587-589) were shared by all CDVs (Haas et al., 1997; Hashimoto et al., 2001). The latter glycosylation site (positions 309-311) was found in all recent virus isolates but was absent in the older Snyder Hill strain. The glycosylation sites at the extreme C-terminus (positions 603-605) is unlikely to be used. Slightly more amino acid changes were noted in the the region around and between the two positions 422-424 and 456-458, which indicate sites in the protein subject to a greater antigenic pressure and selective force (Haas et al.,1997).

The strains in the same clade of the phylogenetic analysis showing more than 95 % amino acid similarity in the H gene may be considered to the same genotype (Mochizuki et al., 1999).Using this criterion, the results of our study indicates only one type of field CDVs in Taiwan based on similarities in the H genes. However, based on the change of the nucleotide at the position 8137 (1059 of H gene) of the viral genome from thymine (T) in the vaccine strains to cytosine(C) in the wild-type strains. This point mutation can be found in the nucleotide sequences of all wild-type CDVs (Demeter et al., 2007) and also found in all six strains of our thesis.

Pathological lesions and associated lesions analysis

1. The lymphoid depletion, pneumonia and CNS demyelination were the most

common CDV-infected principal lesions, and occurrence of inclusion bodies had high lymphotropism and epitheliotropism. However, its positive rate was high in younger clinic group.

2. The distribution of inclusion bodies also showed significant difference in urinary bladder, lymphoid tissues, lung and alimentary tract between two groups(Chapter VI). A variety of 29 co-infections and other associated lesions (Chapter VI) were identified. However, no significant difference in frequency of occurrence between two groups, the exception was interstitial nephritis.

Questions yet to be investigated

In this study, we have provided important information about the CDV viral isolation method, H gene phylogenetic analysis results of Taiwan strains, better immunohistochemical and ISH labeling of CDV and MHV infection using formalin-fixed, paraffin-embedded sections, and realized the tissues distribution of inclusion bodies in different group. However, we still not solve the question, why CDV induced demyelination but CDV antigen was found chiefly in astrocytes rather than myelin-making oligodendrocytes (Vandevelde and Zurbriggen, 2005). The increasing incidences of canine distemper in pups in suburb areas and in urban kennel shops have recently been noticed in spite of vaccination (Kai et al.,1993; Iwatsuki et al.,2000 ; Lan et al., 2006) in Japan and also noted in this study(7/32, 21%). However,

no direct evidence concerning the amino acid variation of the H gene of the field CDV in Taiwan to the current vaccine protection has been established. Passive immunotherapy with more CDV-infected cases, combined with clinical trial, pathological and immunohistochemical analysis might facilitate and clarified pathogenesis of this disease and save animal life.

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Appendix 1: Five pre-treatments of the ISH protocols

