การแสดงออกร่วมของตัวรับเน็กติน-4 และแอนติเจนของเชื้อไวรัสไข้หัดสุนัข ในสุนัขที่ติดเชื้อตามธรรมชาติ

นาย วทัญญู ประทักษ์พิริยะ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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CO-EXPRESSION OF NECTIN-4 RECEPTOR AND CANINE DISTEMPER VIRAL ANTIGEN IN NATURALLY INFECTED DOGS.

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A Thesis Submitted in Partial Fulfillment of the Requirements

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Thesis Title	CO-EXPRESSION OF NECTIN-4 RECEPTOR AND CANINE DISTEMPER VIRAL ANTIGEN IN NATURALLY INFECTED DOGS.
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วทัญญู ประทักษ์พิริยะ : การแสดงออกร่วมของตัวรับเน็กติน-4 และแอนติเจนของเชื้อไวรัสไข้หัดสุนัข ในสุนัขที่ติดเชื้อตามธรรมชาติ. (CO-EXPRESSION OF NECTIN-4 RECEPTOR AND CANINE DISTEMPER VIRAL ANTIGEN IN NATURALLY INFECTED DOGS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.สพญ.ดร. สมพร เตชะงามสุวรรณ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ. น.สพ. ดร. นพดล พิฬารัตน์, 74 หน้า.

เชื้อไวรัสไข้หัดสุนัข เป็นเชื้อที่คุกคามสัตว์เลี้ยงลูกด้วยนมหลายชนิด โดยก่อให้เกิดอาการป่วยหลายระบบ ซึ่งตัวรับของเซลล์ที่มีความสำคัญคือ ตัวรับ SLAM (signaling lymphocyte activation molecule) ซึ่งจะแสดงออกอยู่บนเซลล์ภูมิคุ้มกัน ในขณะที่เชื้อไวรัสสามารถติดเข้าไปยังเซลล์ที่ไม่ได้แสดงออกของตัวรับดังกล่าว อาทิ เซลล์เยื่อบุ และเซลล์ในระบบประสาทส่วนกลาง ทั้งนี้มีการค้นพบตัวรับชนิดใหม่ที่มีความจำเพาะต่อเชื้อไวรัสไข้หัดสุนัข คือ ตัวรับเน็กติน-4 ดังนั้นจุดประสงค์ในการศึกษาครั้งนี้คือ ตรวจสอบการกระจายตัวของตัวรับเน็กติน-4 ในเนื้อเยื่อของสุนัขปกติ และการแสดงออกร่วมกันของเชื้อไวรัสไข้หัดสุนัขและตัวรับเน็กติน-4 ในเนื้อเยื่อของสุนัขที่ติดเชื้อตามธรรมชาติด้วยวิธีอิมมูนโนฮีสโตเคมี และ อิมมูนโนฟลูออเรสซีน นำชิ้นเนื้อเยื่อของสุนัขฝังพาราฟิน จำนวน 20 ตัวอย่างจากประเทศไทยและประเทศเวียดนาม มาทำการศึกษารอยโรคทางจุลพยาธิวิทยา และศึกษาด้วยการย้อมอิมมูนโนฮีสโตเคมีที่จำเพาะต่อเชื้อไข้หัดสุนัขในหลายๆอวัยวะ ทั้งนี้เนื้อเยื่อจากสมองได้ถูกนำมาย้อมทับด้วยแอนติบอดีที่จำเพาะต่อเซลล์แต่ละชนิดเพื่อจำแนกชนิดของเซลล์ในสมอง หลังจากนั้นนำอวัยวะต่างๆมาศึกษาโดยการย้อมอิมมูนโนฮีสโตเคมีที่จำเพาะต่อตัวรับเน็กติน-4 และย้อมทับด้วยแอนติบอดีที่จำเพาะต่อเชื้อไข้หัดสุนัข และจำเพาะต่อเซลล์ชนิดต่างๆในสมอง ผลการตรวจพบรอยโรคที่มีความจำเพาะต่อโรคไข้หัดสุนัข โดยเฉพาะก้อนอินคลูชั่นในไซโตพลาสซม หรือในนิวเคลียสของเซลล์ ในอวัยวะต่างๆ เมื่อศึกษาด้วยวิธีอิมมูนโนฮีสโตเคมี และอิมมูนโนฟลูออเรสซีน พบว่ามีการติดเชื้อของเซลล์หลากหลายชนิดในสมอง พบการแสดงออกร่วมกันของตัวรับเน็กติน-4 และเชื้อไข้หัดสุนัข ในเซลล์เยื่อบุต่อม เซลล์เยื่อบุชนิดต่างๆ และเซลล์ประสาท ทั้งนี้จากการย้อมด้วยแอนติบอดีที่จำเพาะต่อเซลล์ต่างชนิดกันในสมอง สามารถยืนยันได้ว่าตัวรับเน็กติน-4 มีการแสดงออกในเซลล์ประสาท การย้อมด้วยอิมมูนโนฟลูออเรสซีนก็มีการแสองออกของตัวรับและไวรัสในอวัยวะดังที่กล่าวมา ดังนั้นจึงสามารถสรุปได้ว่าเชื้อไข้หัดสุนัขสามารถใช้ตัวรับเน็กติน-4 เป็นตัวรับบนเซลล์ ที่มีความสำคัญ ในการเข้าสู่เซลล์ของสัตว์ป่วย โดยเฉพาะเซลล์เยื่อบุและเซลล์ประสาท การค้นพบครั้งนี้นำไปสู่การศึกษาทางพยาธิกำเนิดของโรคไข้หัดสุนัขในเชิงลึก

ภาควิชา : .....พยาธิวิทยา……..... ลายมือชื่อ …….....

สาขาวิชา : .....พยาธิวิทยาทางสัตวแพทย์..... ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา : ……2555…… ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม .....

##5375560131 : MAJOR VETERINARY PATHOBIOLOGY
KEYWORDS : CANINE DISTEMPER VIRUS / NECTIN-4 RECEPTOR / DOUBLE IMMUNOHISTOCHEMISTRY / IMMUNOFLUORESCENCE

WATANYOO PRATAKPIRIYA : CO-EXPRESSION OF NECTIN-4 RECEPTOR AND CANINE DISTEMPER VIRAL ANTIGEN IN NATURALLY INFECTED DOGS. ADVISOR : ASST. PROF. SOMPORN TECHANGAMSUWAN, Ph.D., CO - ADVISOR : ASSOC. PROF. NOPADON PIRARAT, Ph.D., 74 pp.

Canine distemper virus (CDV) is a virulent infected virus which threatens many mammalian species rendering multisystemic symptoms. The crucial cellular receptor for this virus is signaling lymphocyte activation molecule (SLAM) which expressed on immune cells. It can infect in silencing SLAM expression cells including epithelium and central nervous system. The newly cellular receptor for Measles virus (MV), the closely related morbillivirus, has been investigated so-called nectin-4 receptor. The aims of this study were to investigate the distribution of nectin-4 receptor in canine tissues and to exhibit the co-localization of nectin-4 receptors and CDV in naturally infected canine tissues by double immunohistochemistry and immunofluorescence. The 20 paraffin-embedded canine tissues were collected from Thailand and Vietnam. The routine histopatological process and CDV immunochemistry staining were done in various infected organs. For brain, the double immunohistochemistry between CDV and several brain markers were performed. Subsequently, the dual immunohistochemistry of nectin-4 receptor with CDV and several brain markers were processed. The pathognomonic lesions especially eosinophillic intranuclear or intracytoplasmic inclusion bodies were seen in various organs. The CDV immuno-labeling cells were also noticed abundantly. In brain, the different cell infection was seen. There were co-harboring CDV and nectin-4 cells in glandular cells, various epithelium and neuron using both methods. The brain markers confirmed that nectin-4 expressed in neurons. The co-expression was also seen as same organs by immunofluorescence method. In conclusion, CDV also utilize the nectin-4 as an alternative potential cellular receptor through host cells especially various epithelial cells and neuronal cells. These findings might play a key role to elucidate the pathogenesis of canine distemper infection in depth.

Department : …..Veterinary Pathology…… Student's Signature……
Field of Study : …Veterinary Pathobiology.... Advisor's Signature……
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CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

LIST OF GRAPHS

LIST OF ABBREVIATIONS

^{O}C	=	degree Celsius (centrigrad)
CD	=	cluster of differentiation protein
CDV	=	canine distemper virus
CNS	=	central nervous system
CPE	=	cytopathic effects
DAB	=	3'3-diaminobenzidine
GFAP	=	glial fibrillary acidic protein
H&E	=	hematoxylin and eosin staining
Iba-1	=	ionized calcium binding adapter molecule 1
IHC	=	immunohistochemistry
IFC	=	immunofluorescence
kDa	=	kilodalton
MV	=	measles virus
NeuN	=	neuronal nuclei protein
PBS	=	phosphate buffer saline
SLAM	=	signaling lymphocyte activation molecule
TBS	=	tris buffered saline

CHAPTER I

INTRODUCTION

Canine Distemper is a serious fatal infectious disease which threatens worldwide range of mammal species (Deem et al., 2000). The causative pathogen is canine distemper virus (CDV) which belongs to genus *Morbillivirus* of the Paramyxoviridae family. The closely related viruses in this genus have been reported including measles virus (MV) in humans. CDV is a lipid enveloped, negative single-stranded RNA virus containing non-overlapping six important encoded structural protein genes; hemagglutinin (H), fusion (F), nucleocapsid (N), phosphoprotein (P), matrix (M) and large (L) protein gene. It also composes of 2 non-structural proteins, C and V encoded genes, which related to P gene. N protein composes of conserved region for transcription and replication. Besides, the H and F proteins serve as initially viral surface proteins which mediate receptor binding and promote fusion, respectively, through the host cells (von Messling et al., 2004).

CDV transmit by inhalation and contacting discharges of infected animals. It primarily replicates in lymphocytes and macrophages in oral and upper respiratory tracts and then disseminates (primary viremia), to other lymphoid organs. Subsequently, the hemopoietic tissues including spleen, lymph nodes and associated lymphoid tissues in several organs, which CDV tropism, are infected resulting in generalized lymphoid depletion and lymphopenia. Secondary viremia is evident in epithelial cells of respiratory, urinary and gastrointestinal system as well as central nervous system. The multisystemic symptoms are shown as respiratory, gastrointestinal, neurological sign with immunosuppressive rendering secondary infection from other pathogens (Beineke et al., 2009). Despite the success of using the live-attenuated CDV vaccine for worldwide controlling the disease, CDV still remains one of the majority contagious diseases in many countries including Thailand.

Though our knowledge, the CDV initial receptor has been described, a membrane glycoprotein called signaling lymphocyte activation molecule (SLAM) or CD 150, which also functioning as other Morbilliviruses' receptor (Tatsuo et al., 2001). These molecules mostly express on immune cells such as dendritic cells, macrophages and lymphoid tissues (Sidorenko and Clark, 2003). In contrast, CDV is able to infect SLAM-independent expressed cells such as epithelial lining cells and cells in the nervous system. Thus, CDV might have capacity to other cellular receptors which remained poorly elucidated. Alternative cell receptors have been reported as related to Morbillivirus. CD46, a membrane cofactor protein, is rather MV vaccine strain tropism receptors (McQuaid and Cosby, 2002). Heparin sulfate (HS) molecules, which only express in human 293 cell line, have been revealed as receptor of CDV (Fujita et al., 2007). CD9 have been described as cell to cell fusion and syncytial formation associated protein (Schmid et al., 2000). Recently, there are studies exhibiting the other receptors of CDV in chicken embryo fibroblast and Vero cells, as the 57-kDa and the 42-kDa protein, respectively (Chen et al., 2011). Hence, further identification of the cellular receptor for CDV should be performed.

Nectins, the member of immunoglobulin superfamily (ISF), are known as a Ca^{2+} independent immunoglobulin (Ig)- like cell-cell adhesion molecules (CAM). It comprises four members; nectin-1,-2,-3, and -4. The essential function of nectins is cell-cell organizing intracellular junction by using many activities (Takai et al., 2003). Interestingly, nectins have been identified as viral receptors which expressed broadly in human tissues. The molecular structure of nectins is homologous to Poliovirus receptor (PVR; CD155). Nectin-1 and -2 serve as Herpes simplex virus (HSV) type 1, type 2 and alpha herpes virus receptors, through the host cell and cell-to-cell viral spread (Cocchi et al., 2001). Interestingly, nectin-4 has been recently reported as an epithelial cell receptor for Measles virus (MV) *in vitro* by usage of H protein as similar as other receptors; SLAM, CD46 (Noyce et al., 2011). In addition, tracheal epithelium of inoculated MV in primates revealed nectin-4 positive expression (Mühlebach et al., 2011).

Based on our knowledge, there is no report of nectin-4 serving as receptor for other species of virus belonging to genus Morbillivirus, especially canine distemper virus. The aim of this study is to elucidate the expression of nectin-4 receptor which may possible act as a cellular receptor for CDV in naturally infected dogs by using double immunohistochemistry staining.

Objectives of Study

1. To investigate the distribution of nectin-4 receptor in tissues of non-CDV infected dogs by immunohistochemistry staining.
2. To investigate the co-localization of nectin-4 receptors and CDV infection in various tissues of naturally CDV-infected dogs by double immunohistochemistry and immunofluorescence.
3. To identify the cell type which co-expressed Nectin-4 and CDV viral antigen particularly in affected brain tissues.

CHAPTER II

LITERATURE REVIEW

Canine distemper (CD) is a serious fatal infectious disease that threatening wide range of mammal species. It is not only a worldwide occurring disease among domestic dogs, but also those in captive or free ranging wildlife animals and non-human primates (Deem et al., 2000). The causative pathogen of this immunocompromised disease is canine distemper virus (CDV) which belongs to genus *Morbillivirus* of the Paramyxoviridae family. In addition, the closely related viruses in this genus have been reported as highly infectious diseases including Measles virus (MV) in humans and primates, Phocine distemper virus (PDV), Rinderpest virus (RPV) and Peste des petits ruminant virus (PPRV).

Despite using of live attenuated CDV vaccines for disease controlling and prevention, CD is still one of the majority infectious diseases in many countries including Thailand. Recently, the prevalence of several associated canine respiratory viruses have been investigated during 2008-2009 in Thailand. The results elucidated, that CDV were incidence approximately 2.94% in healthy dogs and 1.83% in dogs with respiratory clinical signs (Posuwan et al., 2010). The unsuccessful disease protection following vaccine administration may count on several factors such as improper vaccination, remaining high titer of maternal immunity or CDV infection prior vaccination. Moreover, the vaccinated dog also could be infected with CDV field strain (Keawcharoen et al., 2005).

Canine distemper virus properties

Canine distemper virus is a lipid enveloped, negative single-stranded RNA virus with approximately 150-300 nm spherical virion. Its genome is 15.7 kb in length and contains non-overlapping six important genes which encode the structural genes; haemagglutinin (H), fusion (F), nucleocapsid (N), phosphoprotein (P), matrix (M) and large

(L) proteins (Figure1). Indeed, it also consists of 2 non-structural genes, C and V encoded genes, which associated to the P gene. The N-, P- and L- proteins are helical nucleocapsid core, called replication complex. The M protein anchors nucleocapsid and glycoproteins. Besides, the H and F proteins serve as viral surface proteins which mediate receptor binding and promote fusion, respectively, through the host cells for entry and exit. The H protein, well known as an important attaching glycoprotein with cellular receptor, shows a variability of amino acid sequence as a consequence of inducing humoral immune of host (von Messling et al., 2004).