1. The sections were initially deparaffinised, rehydrated, and immersed in 0.2 M HCl at room temperature for 20 min, rinsed with 0.2% glycine in PBS, and digested with 20 μg/ml proteinase K at 37℃ for 15 min. After washing with 0.2% glycine, the sections were subjected to five different pre-treatments;
2. In pre-treatment a, the sections were each immersed in a 10% concentrated stock Tris-buffered saline (TBS S3006), 2× SSC for 10 min, and prehybridised and hybridized as described by Chueh et al. (1999). No optional protocol was changed.
3. In pre-treatment b, the Trilogy solution plus the slide sections were heated in an autoclave in the first dish for 15 min at 121℃. The sections (slides) were then taken out, put into another fresh Trilogy solution, pre-heated to 80℃ by microwave, and left to stand for 10 min at 80℃;
4. In pre-treatment c, the serial sections were processed as described in pre-treatment b; however, the solutions used in both the autoclave and microwave were changed to (c) 10% stock Tris-buffered saline (TBS S3006) in Q water;
5. In pre-treatment d, the serial sections were processed as described in pre-treatment b; however, the solutions used in both the autoclave and microwave were changed to (d) a 1% vector citrate-based stock antigen unmasking solution (H-3301, Vector Laboratories) in Q water;
6. In pre-treatment e, the serial sections were processed as described in pre-treatment b; however, the solutions used in both the autoclave and microwave were changed to (e) a target retrieval solution (S1700; Dako Cytomation);
7. After the different pre-treatments, the sections were then immersed in a 10% concentrated stock Tris-buffered saline solution (TBS S3006; Dako Cytomation) in Q water at room temperature for 10 min, rinsed in 2x SSC for 10 min, and prehybridised and hybridised as previously described by Chueh et al. (1999);
8. The following protocols were the same in each of the five pre-treatment groups. Each section was prehybridised at 37℃ for 60 min with prehybridization buffer (50% formamide, 2× SSC, 1× Denhardt's solution, 1 mM EDTA, and 100 μg/ml heat-denatured salmon sperm DNA);
9. Hybridisation was carried out in a hybridization buffer (50% formamide, 2× SSC, 1× Denhardt's solution, 1 mM EDTA, 10% dextran sulphate, and 350 μg/ml heat-denatured salmon sperm DNA);
10. Before being added to the solution, the labelled DNA probe and salmon sperm DNA were denatured for 10 min at 95℃ and immediately transferred onto ice for 3 min;

11. Hybridization was carried out in a humid chamber at 37°C for 16 h;
12. Post-hybridization washes consisted of 2× SSC, 1× SSC, 0.5× SSC, and 0.1× SSC. All washes were performed twice at 42°C for 15 min;
13. In order to detect the bound probes, the slides were briefly rinsed in a washing buffer (0.1 M maleic acid, 0.15 M NaCl, and 0.3% Tween-20, pH 7.5) for 2 min and were then immersed in a blocking buffer for 30 min (0.1 M maleic acid, 0.15 M NaCl, 1% blocking reagent, pH 7.5);
14. The probes were detected using an alkaline phosphatase-conjugated anti-digoxigenin antibody that had been diluted to 1:250 in a blocking buffer. Slides were incubated for 40 min at room temperature with the antibody, washed three times for 10 min in a maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) and then immersed in 500 μl of detection buffer (100 mM Tris–HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) at room temperature for 2 min;
15. The reactions were developed by incubating with a mixture of 200 μL of nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrates (18.75 mg/ml NBT and 9.4 mg/ml BCIP, toluidine salt in DMSO, Roche, Mannheim, Germany) in 10 ml of detection buffer (100 mM Tris–HCl, 100 mM NaCl, and 50 mM $MgCl_2$, pH 9.5) overnight at room temperature in ISH pouches (402 Sealpak pouches, Kapak, MN, USA) with 2 ml aforementioned solution added to each pouch;
16. The reactions were stopped and the slides were washed with Q water for 5 min;
17. Counterstaining of the NBT/BCIP reaction slides with methyl green was carried out for 1–5 min (H3402, Vector, Burlingame, CA, USA) followed by dipping the slides in 0.05% acetic acid in acetone 5–10 times before dehydration;
18. After dehydration in 95% and 100% ethanol, the slides were mounted and stored until examination; and
19. Substituting the probe on sections of CDV-infected spleen, cerebrum, and cerebellums served as a non-specific negative control. Cerebellar and splenic sections from dogs with no evidence of distemper infection served as a specific negative control.