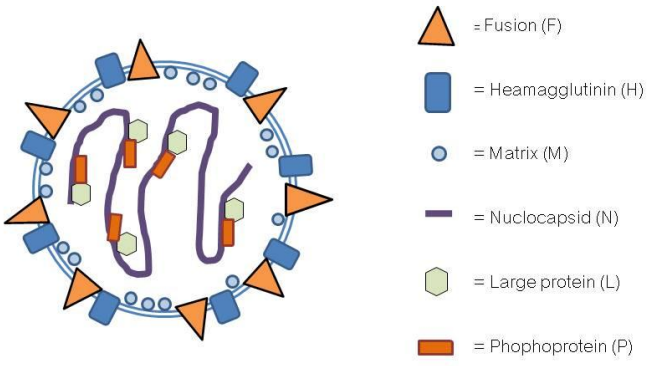


Figure 1: Basic structure of canine distemper virus

Clinical manifestations and pathogenesis

CDVs transmit by inhalation and contacting discharges of infected animals. Through aerosol infection, droplets of viral particles primarily replicate in oral and upper respiratory lymphocytes and macrophages in where the viral particles immensely and rapidly replicate. The viral particles predominately attach with specific SLAM receptor via H protein through tropism host cell. Then the viral progenies disseminate to other lymphoid organs through

lymphatic and hematogenous route via peripheral blood mononuclear cells (PBMCs) causing the first viremia. Subsequently, the peripheral lymphoid tissues; spleen, lymph nodes and mucosal-associated lymphoid cells in several organs become infected, so that CDVs induce lymphoid depletion and lymphoid apoptosis leading to lymphopenia and inhibition of lymphoproliferation and resulting in generalized immunosuppression. The secondary viremia is incident in epithelial cells of respiratory, urinary and gastrointestinal system and also central nervous system including glia cells, astrocytes, oligodendrocytes and neurons (von Messling et al., 2004). However, the pathway of CDV infection in brain tissue remained unclear. It has been explained that the mechanisms are either (i) hematogenous route by infected peripheral blood mononuclear cells (PBMCs) invasion through choroid plexus and cerebral blood vessels, or (ii) an anterograde pathway by utilizing olfactory bulb as a primarily viral target organ and invading to cerebellum, hippocampus and brainstem (Rudd et al., 2006). Various incubation periods, severity and clinical manifestation, which depend on susceptibility, age and immune status of host, have been described. The infected animals exhibit variable clinical signs. At first, they show non-specific clinical characters e.g. lethargy, anorexia, dehydration and fever. Then, mainly symptoms are shown in respiratory (nasal and ocular discharge), gastrointestinal sign (catarrhal diarrhea) with immunosuppressive rendering secondary infection from other pathogens. Some reveals skin lesion (skin rash, hyperkeratosis of nasal planum and foot pad) and neurological sign (convulsion and myoclonus). Histologically, the distinguished lesion is eosinophillic intranuclear or intracytoplasmic inclusion bodies in infected cells of many organs; for example lymphocytes in lymphoid organs, bronchiolar epithelial cells, transitional cells of urinary bladder, glia and neuron cells. The multisystemic lesions are revealed for instance interstitial pneumonia, bronchiolitis and generalized lymphoid depletion. Besides, the responses of secondary bacterial infection are shown e.g. purulent bronchopneumonia. In brain, nonsuppurative encephalitis and demyelination are seen (Beineke et al., 2009).

Associated cellular receptor for CDV

Though our knowledge, the CDV initial receptor has been described, a membrane glycoprotein called signaling lymphocyte activation molecule (SLAM, CD150). Moreover, SLAM also serves as other Morbilliviruses receptors (Tatsuo et al., 2001). This molecule is mostly expressed on immune system cells such as dendritic cells, macrophages, immature thymocytes and lymphoid tissues (Sidorenko and Clark, 2003). In human, the SLAM are expressed in white blood cells and germinal center of lymphoid cell and lacked in epithelial cells, by immunohistochemical labeling (McQuaid and Cosby, 2002). In several organs of dogs including lung, stomach, intestine and transitional cell of the urinary bladder, the expression and distribution of SLAM positive cells, which resembled lymphocyte and macrophage, are identified. Interestingly, the up-regulation of SLAM receptors in many organs is shown during acute CDV infection indicating the progression of viral amplification, whereas MV-infected cells in human showed the down-regulation of SLAM receptors (Techangamsuwan et al., 2009[2]; Wenzlow et al., 2007). On the other hand, CDV is able to infect SLAM-independent expressed cells such as epithelial cells and central nervous system. Moreover, Vero cells, principle cells for Morbillivirus isolation including CDV, do not express SLAM. Thus, CDV might have capacity to other cellular receptors which poorly elucidated.

Alternative cellular receptors have been reported as related Morbillivirus receptors. CD46, membrane cofactor protein, is rather MV vaccine strain tropism receptors and expresses in all nucleated cells (McQuaid and Cosby, 2002). However, only the neoplastic lymphoid cells of dog have been exhibited CD46 *in vitro*. Heparin sulfate (HS) molecules have been revealed as CDV receptor, despite that it expresses only in human 293 cell line (Fujita et al., 2007). CD9, a tetraspan transmembrane protein (TM4), has been described as CDV-induced cell to cell fusion leading to the syncytial formation, even though it has not been accepted as a CDV specific receptor (Schmid et al., 2000). Recent study exhibited

the other receptor of CDV in chicken embryo fibroblast and Vero cells, as 57-kDa and the 42-kDa protein, respectively (Chen et al., 2011).

Nectin receptor

Nectin-4 is the adhesion molecule with molecular weight approximately 55.5 kDa and encodes 510 amino acids. It belongs to nectins family that are the member of immunoglobulin superfamily (ISF), also known as a Ca^{2+} independent immunoglobulin (Ig)-like cell-cell adhesion molecules (CAM). The molecular structure of nectins is homologous to Poliovirus receptor (PVR; CD155) accordingly; it has been originally reported as poliovirus receptor related (PRRs) or poliovirus receptor-like protein (PVRLs). Nectin family classifies in four members; -1,-2,-3, and -4. All nectin, except the nectin-4, has two to three splicing variants (Reymond et al., 2001). Nectins distribute in several cells e.g., hematopoietic, neuronal, endothelial, and also epithelial cells (Irie et al., 2004). Nectins comprise three main molecular structures; (i) the extracellular domain which is immunoglobulin-like with three loop domains of V, C and C type, (ii) the transmembrane domain and (iii) the short cytoplasmic tail, respectively (Figure 2). Moreover, the terminal of cytoplasmic tail, except the nectin -4 and some variant splice of other nectin, have conserved motif of four amino acid residues (Glu/Ala-X-Try-Val) (Takai et al., 2003).

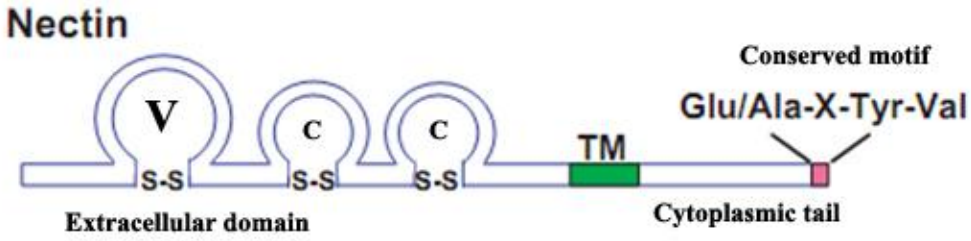


Figure2: The schematic of nectin including extracellular domain which is immunoglobulin-like loop domains of V, C and C type, the transmembrane (TM) domain and cytoplasmic tail. The terminal region is conserved motif of four amino acid residues, except nectin-4 (adapted from Takai et al., 2003).

The essential function of nectins is cell-cell organizing intracellular junction, especially in epithelial junctions, by using many activities. Formation of homophillic cis and trans-dimer of each nectins has been explained (Figure 3) whereas heterophillic trans-dimer constructions are restricted, for example nectin-4 is only hetero trans-dimer forming with V domain of nectin-1. The affinity of that hetero trans dimerization is stronger than homo dimer formation (Reymond et al., 2001). Moreover, the conserved motif of each nectin binds with PDZ domain of afidin, which an actin-filament binding proteins, leading to linkage of nectin with actin cytoskeleton. Though, without specific motif, nectin-4 still binds to PDZ domain by using carboxyl terminal amino acid; valine. Besides, activation of Cdc42 and Rac, small G proteins related signal transduction, promotes cell-cell adherens. There also combines with nectin-like molecules (Necls) that comprises immunoglobulin-like loop domain structures resemble in those of nectins, to construct cellular junction (Irie et al., 2004).

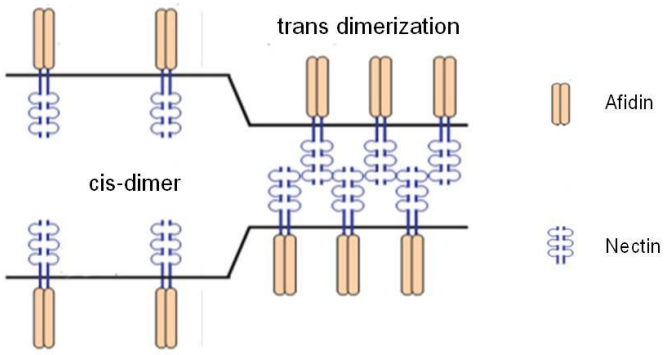


Figure 3: Formation of homophillic cis and trans-dimer of nectins family. Moreover heterophillic trans-dimer formation is constricted. The conserved motif of nectin binds with afidin (modified from Takai et al., 2003).

In addition, Herpes simplex virus (HSV) type 1, type 2 and alphaherpes virus mediate with nectin-1, known as herpes immunoglobulin receptor (HIgR) or herpes virus entry (HveC), through the host cell (Cocchi et al., 2001). That also allows entry of other alpha herpes viruses e.g. pseudorabies virus and bovine herpesvirus. Besides, nectin-2, called HveB, is also described as receptor for entry of clinical and laboratory HSV-1 and -2 strains. The mechanism of HSV binding has been reviewed that envelope glycoprotein D (gD) of HSV utilized nectin-1 and -2 receptor. Subsequently, trigger of membrane fusion is occurred (Krummenacher et al., 2004). Furthermore, nectin-1and -2 are also utilized for HSV cell to cell spread.

Nectin-4 has been intensively studied in human and showed that the expression is particularly in human epithelial cells including trophoblastic cells of placenta, hair and skin of human (Brancati et al., 2010), neuronal cells, bronchial epithelial cells, glandular epithelial cells of several organs and renal tubular epithelial cells (Human Protein Atlas Project, www.proteinatlas.org). Furthermore, it is broadly expressed in mouse and those embryo tissues hence it relates to embryogenesis (Reymond et al., 2001). In contrary, there is a single study reported that nectin-4 was hardly found in normal human tissues for instance, heart, lung, liver, kidney and trachea (Takano et al., 2009). However, the distribution of necti-4 on canine tissues has not been described yet. Interestingly, the role of nectin-4 associated with several disorders gains more attention. The ectodermal dysplasia-syndactyly syndrome (EDSS) in human is caused by the mutation of nectin-4 gene (Brancati et al., 2010). Usefully, nectin-4 receptor is rather a new tumor marker in many types of cancer including breast cancers (Fabre-Lafay et al., 2007), lung cancers (Takano et al., 2009) and ovarian cancers (DeRycke et al., 2010). Recently, only nectin-4 is reported as an epithelial cell receptor for Measles virus *in vitro* by binding with the viral H protein similarly with other receptors; SLAM, CD46 (Noyce et al., 2011). Moreover, the tracheal epithelium of MV inoculated experimental primate reveals a positively immunoreactivity against nectin-4 (Mühlebach et al., 2011).

CHAPTER III

MATERIALS AND METHODS

1. Animal tissue samples

Twenty paraffin embedded tissues of dogs were collected from Thailand (Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University) and Vietnam (Department of Pathology, Faculty of Veterinary Medicine, Hanoi University of Agriculture) since 2007-2011. Those were diagnosed as canine distemper virus infection by history, macroscopic and microscopic findings. In addition, some samples were positively confirmed CDV infection by RT-PCR assay. The two non-CDV infected dogs were used as negative controls and for studying the expression and distribution of nectin-4 in various organs.

2. Histopathology

Sections (4 μm thick) were cut from paraffin wax and routine Hematoxylin and Eosin staining. The histological results were analyzed descriptively for CDV infection by finding pathognomonic lesions. Additionally, histopathological findings of central nervous system were classified in three groups (adapted from Seehusen et al., 2007 and Amude et al., 2010), group I (no pathological lesion), group II (acute lesions; mild gliosis, eosinophillic inclusion bodies and mild demyelination), group III (chronic lesion; severe demyelination, non-suppurative polioencephalitis, diffuse eosinophilic inclusion bodies, gliosis, gitter cells invading and mononuclear perivascular cuffing).

3. Immunohistochemistry staining for CDV detection

The immunohistochemical CDV detection was a confirmation method for positivity sample. The paraffin-embedded tissues were prepared as 4 μm section on silane coated slide. Deparaffinization and rehydration were performed. They were subjected to heat-

induced antigen retrieval by using autoclave in citrate buffer pH 6. Subsequently, endogenous peroxidase blocking was performed by immersion in hydrogen peroxidase (3% in methanol) for 30 minutes at room temperature. Primary antibody was monoclonal mouse anti-CDV antibody (Monotope Virostat®, USA) at 1:200 dilution in 37 ^{O}C moist chambers for 40 minutes. Envision polymer (Dako, Denmark) was utilized as secondary antibody and incubated in the same condition. For each process between incubation, tissues were washed three times in PBS for 5 minutes at room temperature. The chromogen immunoreactivity was visualized by 3'3-diaminobenzidine (DAB; Sigma, USA) in horseradish peroxidase (HRP) system. Tissues were counterstained with Meyer's hematoxylin. The previous positive CDV tissue was used as a positive control.

4. Immunohistochemistry staining for nectin-4 detection

The non-CDV infected paraffin-embedded tissues were processed as mentioned above. Primary antibody was affinity-purified polyclonal goat antibody against human nectin-4 antigen (10 µg/ml dilution, R&D system, USA) incubation in moist chambers at 4 ^{O}C. Universal immuno-peroxidase polymer anti-goat (Histofine® simple stain MAX –PO (G); Nichirei, Japan) was secondary antibody and incubated in 37 ^{O}C moist chambers for 30 minutes. Tissues were visualized by DAB (Sigma, USA) in HRP system and counterstained with Meyer's hematoxylin. Positive control of nectin-4 was mouse's brain section (Reymond et al., 2001). The result of expression of nectin-4 in tissues was exhihited as descriptive analysis.

5. Double immunohistochemistry staining for CDV and Nectin-4 co-expression

Four-µm thick affected tissues including brain, lung, intestine, kidney and urinary bladder were deparaffinized, rehydrated and antigen retrieved by autoclave in citrate buffer pH 6. Blocking endogenous peroxidase and primary monoclonal mouse anti-CDV antibody were done as mentioned above. After that, Universal immuno-alkaline-phosphatase

polymer, anti-mouse and anti-rabbit (Histofine® simple stain AP (MULTI); Nichirei, Japan) was utilized as secondary antibody. Before using chromogen for visualization, sections were dipped into Tris Buffered Saline (TBS) and colorized by Fast red II in naphthol phostphate (Nichirei, Japan) in alkaline phosphatase system.

For double staining, the primary antibody was polyclonal goat antibody against human nectin-4 antigen (10 µg/ml dilution, R&D system, USA) and the secondary antibody was Universal immuno-peroxidase polymer anti-goat (Histofine® simple stain MAX –PO (G); Nichirei, Japan). Tissues were visualized by DAB (Sigma, USA) in peroxidase system and counterstained with Meyer's hematoxylin.

6. Double immunohistochemistry staining for CDV and brain markers

Four-µm thick affected brain in various part including cerebrum, cerebellum, mid brain and spinal cord were deparaffinized, rehydrated and antigen retrieved by autoclave in citrate buffer pH 6. Blocking endogenous peroxidase and primary brain marker antibody including rabbit polyclonal anti-neuronal nuclei (NeuN; 1:500; Millipore, USA) and anti-GFAP (Glial fibrillary acidic protein; 1:2,000; Dako, Denmark) were separately done in 37 ^{O}C moist chambers for 40 minutes. Additionally, selected slide without prior retrieval were incubated by rabbit polyclonal anti-Iba-1 (ionized calcium binding adapter molecule 1; 1:250; Wako Chemicals, USA), which specifically against microglia and macrophage, in 4 ^{O}C moist chambers for overnight. After that, Universal immuno-alkaline-phosphatase polymer, anti-mouse and anti-rabbit (Histofine® simple stain AP (MULTI); Nichirei, Japan) was utilized as secondary antibody. The chromogen for visualization was Fast red II in naphthol phostphate (Nichirei, Japan) in alkaline phosphatase system. For double staining, the mouse anti-CDV antibody and Envision polymer (Dako, Denmark),as secondary antibody,) were performed as mentioned above. Tissues were visualized by DAB (Sigma, USA) in HRP system and counterstained with Meyer's hematoxylin. The positive control of several brain markers was mouse brain.

7. Double immunohistochemistry staning of necin-4 and brain markers

To identify the specific brain cell type that expressed nectin-4, immunohistochemistry technique was done in serial section. As mentioned method, deparaffinization, rehydration, antigen retrieval and endogenous peroxidase blocking were performed. For each slides, primary brain marker antibody including rabbit polyclonal anti-neuronal nuclei (NeuN; 1:500; Millipore, USA) and anti-GFAP (Glial fibrillary acidic protein; 1:2,000; Dako, Denmark) were incubated in 37 ^{O}C moist chambers for 40 minutes. Conversely, rabbit polyclonal anti Iba-1 (ionized calcium binding adapter molecule 1; 1:250; Wako Chemiclas, USA) was used for incubation in 4 ^{O}C for overnight. Afterwards, Universal immuno-alkaline-phosphatase polymer, anti-mouse and anti-rabbit (Histofine® simple stain AP (MULTI); Nichirei, Japan) was utilized as secondary antibody. The chromogen immunoreactivity was visualized by Fast red II (Nichirei, Japan) in alkaline phosphatase system. The Nectin-4 antibody and secondary antibody of consensus IHC staining and chromogen revealing were preformed as mentioned in double staining. Subsequently, slides were counterstained with Meyer's hematoxylin.

8. Immunofluorescence (IFC) double labeling

The IFC protocol was adapted from Robertson and colleagues (2008) to definitely ensure co-harboring nectin-4 and CDV antigen. The chosen tissues (brain, lung, intestine, stomach, kidney and urinary bladder) were section, 4 micron thickness, on the coated slide. The slides were rehydrated through gradient alcohol and immersed in PBS for 5 minutes. Prior heat induced antigen retrieval was done by microwave. The blocking one agent (Nacalai Tesque; Japan.) was incubated in room temperature for 10 minutes for blocking non-specific antigen. First primary antibody (CDV; as mentioned above) and 10 µg/ml diluted immunofluorescent secondary antibody; Alexa Fluor donkey anti-mouse IgG 594 (Invitrogen; USA), were incubated for 1 hour in 37 ^{O}C and RT respectively. For each steps,

immersion into PBS for 5 minutes 3 times was done. The incubation of IFC antibody was prevented from light by placing in a dark box. Subsequently, second primary antibody (nectin-4; as mentioned above) and 10 µg/ml diluted Alexa Fluor donkey anti goat IgG 488 (Invitrogen; USA) were performed without light for overnight 4 ^{O}C and 1 hour in room temperature, respectively. The stained sections were sunk into PBS before covered by 25 µl Vectersheild H-1500 with counterstained 4', 6-diamidino-2-phenylindole (DAPI) and cover slip. There were kept in dark box and stored in 4 ^{O}C for hardening. The LSM 700 confocal laser scanning microscopy (Carl Zeiss) was used for visualizing fluorescence in samples immediately with ZEN 2010 LSM software.

9. Data analysis

The histological and immunohistochemical results were discussed in descriptive analysis as semi-quantitative including the areas of affected organs, severity of lesions, distribution and co-localization of CDV antigen and nectin-4 receptor as well as the tropism of CDV infection in nervous tissue.

CHAPTER IV

RESULTS

Clinical data

Twenty necropsied cases (13 dogs from Thailand and 7 dogs from Vietnam) were retrospectively studied. Accumulating clinical signalments were retrieved; however, some of them were fragmentary due to missing data (Table 1). The susceptible age of infected dogs ranged from 2 month to 5 years with various breeds. The clinical symptoms suggesting CDV infection were presented in most ill dogs predominantly involved the neurological system (75%; convulsion and myoclonus) and respiratory system (66.67%; oculonasal discharge and lung edema). Besides, conjunctivitis, pustular dermatitis and diarrhea were occasionally presented in some cases.

To ensure the CDV infected status, the selected samples from Thailand (TH 09/03, TH 10/01, TH 10/02, TH 10/03, TH 10/04, TH 10/05, TH 11/01, TH 11/02 and TH 11/03) and Vietnam (VN 11/01, VN 11/02 and VN 11/03) were determined by RT-PCR targeting phosphoprotein (P) gene (Radtanakatikanon, 2011.). In contrast, the negative samples (TH07/02 and TH07/03) were determined by using immunohistochemical method to confirm non CDV infection.

Table 1 General signalments and clinical signs of necropsied case

Case no.	Breed	Age	Sex	Clinical sign	PCR
TH 07/01	Pug	MD	M	Cough, nasal discharge, depress, anorexia	ND
TH 08/01	MD	3 m	F	Nasal discharge, convulsion and negative for CDV antigen test kit	ND
TH 09/01	Chi hua hua	MD	M	Serous nasal discharge, nervous sign, blindness	ND
TH 09/02	Yorkshire terrier	MD	F	MD	ND
TH 09/03	Rottwiler	3 m	M	Convulsion, myoclonus, presence of inclusion body in red and white blood cells	+
TH 10/01	Mongrel	MD	F	MD	+
TH 10/02	Pomeranian	2 m	F	Serous nasal discharge, dyspnea, lung edema, diarrhea	+
TH 10/03	Miniature pincher	5 y	F	Oculonasal discharge, increased lung sound, ataxia, uveitis, pustular dermatitis	+
TH 10/04	Chi hua hua	MD	M	MD	+
TH 10/05	Golden retriever	2 m	F	Purulent oculonasal discharge, convulsion, conjunctivitis,	+
TH 11/01	Golden retriever	2 m	MD	MD	+
TH 11/02	Saint Bernard	1 y	M	Dyspnea, convulsion, vomit, chronic dermatitis	+
TH 11/03	Dachshund	1 y 5 m	MD	Lung edema, dyspnea, diarrhea	+
VN 11/01	Mongrel	MD	MD	Fever, convulsions, drooling	+
VN 11/02	Mongrel	MD	MD	Fever, convulsions, drooling	+
VN 11/03	Mongrel	MD	MD	Fever, convulsions, shaking, drooling	+
VN 12/05	MD	MD	MD	MD	ND
VN 12/08	MD	MD	MD	MD	ND
VN 12/09	MD	MD	MD	MD	ND
VN 12/27	MD	MD	MD	MD	ND

MD = missing data; M = Male; F = Female; m = months; y = years, ND = not done

TH = Thailand; VN = Vietnam

Microscopic examination

Following routine histological process, as expected, those samples individually revealed various pathothological lesions of CDV infection in many tissues which were summarized in table 2.

The infected dogs mostly showed respiratory symptoms. The bronchointerstitial pneumonia (47%; 8/17) was showed with or without invading massive neutrophils (Figure 4a). The interstitial pneumonia was 35% (6/17) with fibrinosuppurative exudate. Furthermore, not only intracytoplasmic, but also intranuclear eosinophillic CDV inclusion bodies were notably seen in bronchial, bronchiolar epithelial cells and pulmonary alveolar macrophages (PAM) of all samples (Figure 4b). The syncytial formation in bronchiolar epithelium and proliferation of PAM were also detected (Figure 4c-d).

For gastrointestinal tract tissue, the intestines were mostly revealed catarrhal enteritis (57%; 8/14) with necrotic villi (Figure 5a). The infiltrated inflammatory cells; lymphocyte and plasma cells were also presented in 43% (from fourteen samples). The eosinophillic inclusion bodies were detected in enterocyte and lamina propria (Figure 5b). In addition, two gastric samples from four tissues were infiltrated by lymphocytes (50%; 2/4) and one sample (TH10/01) revealed inclusion bodies in gastric glandular cells (Figure 6).

The lymphoid cells in various organs including spleen, lymph node, tonsil and Payer's patch in large intestine, showed majority of mild to marked lymphoid depletion (60%; 9 samples from 15 specimens; Figure 7a-b). Moreover, there were inflammatory cells infiltration such as histiocytes in spleen (20%; 3/15) and lymph node (50%; 4/8), neutrophils (20%; 3/15 for spleen and 25%; 2/8 for lymph node). The distinguishing eosinophillic intranuclear inclusion bodies were noticed in all samples from lymphoid tissues (Figure 7c).

For urinary bladder, four samples showed CDV inclusion bodies (67%) in transitional cells epithelia (Figure 8) accompanied with lymphocytes and plasma cells infiltration in two samples. The kidney sample exhibited almost mild pathological change including

congestion, except remarkable lesion in renal pelvis. There were evident of eosinophillic intranuclear inclusion bodies in renal pelvis epithelium (43%; 6/14; Figure 9).

For the neurological system, nineteen brains were characterized according to the previous reports with some modifications (Seehusen et al., 2007; Amude et al., 2010).Based on histopathology combined with immunohistochemistry results, they were divided into three groups (Figure 10a-c). Group I; histological finding showed no pathological lesions while the evidence of CDV immunopositivity in many cells was detected as seen in six samples (TH09/02, TH10/02, TH10/04, TH11/01, VN12/05, VN12/09). Group II; acute group characterized by mild gliosis, eosinophillic inclusion bodies in neuron and glial cells, and mild demyelination lesion as shown in nine samples (TH08/01, TH09/03, TH 10/05, TH11/02, TH11/03, VN11/02, VN11/03, VN12/08, VN12/27). For chronic group (Group III), the severe demyelination, leukoencephalomalacia, non-suppurative polioencephalitis, meningoencephalitis, distinguished eosinophilic inclusion bodies in neurons and glial cells, gliosis, gitter cells invading and mononuclear perivascular cuffing were overtly evidenced (TH07/01, TH09/01, TH10/01, TH10/03; Figure 10d-f). The conclusion of microscopic diagnosis of CDV induced lesions is demonstrated in Table 3.

Immunohistochemistry stanning

1) Single immunohistochemisty

1.1) Canine distemper virus

Total specimens were confirmed for viral infection by immunohistochemical procedure utilizing DAB as a chromogen showing brownish when positive. Many samples were histologically no remarkable lesions, while expressed immunoreactive of CDV antigen especially in central nervous tissues (TH09/02, TH10/02, TH10/04, TH11/01, VN12/05 and VN12/09).

Immunohistochemically, all samples, the lymphoid tissues in various organs including spleen, lymph node, tonsil and also Payer's patch, exhibited dispersedly CDV expressing lymphocytic cells. The viral antigen were visualized and located in nuclei and cytoplasm of infected lymphocytes and histiocytes.

In the brain, various infected cells were morphologically visualized by DAB staining (Figure 11a-f). The most positive cells were astrocytes in all infected groups. CDV labeling astrocye-like notably disseminated in many parts of brain including cerebrum, cerebellum, mid brain and spinal cord, whereas the CDV labeling neuron-like cells were sporadic seen. Moreover, the ependymal cells, choroid plexus, Purkinje's cells and meningeal cells were colorized in some positive samples. The distribution of positive cells was observed in white matter and grey matter of each part of brain. The distribution and intensity of immunopositive CDV antigen depended on stage of infection (Table 4).

The exhibited viral antigens were also viewed in other organs. All of seventeen lungs from twenty samples showed immunoreactivity in varied cells especially bronchial and bronchiolar epithelium (Figure 12). The pulmonary alveolar macrophages (PAM) and pneumocytes were also positively locateded in nucleus and cytoplasm. Total samples from gastrointestinal system demonstrated positively brownish stained CDV in nucleus and cytoplasm of glandular epithelial cells with infiltrative lymphocyte in the lamina propria (Figure 13-14). The transitional cells of urinary bladder were immunologically accorded with another in cytoplasm and nucleus (Figure 15). In addition, the renal pelvis, which microscopically showed eosinophilic inclusion bodies, were also recognized harboring viral protein in cytoplasm and nucleus (Figure 16). Moreover, the focally positive renal tubular cells were noticed. Additionally, epithelia of tonsils (Figure 17) and epidermis (keratinocytes) were detectably positive for CDV.

The viral antigens persisted in varied organs which conventionally revealed by immunohistochemical method, for instance gastrointestinal tract, respiratory, lymphoid

organs, urogenitral tract and also brain. The conclusion of CDV labeling cells in various organs is demonstrated in Table 5.

1.2) Nectin-4

Immunological staining of nectin-4 was performed in several tissues from negative CDV samples including brain, lung, stomach, intestine and kidney compared with mouse brain as a nectin-4 positive control (Reymond et al., 2001). DAB-stained positive nectin-4 receptor was demonstrated in cytoplasm of effective cells. There were strong positive in gastric and intestinal glandular epithelial cells. The moderate intensity of positivity was revealing in bronchial and bronchiolar epithelium in lung, renal tubular and pelvis epithelial cells. There was sporadically moderate to weak intensity revealing in neuron-like cells in cortico-medullary junction of cerebrum, while in cerebellum, interestingly, the weak positive of nectin-4 was detected in Purkinje's cells. The lymph node and spleen were rarely observed. According with CDV naturally infected specimens which were randomly selected for performing a single nectin-4 immunohistochemistry staining, the distribution of nectin-4 were expressed in several organs as similar as shown in a negative CDV one (Figure 18-23).

Table 2 Histopathological findings in various organs

Case no.	Hemopoietic organ			Central nervous system[a]					Lung		Gastrointestinal tract		Urogenital tract	
	SP	LMD	HC	gliosis	DM	MNPC	meningitis	IB	BINP	INP	catarrhal	LM/LMP	IB	UB MNi
TH 07/01	-	-	+	++	+++	+	++	+++	-	FNP	+	-	-	NS
TH 08/01	+	+	-	++	++	-	-	+	+	-	+	+	-	+
TH 09/01	-	-	-	+++	+++	-	-	+++	-	+	NS		-	NS
TH 09/02	-	+	-	-	-	-	-	-	-	+	+	-	+	NS
TH 09/03	-	+	+	+	-	-	-	+	BNP	-	+	-	-	-
TH 10/01	-	+	+	+++	+++	+	+++	++	+	-	+	+	+	-
TH 10/02	-	+	+	-	-	-	-	-	+	-	NS		+	-
TH 10/03	-	+	+	++	+++	++	++	+++	+	-	+	+	+	-
TH 10/04	-	+	+	-	-	-	-	-	-	+	+	+	-	-
TH 10/05	NS			+	+	+	-	+	NS		NS		NS	

[a]: The severity of lesion in CNS was semi-quanlitatively scored as +++ severe, ++ moderate, + mild lesion.

Abbreviation: BIPN: bronchointerstitial pneumonia, BPN: bronchopneumonia, DM: demyelination, FPN: fibrinopurulent pneumonia, HC: histiocytic infiltration, IB: inclusion body, IPN: interstitial pneumonia, LMD: lymphoid depletion, MNPC: mononuclear perivascular cuffing, MNi: mononuclear cell (lymphocytic/lymphoplasmacytic) infiltration, NS: no sample, SP: suppurative, UB: urinary bladder.

Case no.	Hemopoietic organ			Central nervous system[a]					Lung		Gastrointestinal tract		Urogenital tract	
	SP	LMD	HC	gliosis	DM	MNPC	meningitis	IB	BINP	INP	catarrhal	LM/LMP	IB	UB MNi
TH 11/01	-	+	-	-	-	-	-	-	+	-	+	-	+	-
TH 11/02	+	+	-	+	+	+	+	+	BNP	-	NS		-	NS
TH 11/03	-	NR	-	+	-	-	+	+	+	-	NS		NS	
VN 11/01	-	+	-			NS			+	-	+	-	-	NS
VN 11/02	+	+	-	+	+	-	-	+	+	-	+	-	-	NS
VN 11/03	-	+	-	+	-	-	-	+	NS		+	-	+	-
VN 12/05	-	+	+	-	-	-	-	-	-	+	+	-	NS	
VN 12/08		NS		+	+	-	+	+	-	+	+	+	NS	
VN 12/09	+	-	-	-	-	-	-	-	-	+	+	+	NS	
VN 12/27	+	-	-	+	+	+	+	+	NS		+	+	NS	

[a]: The severity of lesion in CNS was semi-quanlitatively scored as +++ severe, ++ moderate, + mild lesion.