Appendix 2: Details of the ISH protocol on B95a cells

The ISH performed on B95a cells was basically the same as that performed on the tissue sections, except that CDV-infected B95a cells were cultured on 15-mm circular cover slips, fixed in 4% paraformaldehyde for ISH labeling, and the lower final concentration of the proteinase K (1 μg/ml) and labelled probe (100 ng/ml) were compared to tissue sections (400 ng/ml of a dsDNA probe (287 bp) and 20 μg/ml proteinase K). The ISH protocol is as follows:

1. All of the ISH procedures were performed in the six-well chamber;
2. The 15-mm circular cover slips sections were rehydrated using DEPC-treated PBS for 1 hour;
3. The sections were immersed in 0.2 M HCl at room temperature for 20 min, rinsed with 0.2% glycine in PBS, and digested with 500 μl (1 μg/ml) of proteinase K at 37°C for 15 min;
4. After washing with 0.2% glycine and 2× SSC, each section was prehybridised at 37°C for 60 min with prehybridisation buffer (50% formamide, 2× SSC, 1× Denhardt's solution, 1 mM EDTA, 100 μg/ml heat-denatured salmon sperm DNA). Hybridization was carried out in a hybridization buffer (50% formamide, 2× SSC, 1× Denhardt's solution, 1 mM EDTA, 10% dextran sulphate, 350 μg/ml heat-denatured salmon sperm DNA);
5. Before being added to the solution, the labelled DNA probe and salmon sperm DNA were denatured for 10 min at 95°C and immediately transferred onto ice for 3 min. The final concentration of the labelled probe was 100 ng/ml;
6. The cover slips were put backwards (infected cells facing down) onto an acetone pre-treated slide with 180 μl hybridisation buffer already added;
7. Hybridization was carried out in a humid chamber at 37°C for 16 h;
8. Post-hybridization washes consisted of 2× SSC, 1× SSC, 0.5× SSC, and 0.1× SSC;
9. All washes were performed twice at 42°C for 15 min;
10. In order to detect the bound probes, the slides were briefly rinsed in washing buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3% Tween-20, pH 7.5) for 2 min and then immersed in a blocking buffer for 30 min (0.1 M maleic acid, 0.15 M NaCl, 1% blocking reagent, pH 7.5);
11. The probes were detected using an alkaline phosphatase-conjugated anti-digoxigenin antibody that had been diluted to 1:250 in a blocking buffer;
12. Slides were incubated for 40 min at room temperature with the antibody, washed three times for 10 min in a maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl,

pH 7.5), and then immersed in 500 μl of detection buffer (100 mM Tris–HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) at room temperature for 2 min;

13. The reactions were developed by incubation with a mixture of 200 μL of nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrates (18.75 mg/ml NBT and 9.4 mg/ml BCIP, toluidine salt in DMSO, Roche, Mannheim, Germany) in 10 ml of detection buffer (100 mM Tris–HCl, 100 mM NaCl, and 50 mM $MgCl_2$, pH 9.5) overnight at room temperature in ISH pouches (402 Sealpak pouches, Kapak, MN, USA) with 2 ml added to each pouch;
14. The reactions were stopped and the slides were washed with Q water for 5 min;
15. Counterstaining of the NBT/BCIP reaction slides with methyl green was carried out for 1–5 min (H3402, Vector, Burlingame, CA, USA), followed by dipping of the slides in 0.05% acetic acid in acetone 5–10 times before dehydration; and
16. After dehydration with 95% and 100% ethanol, the slides were mounted and stored until examination.

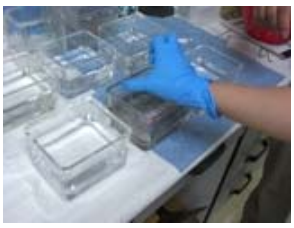
Appendix 3: Super sensitive non-biotin HRP combined with MOM detection system

1. Basically, the host of primary antibodies and the tissues can not be the same species.
2. If you do IHC of dog's tissues or your primary antibodies are different from your tissue sections, please follow the procedures published on 'J Comp Pathol. 136, 57-64, 2007"
3. If you do IHC of mouse tissues, and your primary antibodies are same mouse origin, please use MOM kit and follow the procedures published on "J Comp Pathol 131, 214-220, 2004"

<table>
<tr><th>MOM (mouse on mouse)</th><th>傳統 IHC (rabbit on mouse)</th><th>notice</th></tr>
<tr><td colspan="2">以 55℃烤片，烤至蠟變透明</td><td>使組織更加貼附於玻片上</td></tr>
<tr><td colspan="2">De-paraffin:
1. Xylene (二甲苯)：換缸/5mins，共三次
Rehydration:
2. 100% alcohol 1min ×2 次
3. 95% alcohol 1min
4. 80% alcohol 1min
5. 自來水水洗 1min</td><td>Xylene 第一缸使用至變色(淡黃) 或浮蠟，即可丟棄，將第二缸補至第一缸。

Rehydration: 還水、wash xylene</td></tr>
<tr><td colspan="2">以夾子夾取將 slide 放至耐熱 rank 上，浸泡在 Trilogy (20X dilute with D.W) → Ag retrieving (加蓋塑膠容器) → 不可 reuse

放鐵盤上，置入高壓滅菌鍋內，121℃，15mins
(自來水加至管線下即可)</td><td>達到 121℃才開始計時，在進入"Dry"模式前關閉滅菌鍋，洩壓。(全程約 30 mins)
鐵盤可避免液體濺出腐蝕機器管路。</td></tr>
<tr><td colspan="2">置入另一缸 Trilogy (先以微波爐加熱至 80℃) 10mins
→ 可 reuse 10 次，丟棄前可移至前一步驟使用。</td><td>分次加熱，避免沸騰溢出</td></tr>
<tr><td>Cold PBS 10mins</td><td>Cold TBS 10mins</td><td>Buffer 可 reuse 到最後</td></tr>
<tr><td colspan="2">3% H_2O_2 (10x dilution) in methanol，RT 15mins
→ 現配置冰上避免揮發，以 dropper cover slide
→ 將玻片盒盒蓋蓋上，避免揮發，盒下須加自來水，保持盒內濕度。
  </td><td>Peroxidase blocker: 去除內源性 Peroxidase，避免造成呈色偽陽性</td></tr>
</table>

PBS washing (過三缸)	TBST washing (過三缸) ***加一抗後，每次 wash 5 mins，共三次。**	
先以蠟筆畫線圍住組織切片，再加 blocker		ImmEdge pen (vector)
Mouse Ig blocking reagent RT 1hr	Power Block RT 10 mins **(1hr)** (BioGenex, Cat. HK085-5K) → 10X dilution，不可 reuse	勿過多，覆蓋過組織即可，玻片盒下須加水，避免玻片乾燥
Block 後不需 wash，玻片略倒乾直接加 1° Ab，置於玻片盒 4℃ O/N → 現配，use antibody dilution (Ventana, Lot. 556140) 		150µl/ slide **加一抗後的步驟，絕對不可讓玻片乾掉，避免抗體非專一性黏著於玻片上。**
MOM (mouse on mouse)	**傳統 IHC (rabbit on mouse)**	**notice**
PBS washing (過三缸)	TBS washing (過三缸)	Wash 可用力些
Biotinylated anti-mouse IgG reagent RT 10 mins	---	
---	Super enhancer RT 1hr (Cat. QD420-YIKE, polymer HRP IHC detection system, BioGenex) ***取 98.1 new kit**	最好避光操作，置於玻片盒
PBS washing (過三缸)	TBS washing (過三缸)	
Avidin-based detection system	Poly-HRP reagent(kit) RT 1hr	最好避光操作，置於玻片盒
PBS washing (過三缸)	TBS washing (過三缸)	
	AEC 呈色 **(1min)**	避光操作，置於玻片盒
TBST wash，以 Hemotoxylin 3-5min 背景染色 → (No. 3008-1, Muto Pure Chemicals)		加完避光操作，再開始計數 3 mins
放至流動自來水下，RT 10mins		置於玻璃缸內
置於玻片架上，以冷風吹乾，避免水分造成封片破裂		
封片： 在蓋玻片上滴一滴封片膠，載玻片斜角朝下蓋上蓋玻片，利用重力壓開封片膠，若有氣泡需推開。 (Aqueous mounting medium—without xylene, Dako)		全程須避免氣泡產生，否則會使封片破裂。
Reference:		