Abbreviation: BIPN: bronchointerstitial pneumonia, BPN: bronchopneumonia, DM: demyelination, FPN: fibrinopurulent pneumonia, HC: histiocytic infiltration, IB: inclusion body, IPN: interstitial pneumonia, LMD: lymphoid depletion, MNPC: mononuclear perivascular cuffing, MNi: mononuclear cell (lymphocytic/lymphoplasmacytic) infiltration, NR: necrotic, NS: no sample, SP: suppurative, UB: urinary bladder.

Table 3 Histopathological diagnosis

Case no.	Organs	Microscopic diagnosis
TH 07/01	Brain	Severe non suppurative meningoencephalitis and generalized leukoencephalomalacia
	Lung	Severe fibrinopurulent pneumonia
	Intestine	Mild catarrhal enteritis
	Spleen	Histiocytic splenitis
TH 08/01	Brain	Brain congestion with leukoencephalomalacia
	Lung	Severe diffuse suppurative bronchointerstitial pneumonia with inclusion bodies
	Intestine	Mild lymphocytic gastroenteritis
	Spleen	Suppurative splenitis
	Urinary bladder	Mild lymphoplasmacytic cystitis
TH 09/01	Brain	Moderate focally demyelination
	Lung	Moderate interstitial pneumonia
TH 09/02	Brain	No remarkable lesion
	Lung	Severe fibrinopurulent hemorrhagic interstitial pneumonia with inclusion bodies
	Intestine	Catarrhal enteritis
	KIdney	Inclusion bodies in renal pelvis

Case no.	Organs	Microscopic diagnosis
TH 09/03	Brain	Mild non suppurative polioencephalitis
	Lung	Acute suppurative bronchopneumonia with with inclusion bodies
	Intestine	Mild catarrhal enteritis
	Spleen	Multifocal follicular necrotic splenitis
	Lymph node	Histiocytic lymphadenitis
TH 10/01	Brain	Severe leukoencephalomalacia and non-supurative meningitis
	Lung	Acute suppurative bronchointerstitial pneumonia
	Intestine	Mild lymphocytic catarrhal enteritis
	Stomach	Mild lymphocytic gastritis
	Spleen	Splenic congestion
	Lymph node	Histiocytic lymphadinitis
TH 10/02	Brain	No remarkable lesion
	Lung	Moderate acute suppurative bronchointerstitial pneumonia
	Spleen	Lymphoid depletion of spleen
	Lymph node	Histiocytic lymphadenitis
	Urinary bladder	Inclusion bodies in transitional cells

Case no.	Organs	Microscopic diagnosis
TH 10/03	Brain	Severe generalized leukoencephalomalacia and non-suppurative polioencephalitis
	Lung	Severe suppurative brochointerstitial pneumonia
	Intestine	Lymphoplasmacytic catarrhal enteritis
	Spleen	Histiocytic splenitis and hemosiderosis
	Kidney	Inclusion bodies in renal pelvis
	Urinary bladder	Lymphocytic cystitis
TH 10/04	Brain	No remarkable lesion
	Lung	Moderate suppurative-hemorrhagic interstitial pneumonia
	Intestine	Mild lymphocytic catarrhal enteritis
	Lymph node	Mild histiocytic lymphadenitis
TH 10/05	Brain	Moderate non-suppurative polioencephalitis and focal demyelination
TH 11/01	Brain	No remarkable lesion
	Lung	Acute hemorrhagic bronchointersitital pneumonia
	Intestine	Moderate catarrhal enteritis
	Spleen	Follicular depletion
	Kidney	Inclusion bodies in renal pelvis
	Urinary bladder	Inclusion bodies in transitional cells

Case no.	Organs	Microscopic diagnosis
TH 11/02	Brain	Moderate non-suppurative polioencephalitis and focal demyelination
	Lung	Severe acute diffuse suppurative bronchopneumonia with inclusion bodies
	Spleen	Severe multifocal necrotic suppurative splenitis
	Lymph node	Severe diffuse hemorrhagic suppurative lymphadenitis
TH 11/03	Brain	Mild non-suppurative polionecephaliits and non-suppurative meningitis
	Lung	Moderate subacute suppurative bronchointerstitial pneumonia
	Spleen	Multifocal necrotic splenitis
VN 11/01	Lung	Severe diffuse bronchointerstitial pneumonia
	Intestine	Severe catarrhal enteritis
	Spleen	Severe splenic lymphoid depletion
VN 11/02	Brain	Moderate focally demyelination
	Lung	Moderate bronchointerstitial pneumonia
	Intestine	Moderate catarrhal enteritis
	Lymph node	Mild suppurative lymphadinitis
VN 11/03	Brain	Mild non-suppurative polioencephalitis
	Intestine	Moderate catarrhal enteritis
	Spleen	Moderate follicular lymphoid depletion
	Kidney	Inclusion bodies in renal pelvis

Case no.	Organs	Microscopic diagnosis
VN 12/05	Brain	No remarkable lesion
	Lung	Moderate interstitial pneumonia
	Intestine	Mild catarrhal enteritis
	Spleen	Necrotic histiocytic splenitis
VN 12/08	Brain	Moderate non suppurative meningoencephalitis
	Lung	Severe interstitial pneumonia
	Intestine	Severe lymphocytic catarrhal enteritis
VN 12/09	Brain	Mild non-suppurative encephalitis
	Lung	Moderate interstitial pneumonia.
	Intestine	Lymphocytic catarrhal enteritis
	Spleen	Moderate suppurative splenitis
VN12/27	Brain	Mild non-suppurative meningoencephalitis
	Intestine	Severe lymphoplasmacytic catarrhal enteritis
	Spleen	Mild suppurative splenitis

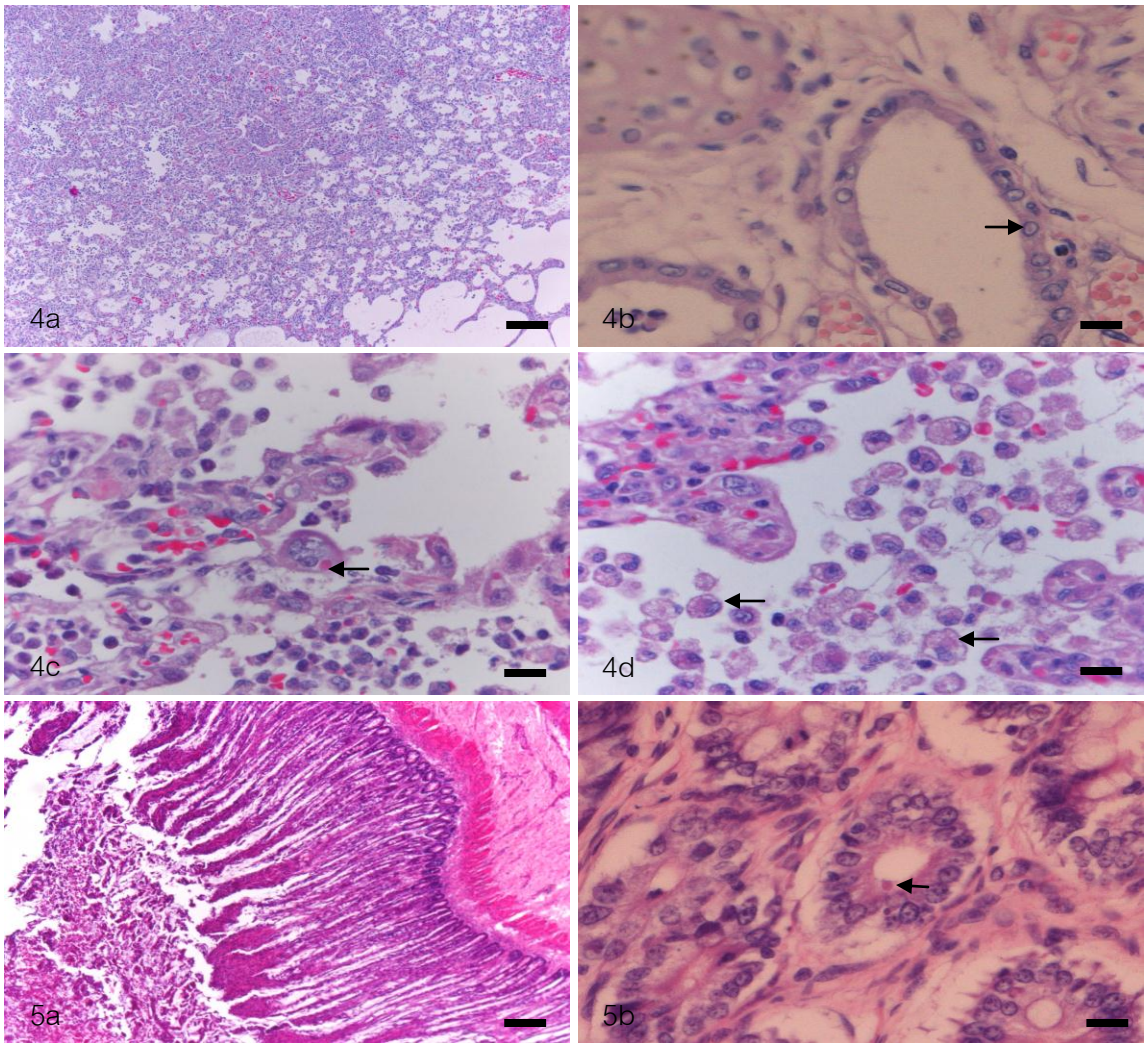


Figure 4a: Severe suppurative bronchopneumonia, lung; TH09/03 (H&E, bar = 100 um).

Figure 4b: Eosinophilic intranuclear inclusion body (arrow) in bronchiolar epithelium, lung; TH09/01 (H&E, bar = 10 um).

Figure 4c: Pulmonary alveolar macrophage (PAM) proliferation and syncytial cell formation with eosinophillic inclusion body (arrow), lung; TH09/03 (H&E, bar = 10 um).

Figure 4d: Pulmonary alveolar macrophage (PAM) proliferation with eosinophillic intracytoplasmic inclusion bodies (arrow), lung; TH09/03 (H&E, bar = 10 um).

Figure 5a: Catarrhal enteritis with necrotic villi, intestine, VN12/08 (H&E, bar = 100 um).

Figure 5b: Eosinophilic intracytoplasmic inclusion body (arrow) in intestinal glandular cell, intestine; VN11/03 (H&E, bar = 10 um).

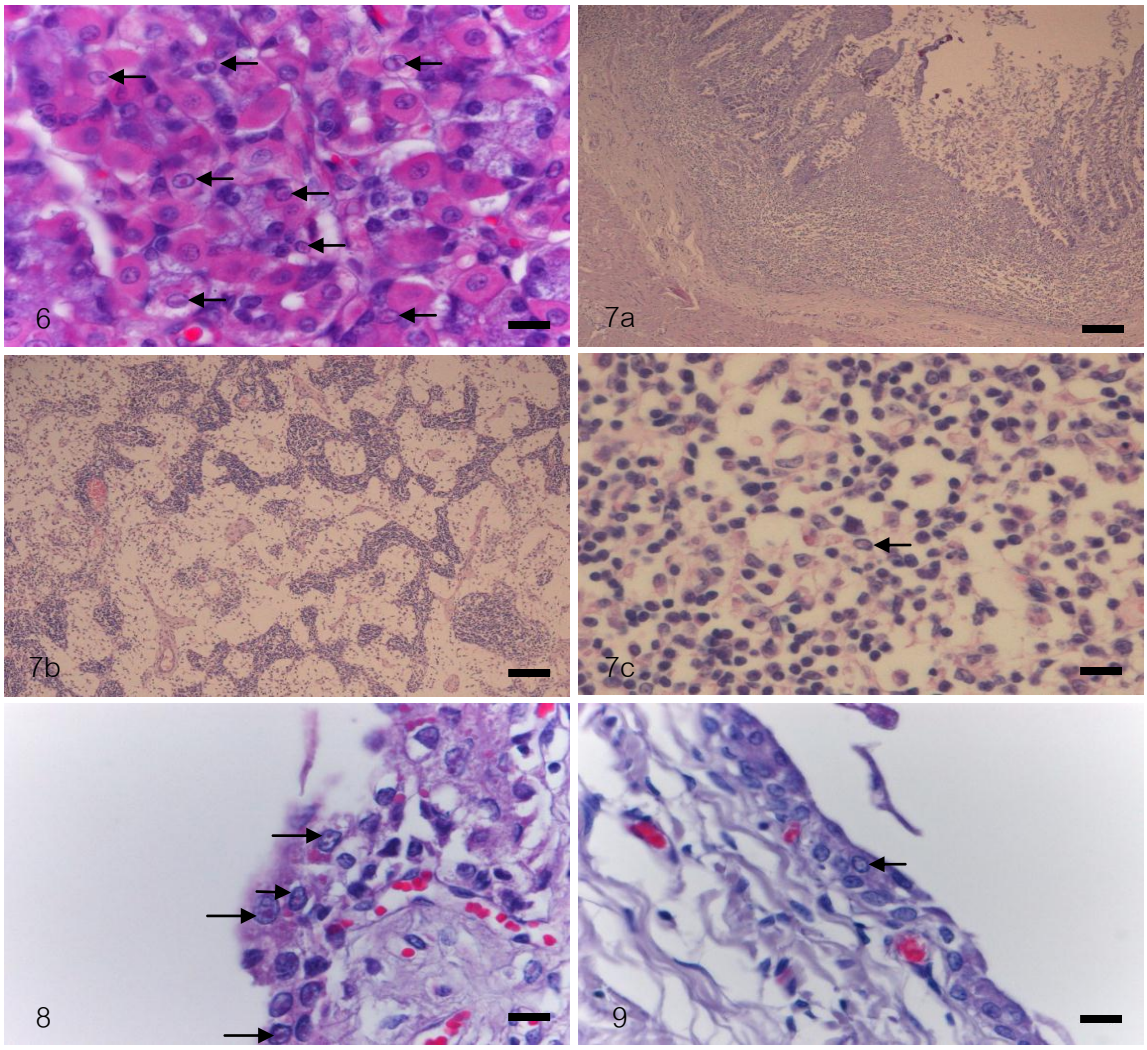


Figure 6: Abundant eosinophilic intranuclear inclusion bodies (arrow) in gastric glandular cell, stomach; TH10/01 (H&E, bar = 10 um).

Figure 7a: Lymphoid depletion in Payer's patch with mild autolysis, intestine; VN11/01 (H&E, bar = 100 um).

Figure 7b: Severe lymphoid depletion, lymph node; VN11/03 (H&E, bar = 100 um).

Figure 7c: Eosinophillic intranuclar inclusion body, lymph node; VN11/03 (H&E, bar = 10 um).

Figure 8: Eosinophilic intranuclear inclusion bodies (arrow) in transitional cells, urinary bladder: TH10/03 (H&E, bar = 10 um).

Figure 9: Eosinophilic intranuclear inclusion bodies (arrow) in renal pelvis epithelium, kidney: TH10/04 (H&E, bar = 10 um).

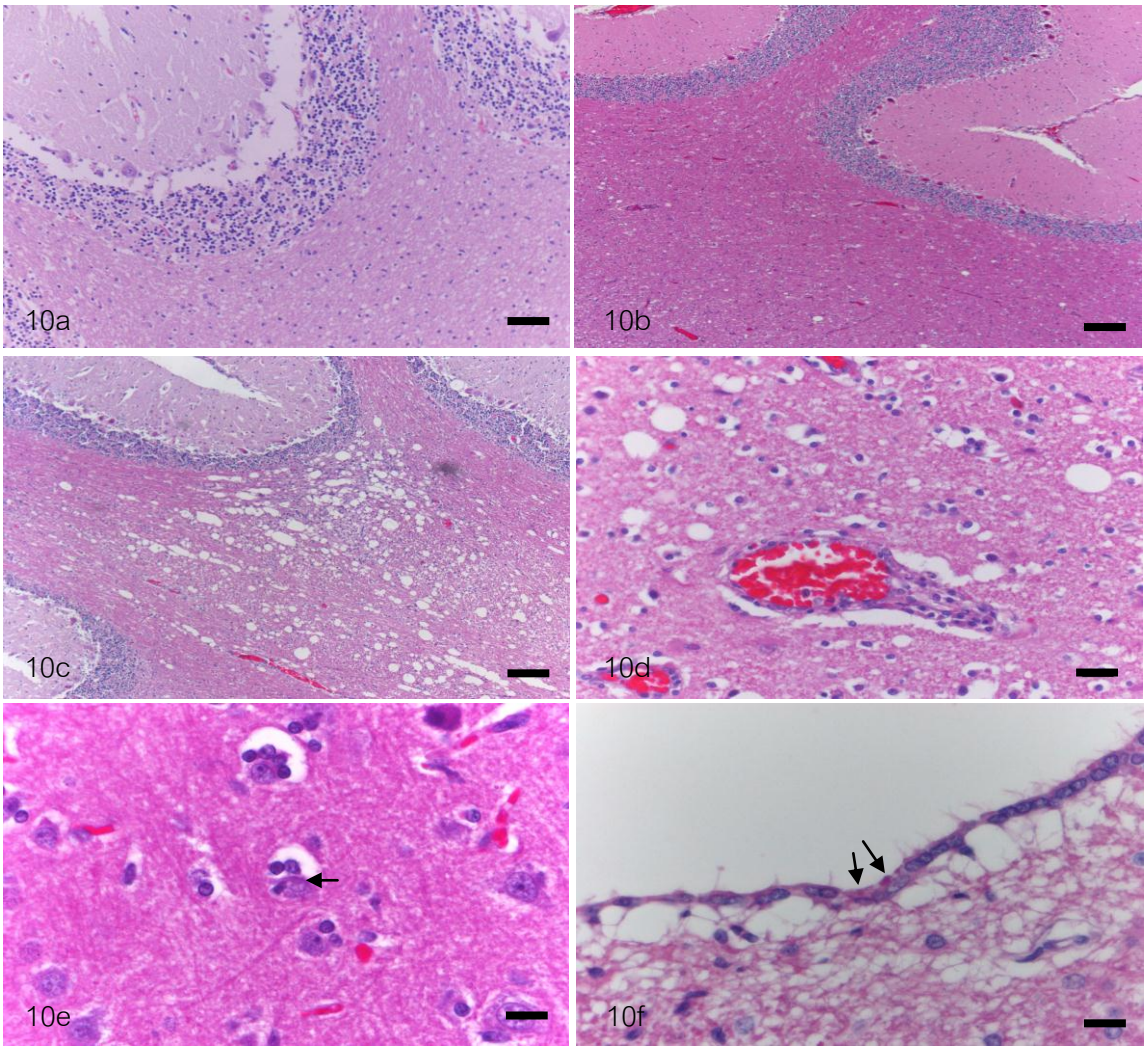


Figure 10a: No pathological lesion group, cerebellum; TH09/01 (H&E, bar = 100 um).

Figure 10b: Acute group with mild multifocal demyelination, cerebellum; TH10/05 (H&E, bar = 100 um).

Figure 10c: Chronic group with severe demyelination, cerebellum; TH07/01 (H&E, bar = 100 um).

Figure 10d: Mononucler perivascular cuffing with edema and gilosis, cerebrum; TH10/03 (H&E, bar =20 um).

Figure 10e: Neuronophagia with eosinophillic intracytoplasmic inclusion body (arrow) in neuron, cerebrum; TH10/01 (H&E, bar = 10 um).

Figure 10f: Eosinophilic intracytoplasmic inclusion bodies (arrow) in ependymal cells, cerebellum; TH09/03 (H&E, bar = 10 um).

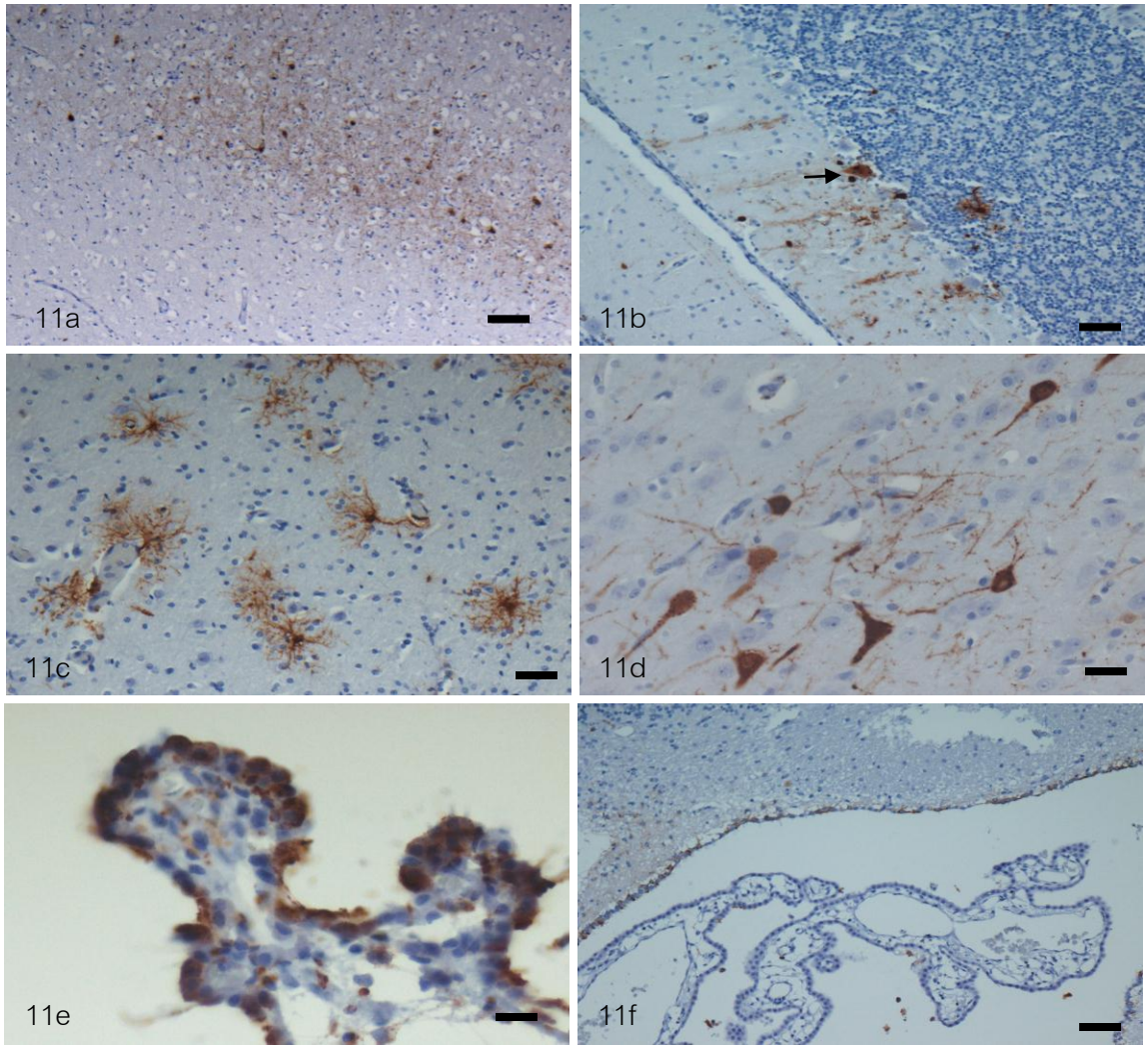


Figure 11a: The CDV antigen positive cells in cortico-medullary junction, cerebrum; TH10/05 (HRP system, bar = 100 um).

Figure 11b: The CDV antigen positive cells including Purkinje's cell (arrow), cerebellum; TH11/03 (HRP system, bar = 40 um).

Figure 11c: CDV positive astrocyte-like cells, cerebrum; TH10/03 (HRP system, bar= 20 um).

Figure 11d: CDV positive neuronal-like cells, cerebrum; TH11/03 (HRP system, bar= 20 um).

Figure 11e: CDV positive in choroid plexus cells, cerebellum; TH10/01 (HRP system, bar = 20 um).

Figure 11f: CDV positive in ependymal cells, cerebellum; TH09/03 (HRP system, bar = 40 um).

Table 4 Immunohistochemical results in central nervous system [a]

Histopathological grouping	Case no.	Cerebrum		Cerebellum		Mid brain		Spinal cord		Meningeal cells	Ependymal cells	Choroid plexus cells
		Nu	As	Nu	As	Nu	As	Nu	As			
No pathological lesion	TH 09/02	+	+++	-	++	+	++	NS		-	+	-
	TH 10/02	+	++	-	-	-	+	-	-	-	+	-
	TH 10/04	-	+	-	-	-	-	-	+	-	-	-
	TH 11/01	-	+	-	-	-	+	-	+	-	++	
	VN12/05	+	+						NS			
	VN12/09	+	-						NS			
Acute lesion	TH08/01	+	++	+	++	-	+	NS		-	-	-
	TH09/03	-	+	-	+	+	+	+	+	-	++	-
	TH10/05	+++	+++	Gr	++	-	++	-	++	-	-	-
	TH11/02	-	++	-	+		+	-	-	-	-	-

[a] The number of CDV positive cells in CNS were scored as +++ marked positive, ++ moderate positive, + slight positive.

Abbreviation: As: astrocyte like cells, Gr: granular cells, NS: no sample, Nu: neuron-like cells, Pu: Purkinje's cells

Histopathological grouping	Case no.	Cerebrum		Cerebellum		Mid brain		Spinal cord		Meningeal cells	Ependymal cells	Choroid plexus cells
		Nu	As	Nu	As	Nu	As	Nu	As			
Acute lesion	TH 11/03	+++	++	Pu	++	++	++	NS		-	++	-
	VN 11/02	NS		-	+					NS		
	VN11/03	-	+						NS			
	VN12/08	+	++			NS				+	-	-
	VN12/27	+	-			NS				+	+	-
Chronic lesion	TH07/01	-	+	-	+++	-	+	NS		-	-	-
	TH09/01	-	++	-	+++	-	++	NS		-	-	+
	TH10/01	-	++	-	+++	-	+++	-	++	+++	++	++
	TH10/03	+	+++	+	+++	+	++	-	++	-	+	-

[a] The number of CDV positive cells in CNS were scored as +++ marked positive, ++ moderate positive, + slight positive.

Abbreviation: As: astrocyte like cells, Gr: granular cells, NS: no sample, Nu: neuron-like cells, Pu: Purkinje's cells

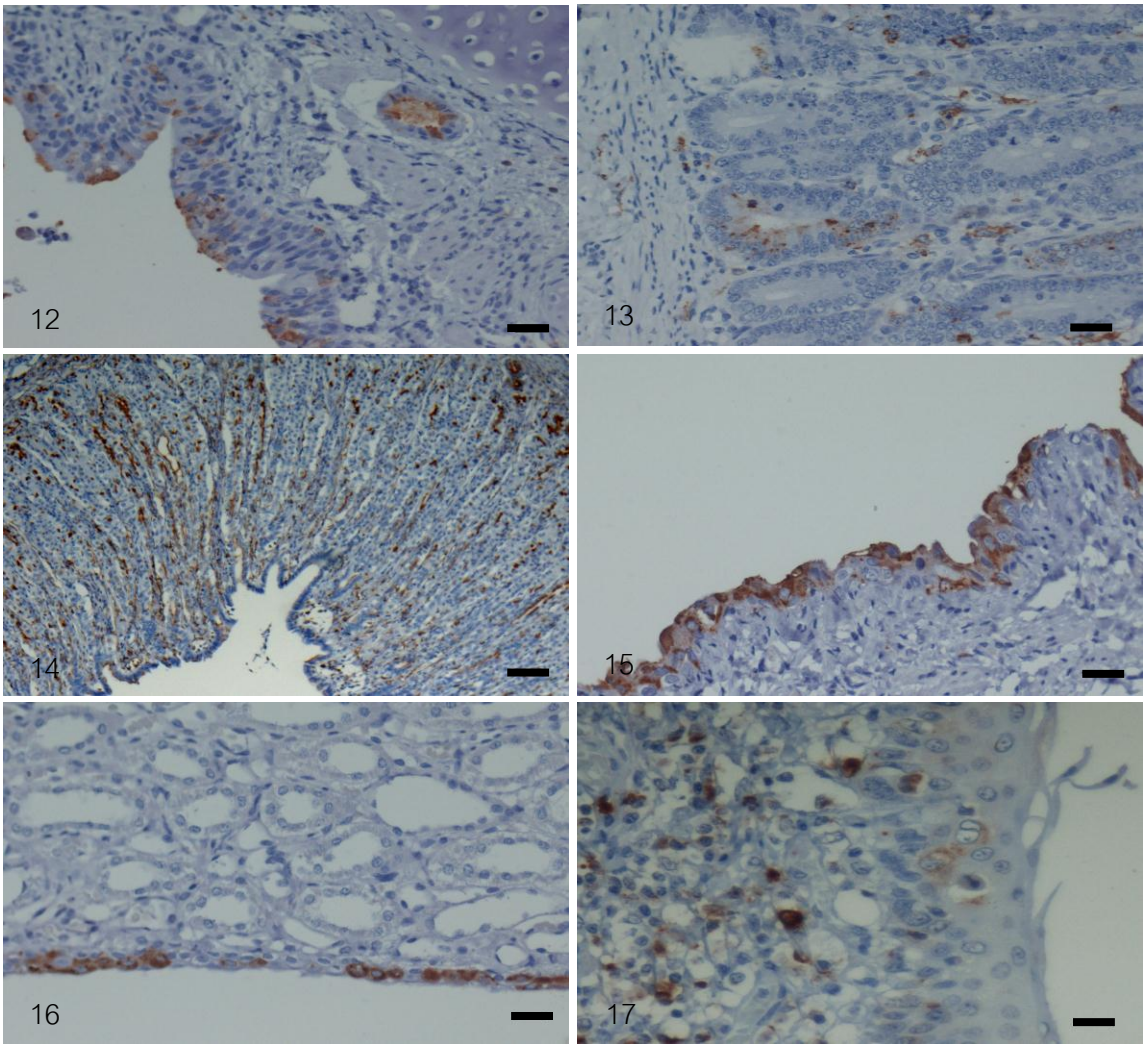


Figure 12: CDV positive cells in bronchial and bronchiolar epithelia, lung; TH10/04 (HRP system, bar = 20 um).

Figure 13: CDV positive cells in glandular cells and lymphocyte in lamina propria, intestine; TH09/03 (HRP system, bar = 20 um).

Figure 14: CDV positive cells in glandular epithelium, stomach; TH10/01 (HRP system, bar = 100 um).

Figure 15: CDV positive cells in transitional epithelium, urinary bladder; TH09/03 (HRP system, bar = 20 um).

Figure 16: CDV positive cells in renal pelvis epithelium, kidney; TH09/03 (HRP system, bar= 20 um).

Figure 17: CDV positive cells in lymphoid cells and epithelium, tonsil; TH11/01 (HRP system, bar= 20 um).

Table 5 Immunohistochemical results in various organs [a]

Case no.	Lymphoid organs			Respiratory		Stomach	Intestine	Kidney	UB	Others
	LN	SP	TS	PAM	BP					
TH 07/01	NS	++	NS	++	+	NS	+	+/RP	NS	-
TH 08/01	NS	++	NS	++	+	+++	+++	+/RP	++	+/KC
TH 09/01	NS	++	NS	++	+	NS	NS	++/RP	NS	-
TH 09/02	++	++	NS	++	++	NS	+++/PP	+/RP	NS	-
TH 09/03	+++	++	++	++	+	NS	++	++/RP	++	-
TH 10/01	++	+++	++	++	+++	+++	+++	++/RP	NS	+/KC
TH 10/02	++	++	NS	+	++	NS		+/RP	+	-
TH 10/03	NS	+++	NS	++	+	++	++	++/RP	++	+/KC
TH 10/04	++	+	NS	++	+++	-	++	+/RP	+	-
TH 10/05	NS									
TH 11/01	NS	+++	+++	++	+	++	++	+/RP	++	-
TH 11/02	++	+++	NS	+	+	NS	NS	+/RP	NS	++/SK
TH 11/03	NS	+	NS	++	+		NS			+/KC
VN 11/01	++	++	NS	NS		NS	+++/PP	-	NS	-
VN 11/02	++	++	NS	++		NS	++	-	NS	-
VN 11/03	++	++	NS	+++	++	NS	+++	++/RP	NS	-
VN 12/05	NS	++	NS	+	++	NS	+	NS	NS	-
VN 12/08		NS		+	++	NS	+/PP	NS	NS	-
VN 12/09	NS	++	NS	++	+	NS	++	NS	NS	-
VN 12/27	NS	+	NS	NS		NS	++	NS	NS	-

[a]: The number of CDV positive cells in each organs were scored as +++ marked positive, ++ moderate positive, + slight positive.