1. Liang CT *et al.*, A non-biotin polymerized horseradish peroxidase method for the IHC diagnosis of canine distemper virus infection. J Comp Pathol. 136:57-64, 2007 (SCI).
2. Liang CT *et al.*, IHC diagnosis of mouse hepatitis virus and Mycoplasma pulmonis infection with murine antiserum. J Comp Pathol. 131:214-220, 2004 (SCI).
3. 梁鍾鼎，王琇真，劉振軒。改良式免疫組織化學染色技術應用於福馬林固定或酒精固定後石蠟包埋豬組織之研究。中華獸醫誌 26:213-222，2000。

***MOM detection system: VECTOR MOM immunodetection kit (Basic Cat. No. BMK-2202)**

CURRICULUM VITAE

David, Chung-Tiang Liang

I. Basic Information

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II. Education

Institution	Nation	Department/Program	Degree	Dates
Veterinary Medicine	Taiwan/ROC	National Taiwan University Department of Veterinary Medicine	Ph. D. program	From 2002 / 9 to 2011 /
Veterinary Medicine	Taiwan/ROC	National Taiwan University Department of Veterinary Medicine	M.S.	From 1985 / 9 to 1988 / 7
Veterinary Medicine	Taiwan/ROC	National Taiwan University Department of Veterinary Medicine	D.V.M.	From 1980 / 9 to1985 / 7

III. Current Position and Working Experience

Institution	Department/Program	Position	Duration
Current Position:	National Laboratory Animal Center, National Applied Research Laboratories	Chief assistant of Director Office	From 2008/ 11 to now /
Past Experiences:	National Laboratory Animal Center, National Applied Research Laboratories	Associate Research Fellow /Attending Veterinarian	From 2002/ 3 to now /
	National Laboratory Animal Center, National Applied Research Laboratories	Assistant Researcher	From1991 / 10 to 2002 /2

IV. Fields of Expertise

1. Veterinary Medicine (Internal medicine and surgery of pet animal)
2. Laboratory Animal Medicine (Health monitoring program and daily inspection)
3. Veterinary Pathology (Swine disease, exotic animal, dog and cats)
4. Laboratory Animal Disease (Infectious and neoplastic disease, GEM pathology phenotyping)
5. AAALAC accreditation(Three time experiences as an attending veterinarian on site visit)
6. Molecular pathology and related techniques(IHC, ISH, IFA, RT-PCR, Cell culture, Virus isolation, Phylogenetic analysis, animal bleeding and anesthesia)

V. Membership and Certificate

1. Chinese-Taipei Society of Laboratory Animal Sciences
2. Certificated Veterinary Pathologist, Chinese Society of Veterinary Pathology
3. Chinese Society of Comparative Pathology
4. Certificated Veterinarian, Taiwan, ROC
5. IACUC member of National Laboratory Animal Center (Taipei and Tainan branch of NLAC), National Health Research Institute (NHRI) and Fu Jen Catholic University.

VI. Major Training

1. Pathology of Genetically Engineered Rodents and Aquatic Species, an Introduction to Phenotyping Workshop, Covance Laboratory Learning Center, USA, 2007.
2. 7th Annual Workshop on the Pathology of Mouse Models for Human Disease. Cornell University, Ithaca, NY, 2008.
3. The Rodent Pathology Phenotyping Workshop, Taiwan (Coordinated and leaded by Johns Hopkins University, School of Medicine , Baltimore, Rodent Phenotyping Core, Dr. Cory Brayton), 2009.
4. American veterinary pathologist residency training, (Baltimore, Jones Hopkins Medical School, Phenotyping Core), 2010 (July 28, 2010-Dec 18, 2010).