Abbreviation: BP: bronchial epithelium, KC: Kupffer cells, LN: lymph node, NS: no sample, PAM: pulmonary alveolar macrophage, PP: Payer's patch, RP: renal pelvis epithelium, SK: skin epithelium, SP: spleen, TS: tonsils.

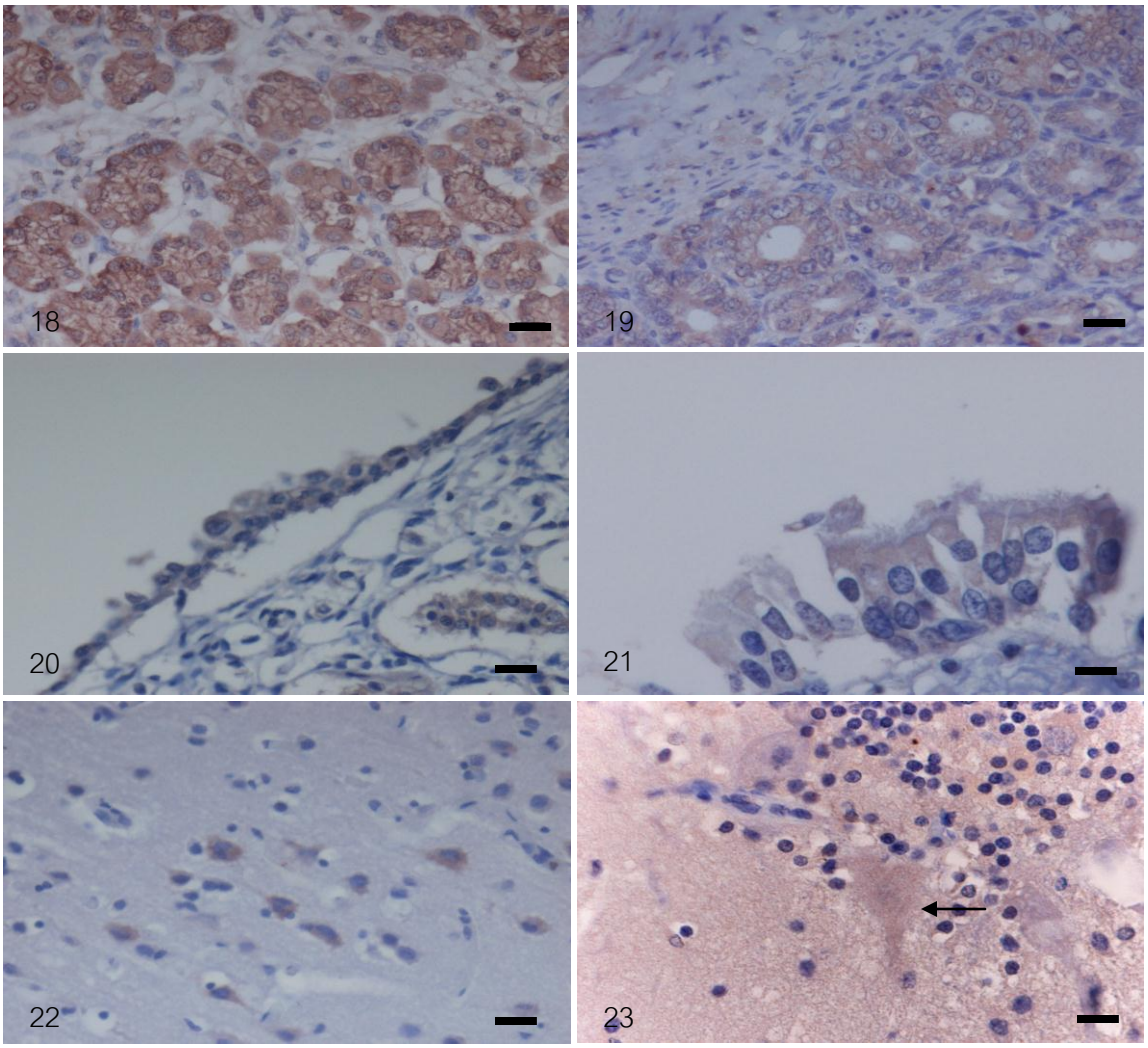


Figure 18: Strong nectin-4 positive in glandular epithelium, stomach; TH07/03 (HRP system, bar = 20 um).

Figure 19: Strong nectin-4 positive in glandular epithelium, intestine; TH07/03 (HRP system, bar = 20 um).

Figure 20: Strong to moderate nectin-4 positive in renal pelvis epithelia and renal tubular cells, kidney; TH07/02 (HRP system, bar = 20 um).

Figure 21: Moderate nectin-4 positive in bronchial epithelia, lung: TH07/02 (HRP system, bar = 10 um).

Figure 22: Moderate to weak nectin-4 positive in neuron-like cells, cerebrum: TH07/03 (HRP system, bar = 20 um).

Figure 23: Weak nectin-4 positive in Purkinje's cells (arrow), cerebellum; TH07/03 (HRP system, bar = 10 um).

2) Double immunohistochemisty

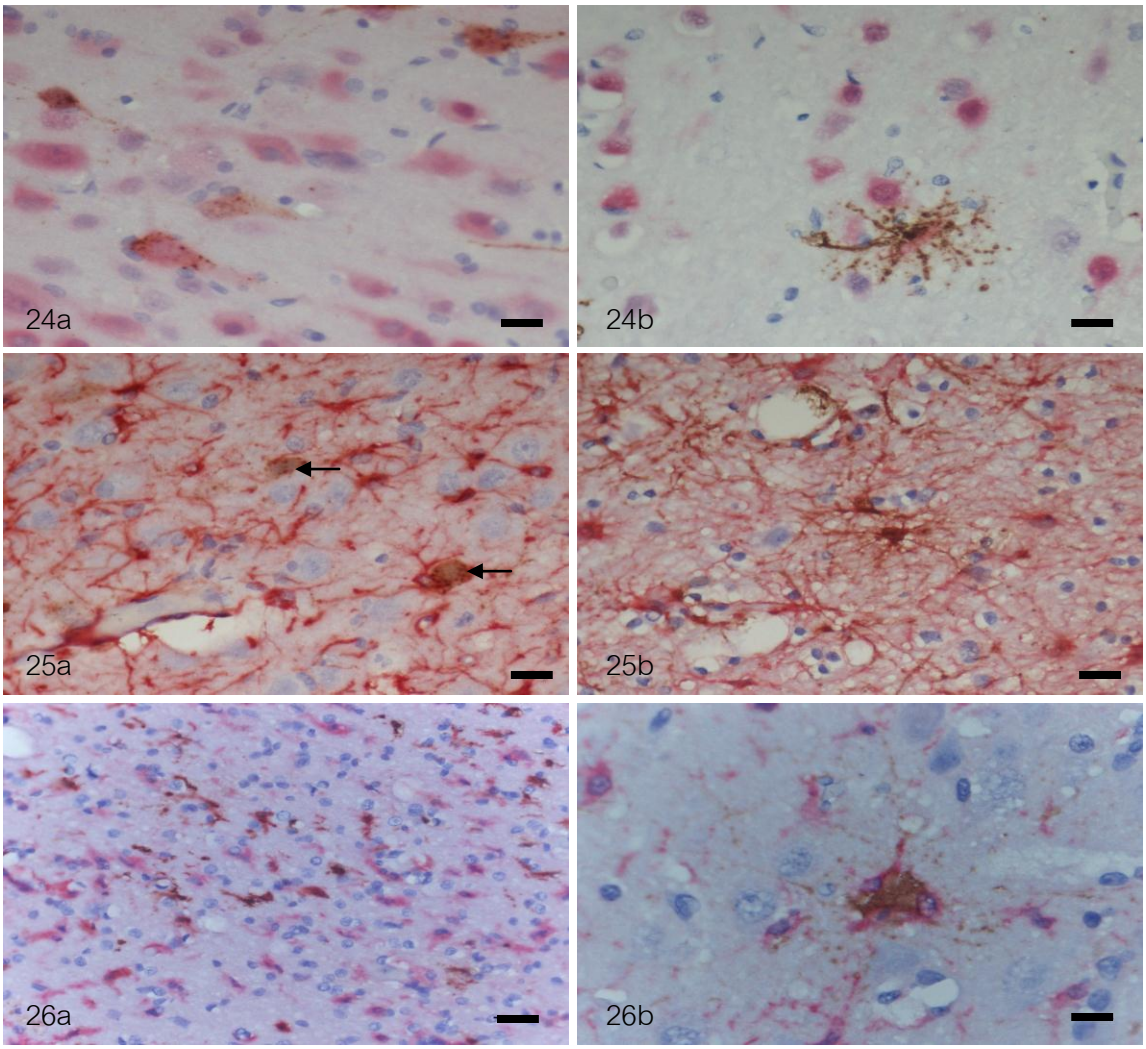
2.1) CDV with brain markers

As already stated, a single immunohistochemical staining against CDV revealed various kinds of infected cells particularly in brain. To ensure the specific cell type that was infected by CDV, the dual immunohistochemistry was done with several brain markers (NeuN for neurons, GFAP for astrocytes, IbaI for microglia). The results showed that CDV could infected neurons, astrocytes and also microglia.

The reddish color of neuronal nuclei protein (NeuN) was stained with Fast red II and presented in nuclei and cytoplasm of neurons in cerebral cortex, granular cells of cerebellum and large neuron in mid brain and spinal cord without Purkinje's cells. The CDV antigen occurrence was also seen in brownish in neurons that co-expressed NeuN (Figure 24a). In addition, the astrocyte-like CDV positive cells and NeuN-expressing cells were separately exhibited. Conversely, astrocyte resembling CDV positive cells and neurons were also isolated presenting (Figure 24b).

In contrary, GFAP in red color staining with Fast red II chromogen was viewed in nuclei and processes of astrocytes. The location of glial protein expressing cells abundantly was in white and grey matter of cerebrum, cerebellum, mid brain and spinal cord. As we aspected, coincidence of CDV and GFAP antigen was also noticed in various areas evaluating by the cellular morphology and co-appearance of reddish and brownish colors (Figure 25a-b).

Without cross reactivity to neuron and astrocyte, Iba-1 positive microglia were ubiquitously seen as red color in both white and gray matter of brain including cerebrum, cerebellum, mid brain and spinal cord. The co-expression of both CDV and Iba-1 was occasionally noticed (figure 26a). Moreover, CDV-infected neurons were surrounded by gathering Iba-1 positive microglia which representative for the neuronophagia lesion (Figure 26b). In addition, the CDV positive astrocyte-like cells were separately evidenced with some aggregating microglia.



The dual immunohistochemistry of several brain markers (red; Fast red II) with CDV (brown; DAB).

Figure 24a: Co expression of NeuN and CDV, cerebrum; TH11/03 (AP&HRP system, bar = 20 um).

Figure 24b: $NeuN^{+}$ cells and CDV^{+} astrocyte like cell; cerebrum; TH10/01 (AP&HRP system, bar = 20 um).

Figure 25a: Scattered $GFAP^{+}$ cells and CDV^{+} neuron like cells (arrow), cerebrum; TH10/03 (AP&HRP system, bar= 20 um).

Figure 25b: Co-labeling GFAP and CDV, cerebrum: TH10/03 (AP&HRP system, bar= 20 um).

Figure 26a: Co-expression of Iba-1 and CDV, cerebrum: TH10/03 (AP&HRP system, bar = 20 um).

Figure 26b: Aggregated microglia surrounded CDV infected neuron, cerebrum: TH11/03 (AP&HRP system, bar = 10 um).

2.2) Nectin-4 and brain marker

As mentioned above, nectin-4 was moderately expressed in cytoplasm of neuron-like cells of both mock- and CDV-infected brains. Therefore, to confirm the particular nectin-4 positive cells, a number of parallel double immunohistochemistry between nectin-4 and brain markers have been done. NeuN and nectin-4 were co-expressed in neuronal cells. NeuN was stained in cytoplasm and nucleus in reddish color following nectin-4 staining and showed as brownish in cytoplasm (Figure 27a). The granule cells in granular layer of cerebellum were stained reddish of NeuN antibody, although hardly labeled by nectin-4. Interestingly, Purkinje's cells were not expressed NeuN, while showed weakly nectin-4 positive (Figure 27b). In white matter of all sections, there were scantly visualized using NeuN and nectin-4. The co-expression of both antigens was also seen in large neuron of mid brain and spinal cords.

However, there were no co-localizations between GFAP or Iba-1 and nectin-4 in all parts of selected brain tissues (cerebrum, cerebellum) (Figure 28-29). In white matter of cerebrum and cerebellum, nectin-4 was rarely detected while GFAP were abundantly seen (Figure 28b). In contrary, in grey matter of spinal cord and mid brain, GFAP was predominately expressed whereas nectin-4 was sporadically showed in cytoplasm of large neurons.

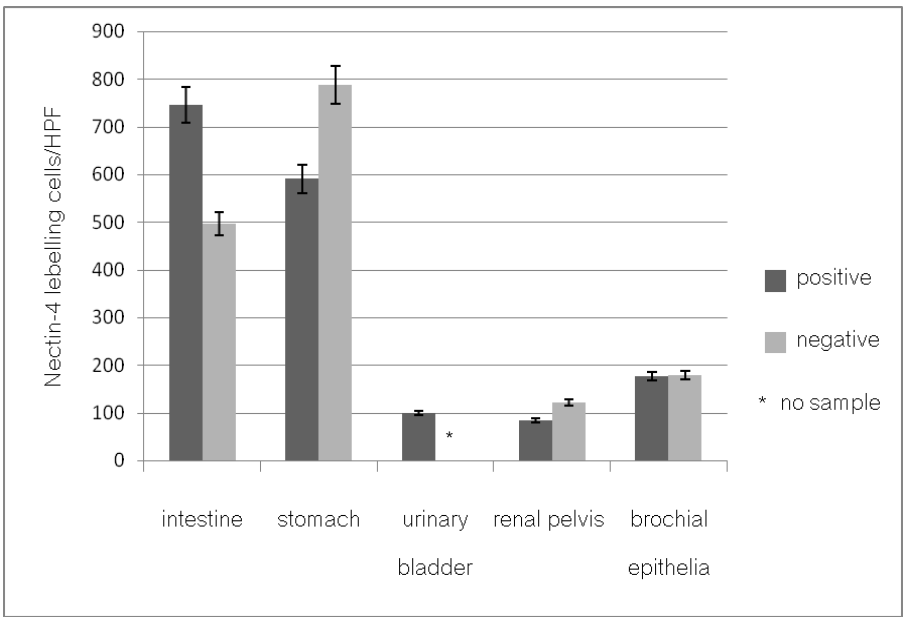
Taking together, it clearly clarified that the expression of nectin-4 was specific to neuron only, notastrocyte and microglia. The greater extent was presented in neurons of cerebrum, especially cortico-medullary junction, mid brain and spinal cord. The lesser extent was observed in Purkinje's cells of cerebellum.

2.3) CDV and nectin-4

In this study, nectin-4 labeling was visualized as brownish staine of DAB in various organs, while viral antigen was revealed in reddish labelling by Fast red II chromogen. The location of nectin-4 expression was mainly restricted in cytoplasm, whereas that of labeled CDVs was in nucleus and cytoplasm.

Nectin-4 positive reactivity was ubiquitously exhibited in glandular epithelial cells of intestine in each group (Figure 30). For CDV immunopositive cells were mostly resided in lamina propria of intestine and also payer's patch, however there was immensely co-evident reactivity with nectin-4. This finding was found also in the gastric glandular epithelial cells that distinctively revealed the coincidence of both antigens (Figure 31). In urinary system, renal tubular epithelial cells and renal pelvis epithelial cells exhibited a moderate extent of co-localization of nectn-4 and viral antigen especially on epithelia of renal pelvis (Figure 32). Moreover, the co-expression was situated in cytoplasm of transitional epithelial cells of urinary bladder (Figure 33). This observation was as similar as in respiratory system particularly in bronchial or bronchiolar epithelia (Figure 34) with a lesser intensity compared with those in urinary and gastrointestinal tract. Interestingly, one sample of the tonsillar epithelial cells (Figure 35) and keratinocytes of epidermis also demonstrated the co-labeling appearance.

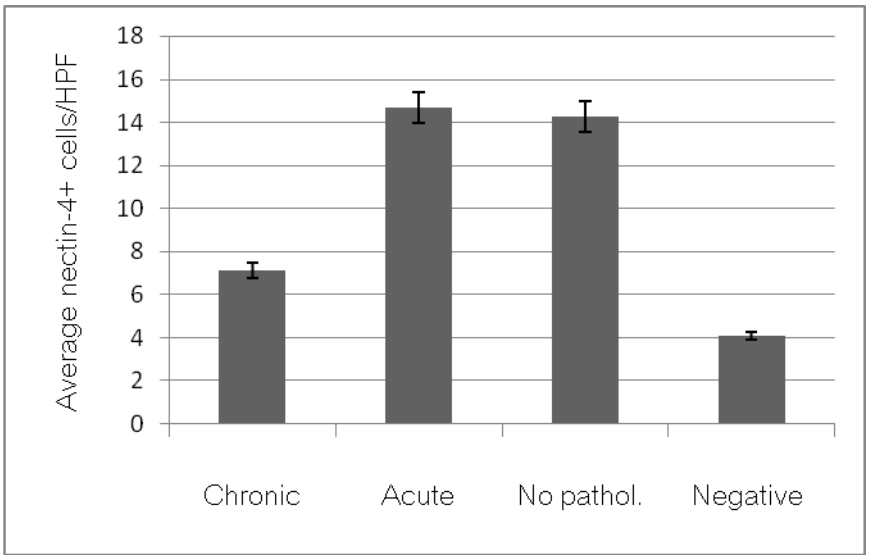
To further evaluate semi-quantitively the up- or down-regulation of nectin-4 comparing between mock- and CDV-infected groups, selected nectin-4 positive tissues (stomach, intestine and lung) were counted as 3, 6, 9 and 12 clockwise fields at high magnification (40X), while urinary bladder and renal pelvis were randomly counted in 4 fields at the same magnification. Results showed the differential expression of nectin-4 particularly in the gastrointestinal system following CDV infection. The striking up-regulation of nectin-4 was elucidated in intestine, but not stomach, of CDV-infected dogs. (Graph 1). In other organs, the expression was not significant difference.



Graph 1: Illustration of semi-quantitative comparison of nectin-4 expression in various organs of CDV-infected (positive) and non-infected (negative) dogs.

To elucidate the relationship of nectin-4 expression and categorized histopathological lesions of brain, selected cerebral section in each mock- and infected group (no pathological, acute and chronic lesion) were randomly counted only in the area of cortico-medullary junction in 10 fields of high magnification. Interestingly, results revealed the up-regulated nectin-4 expression in no pathological and acute lesion assuming as an early phase of infection (Graph 2). Subsequently, the level of expression gradually decreased in chronic lesion indicating later phase of infection, however, it remained higher than negative control. By observing the intensity of positivity, the nectin-4 expressed cells appeared scattering weakly positive signal in neuronal cytoplasm of cerebrum, midbrain and spinal cord, though there were scant in white and gray matter of cerebellum. Despite, the majority of positive nectin-4 and CDV was evident separately in different cells in cerebrum, the coincident staining of both antigens were also seen (Figure 36a-d).

Moreover, the infected Purkinje's cells and ependymal cells of choroid plexus in cerebellum were co-labeled with nectin-4 (Figure 37-38).



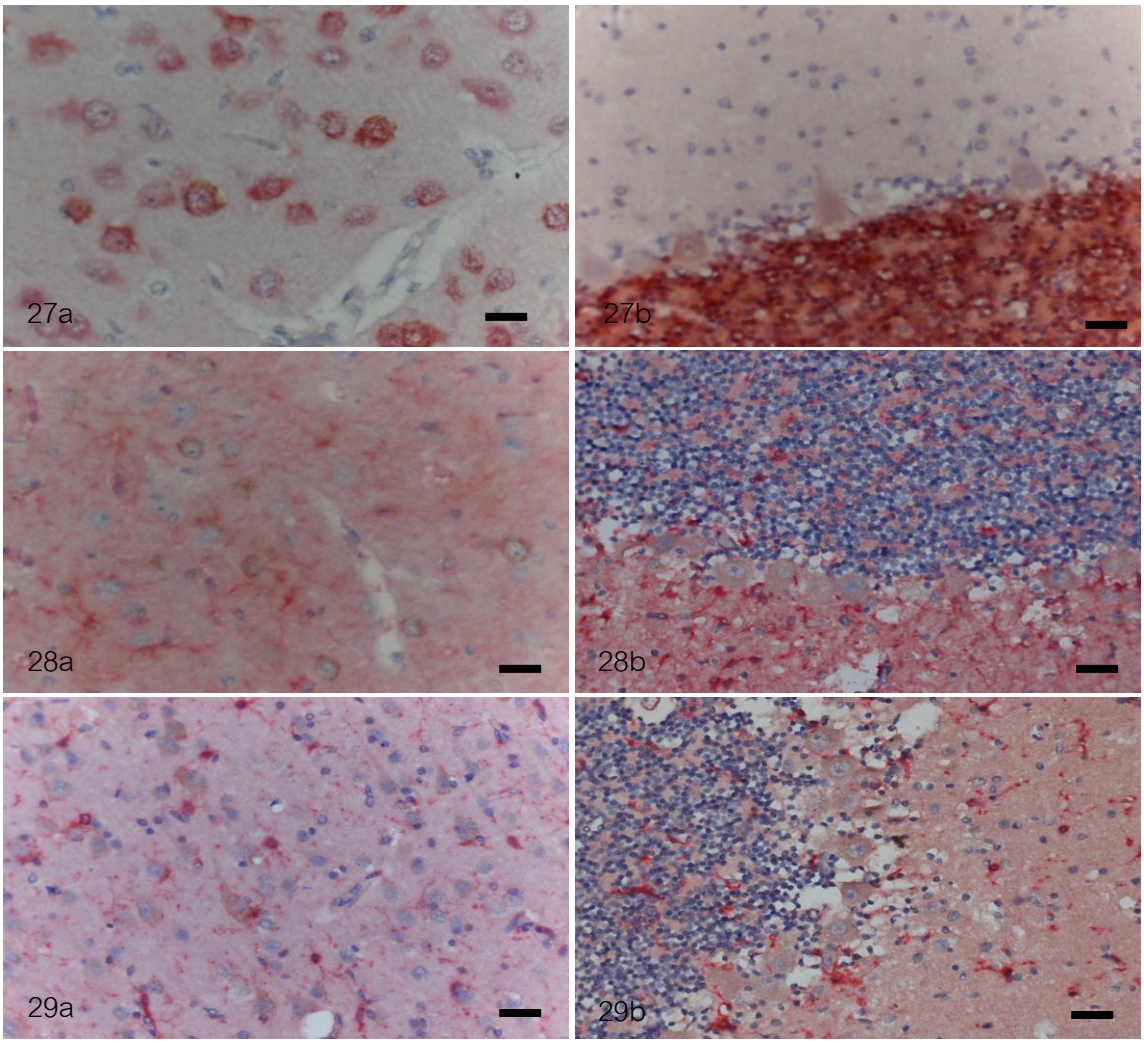
Graph 2: Illustration of semi-quantitative comparison of nectin-4 expression in cerebrum of each histopathological group.

Immunofluorescence double staining

To overcome the uncertainty of co-positive nectin-4 and CDV antigens using immunohistochemistry, the immunofluorescence method has been applied to clearly visualization the co-inhabiting expression of nectin-4 and CDV. Various organs showed green fluorescence signal (Alexa Flour 488) of nectin-4 in cytoplasm of intestinal and gastric glandular epithelial cells (Figure 39-40), transitional epithelial cells in urinary bladder (Figure 41), bronchial epithelium (Figure 42), renal pelvis epithelium (Figure 43) and keratinocytes in epidermis (Figure 44). The obvious red color (Alexa Flour 594) of CDV antigen was notably seen. The co-expression of both colors was revealed as yellow color after merging both figures. The nuclei were counterstained by DAPI (blue). Additionally, brain in negative sample showed the green fluorescence of nectin-4 in neuron and also Purkinje's cells in

cerebellum (Figure 45-46), while co-localization of both colors, as mentioned, was notably showed in neuron (Figure 47-48) and Purkinje's cell (Figure 49) of infected brain. Moreover, ependymal cells of choroid plexus also exhibited the co-expression of both antigens (Figure 50-51).

In conclusion, the expression of nectin-4 was clearly detected in glandular epithelial cells of gastrointestinal tract, epithelial cells of renal pelvis, transitional epithelial cells of urinary bladder, bronchial epithelial cells of lung and keratinocytes ofskin by using immunohistochemistry and immunofluorescence techniques. In the brain, nectin-4 was sporadically noticed in neuron of cerebrum, mid brain, spinal cord with Purkinje's cells of cerebellum. Additionally, the co-expression of CDV antigen and nectin-4 was precisely exhibited in target cells of various organs as mentioned above.



The dual immunohistochistry between brain makers (red; Fast red II) and nectin-4 (brown; DAB).

Figure 27a: Co-expression of NeuN and nectin-4, cerebrum; VN12/27 (AP&HRP system, bar = 20 um).

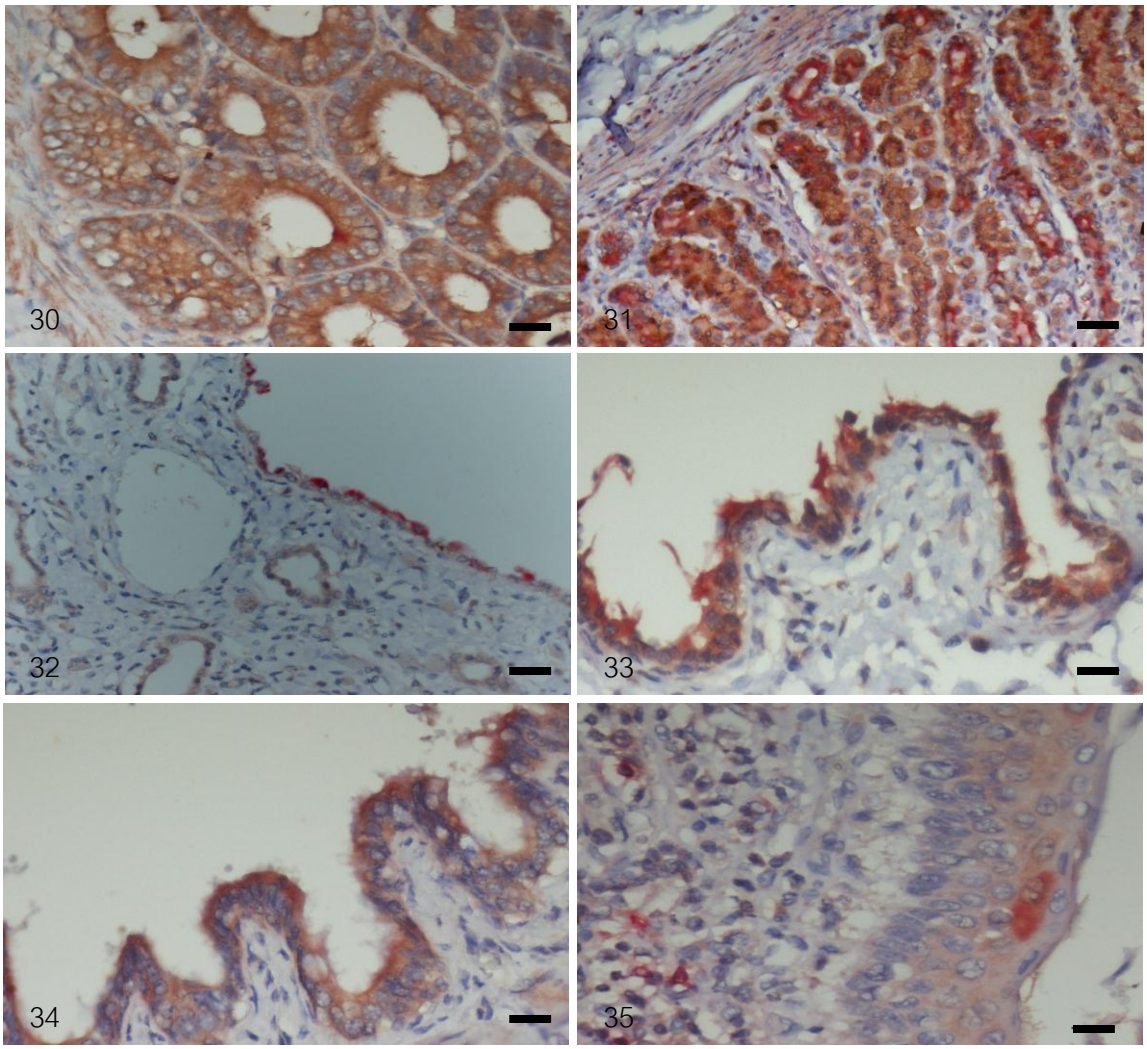
Figure 27b: $NeuN^{+}$ cells in granular cells showed strong nectin-4 positive while Purkinje cells showed weak nectin-4 positive, cerebellum; TH11/03 (AP&HRP system, bar = 20 um).

Figure 28a: Individual expression of GFAP and nectin-4, cerebrum; VN12/27 (AP&HRP system, bar = 20 um).

Figure 28b: Individual expression of GFAP expressed cells and nectin-4 positive Purkinje's cells, cerebellum: TH11/03 (AP&HRP system, bar = 20 um).

Figure 29a: Individual expression of Iba-1 and nectin-4, cerebrum: TH11/03 (AP&HRP system, bar = 20 um).

Figure 29b: Individual expression of Iba-1 expressed cells and nectin-4 positive Purkinje's cells, cerebellum: TH11/03(AP&HRP system, bar = 20 um).



The co-expression of CDV (red; Fast red II) and nectin-4 (brown; DAB) in various organs.

Figure 30: Glandular epithelial cells, intestine; VN12/08 (AP&HRP system, bar = 20 um).