VII. Research Honors

1. 2009 Taiwan Vet J annual best paper award" Isolation and the phylogenetic analysis of canine distemper viruses in Taiwan. Taiwan Vet J 34(4), 198-210, 2008. "
2. 1995 National Science Council annual project award" Congenital abnomalities in piglets from a purebred swine herd. J Chin Soc Vet Sci 21：183-195, 1995", USD 3,000
3. 1997 National Science Council annual project award" The occurrences of

spontaneous tumors in SFP rats and mice. J Chin Soc Vet Sci 23：531-545,1997.”, USD 3,000

4. 1998 National Science Council annual project award” Case report: Histiocytic Sarcoma in a mouse. J Chin Soc Vet Sci 23：506-514, 1997.”, USD 3,000

VIII. Selected Publications:

A. Articles published in periodicals

1. **Liang CT**, Chueh LL, Pang VF, Lee KH, Liang SC, Yu CK, Lee CC, Lee YH and Liu CH. Improving detection of canine distemper virus in formalin-fixed, paraffin-embedded, tissues: using in situ hybridisation with integrated optical density to give a semi-quantitative assessment. Inter J Appl Res Vet Med. 2011.(prepared for submission)
2. **Liang CT**, Chueh LL, Brayton C, Pang VF, Wu SC, Huang SW, Liang SC, Yu CK, Lee CC, Liu CH. Canine Distemper in Taiwan from 2000-2009: co-infections, and use of RT-PCR and immunohistochemistry to detect tissue involvement in two groups of dogs. Inter J Appl Res Vet Med. 2011.(submitted)
3. Huang SW, Ho PY, Cheng PH, Ma WH, Chen YL, Liao SL, Chiu YY, Yu CK, Liang SC, **Liang CT**. Case report: High mortality associated with ketorolac treatment in CByB6F1 mice after embryo transfer surgery. Taiwan Vet J 36(4): 261-268, 2010.
4. **Liang CT**, Shih A, Chang YH, Liu CW, Huang YL, Huang WT, Kuang CH, Lee KH, Zhuo YX, Ho SY, Liao SL, Liang SC, Yu CK. Microbiological contamination of laboratory mice and rats in Taiwan from 2004 to 2007. J Amer Assoc Lab Anim Sci 48(4): 381-386, 2009. **(SCI)**
5. Zhuo YX, **Liang CT**, Lin YH, Chen JF, Tseng YS, Liao HL, Liang SC, Huang YT. Retrospective study of spontaneous lesions in the germfree mice and rats. Taiwan Vet J 35(2): 124-130, 2009. (in Chinese)
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December 17, 2010

Re: Dr. David Liang, Visiting Scientist

To Whom it may concern,

It was pleasure to have Dr. Liang as a visiting scientist training in our Department of Molecular and Comparative Pathobiology and Phenotyping Core from July 28 to December 18, 2010. Unfortunate delays related to visa procurement prevented his participation in our Mouse Pathobiology course July 12-16. But I am confident that his hard work and commitment to participating in all available training, phenotyping and pathology activities compensated for his missing that opportunity.

Training consisted primarily of hands on experience with our pathology and phenotyping cases in diverse biomedical research areas, many opportunities to attend lectures and seminars, and access to our comparative medicine and pathology training materials. He also attended the RTP Rodent Liver Pathology short course, and the American College of Veterinary Pathologists (ACVP) national meeting in Baltimore.

Dr. Liang demonstrates a high level of expertise and collegiality. NLAC is fortunate to have such a motivated, hard working and expert faculty member to support translational research efforts at NLAC. As I expected, his visit was an excellent experience for all involved, and I hope that it will lead to further productive collaboration among colleagues and institutions.

Respectfully and sincerely,

Cory Brayton, D.V.M. Diplomate, A.C.L.A.M., A.C.V.P.
Director, Phenotyping Core
Associate Professor, Molecular and Comparative Pathobiology

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