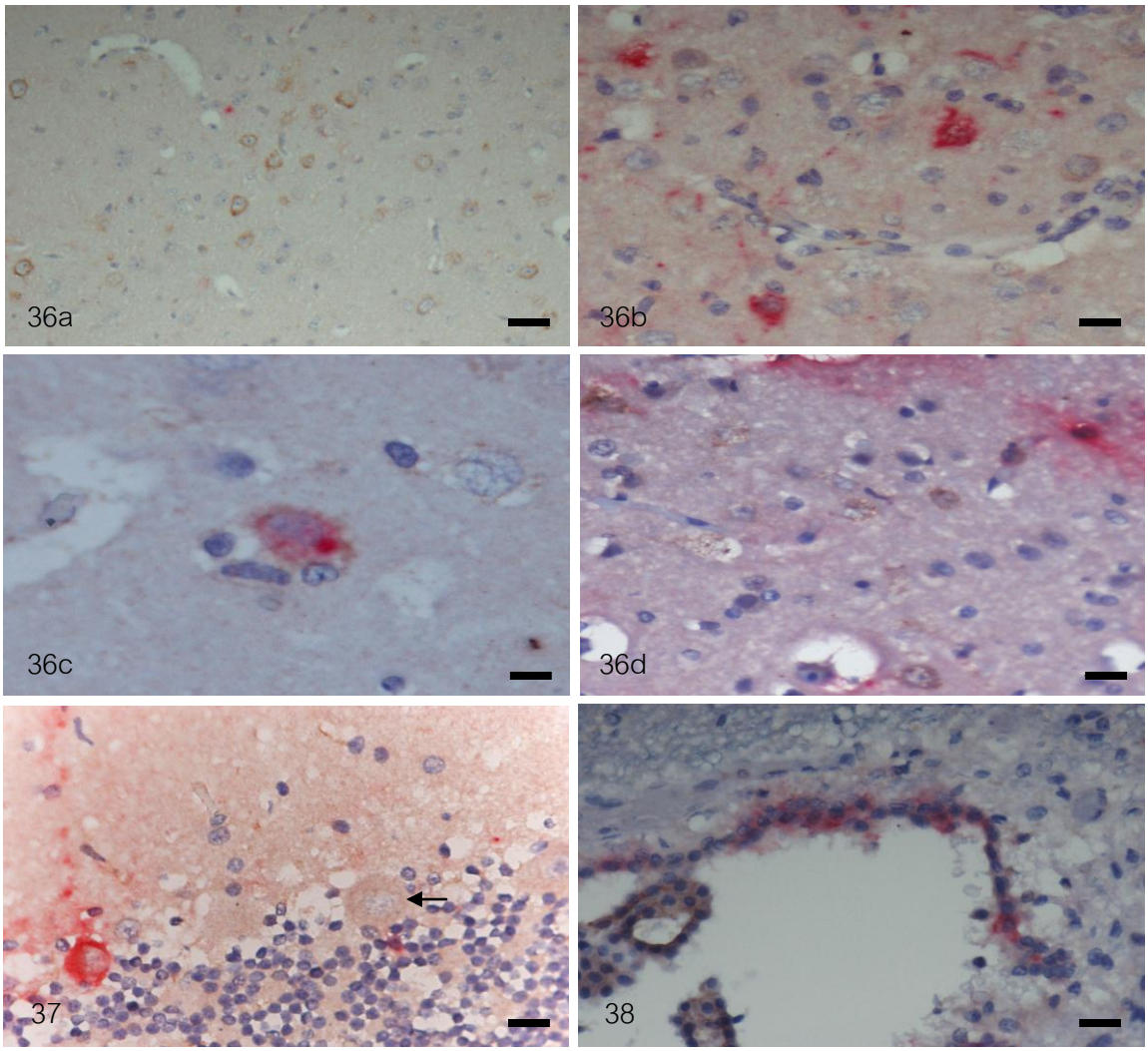
Figure 31: Glandular epithelial cells, stomach; TH10/01 (AP&HRP system, bar = 40 um).

Figure 32: Renal pelvis epithelia, kidney; TH10/02 (AP&HRP system, bar = 20 um).

Figure 33: Transitional epithelia, urinary bladder; TH11/01 (AP&HRP system, bar = 20 um).

Figure 34: Bronchial epithelia, lung: TH10/01 (AP&HRP system, bar = 20 um).

Figure 35: Epithelium of tonsil and CDV positive cells in lymphoid cells without nectin-4 positivity; TH11/01 (AP&HRP system, bar = 20 um).



The dual immunohistochemistry between CDV (red; Fast red II) and nectin-4 (brown; DAB).

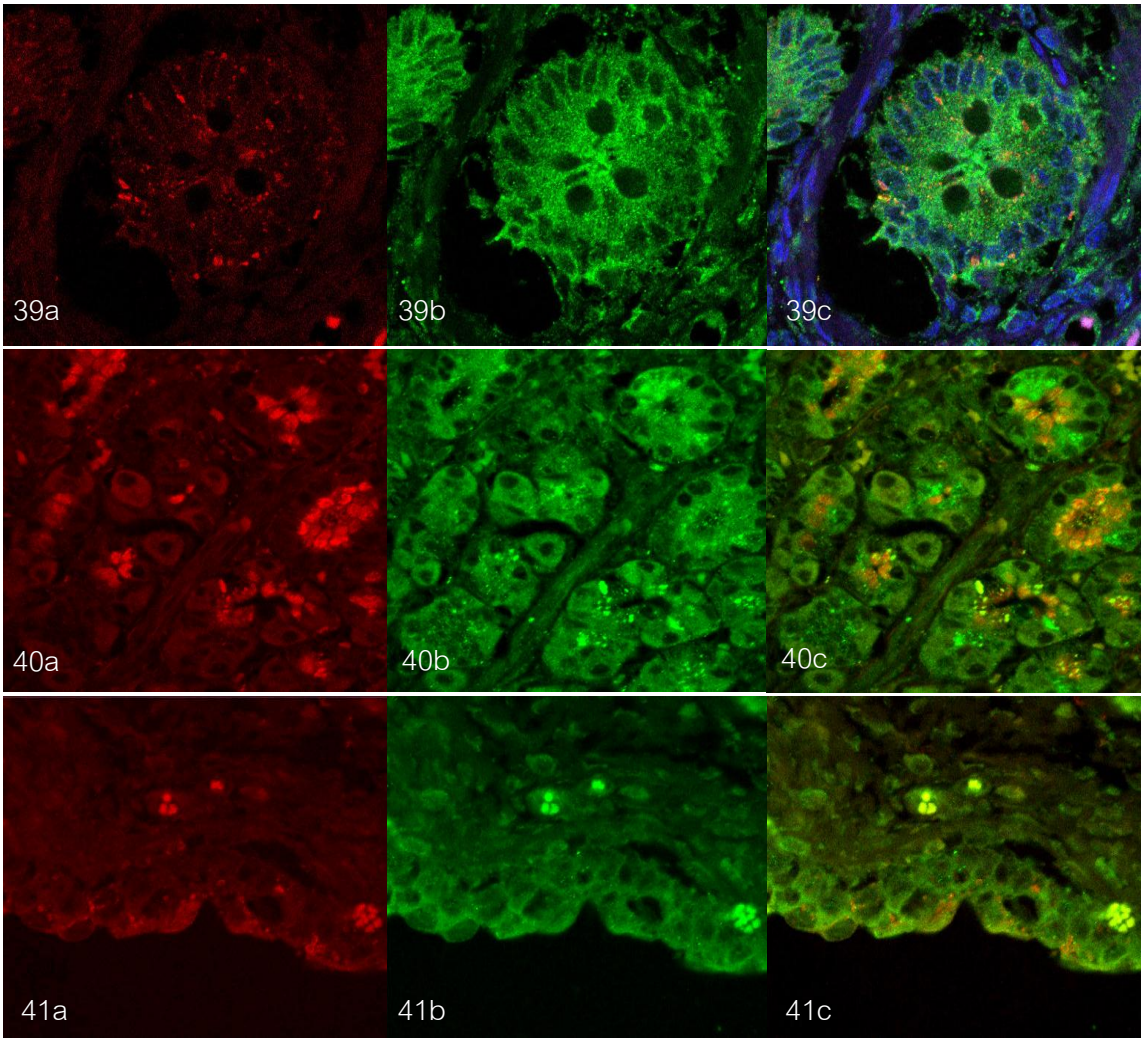
Figure 36a: The expression of nectin-4, cerebrum; VN12/27 (AP&HRP system, bar = 40 um).

Figure 36b-c: The co-expression of CDV and nectin-4, cerebrum; TH11/03 (AP&HRP system, bar = 20 um and 10 um respectively).

Figure 36d: The individual expression of CDV and nectin-4, cerebrum; TH10/03 (AP&HRP system, bar = 20 um).

Figure 37: The co-expression of CDV and nectin-4 in Purkinje's cells with weak nectin-4 positive (arrow), cerebellum, TH11/03 (AP&HRP system, bar =10 um)

Figure 38: The co-expression of CDV and nectin-4 in ependymal cells of choroid- plexus, cerebellum; TH11/01 (AP&HRP system, bar = 20 um)

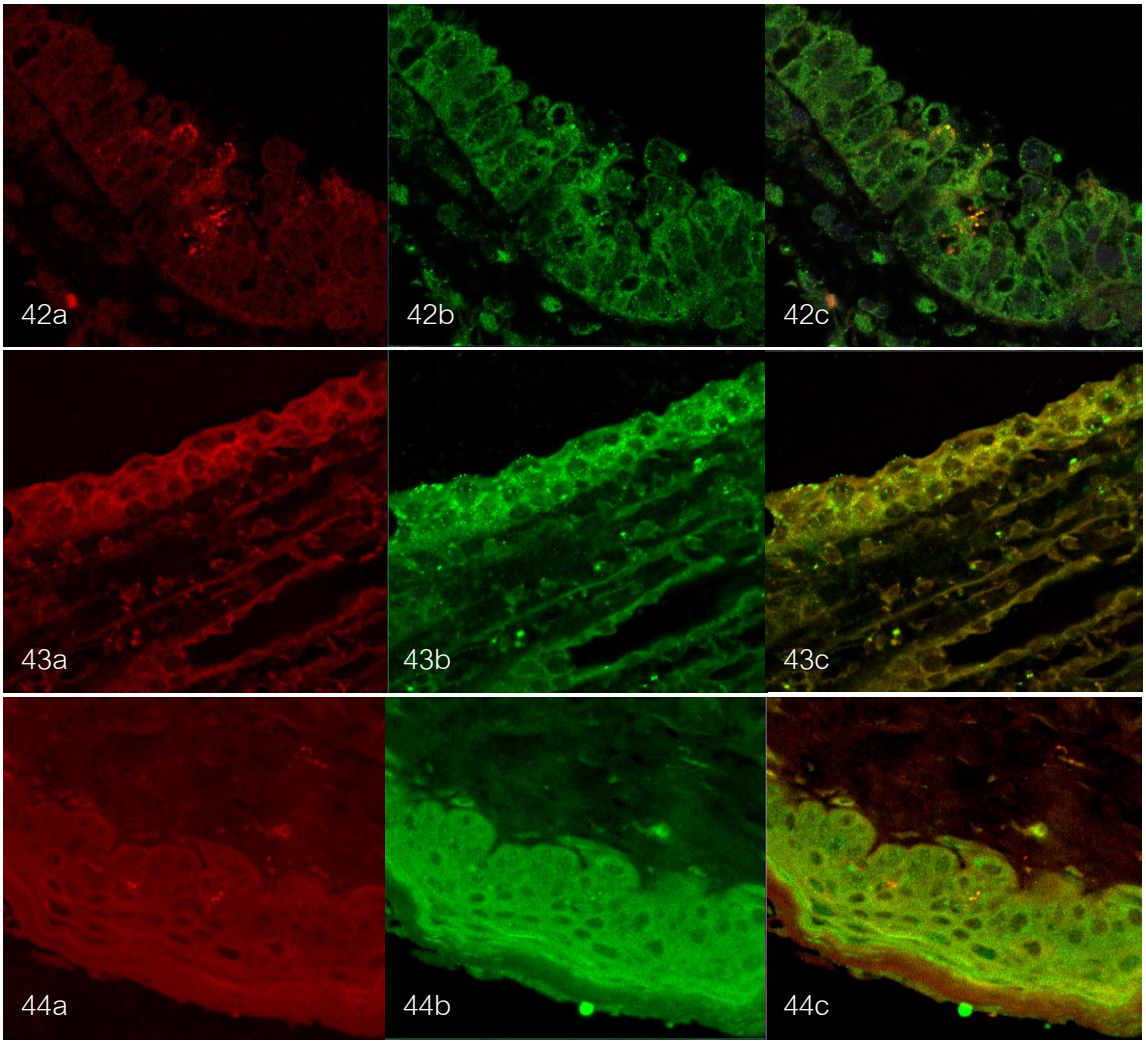


The double immunofluorescence staining between CDV (red; a panel) and nectin-4 (green; b panel) with illustration of overlay (c panel) and nucleus (blue, counterstained DAPI).

Figure 39: glandular epithelial cells, intestine; VN11/03 (4,000 fold magnification).

Figure 40: glandular epithelial cells, stomach; TH10/01 (4,000 fold magnification).

Figure 41: transitional epithelial cells, urinary bladder: TH11/01 (4,000 fold magnification)



The double immunofluorescence staining between CDV (red; a panel) and nectin-4 (green; b panel) with illustration of overlay (c panel) and nucleus (blue, counterstained DAPI).

Figure 42: bronchial epithelial cell, lung; TH07/01 (4,000 fold magnification).

Figure 43: renal pelvis epithelia, kidney; TH10/01 (4,000 fold magnification).

Figure 44: keratinocytes in epidermis, skin: TH11/02 (4,000 fold magnification).

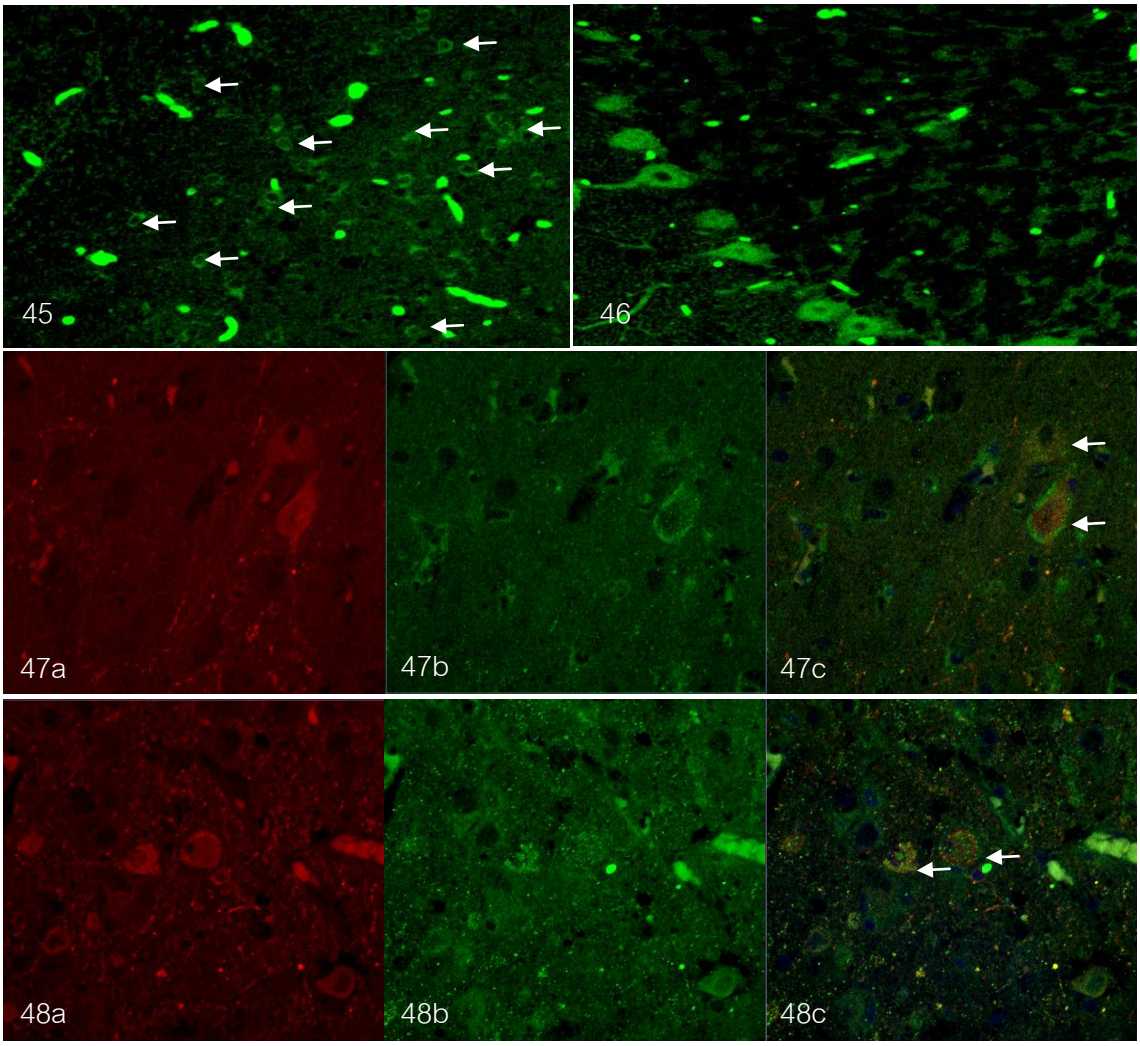
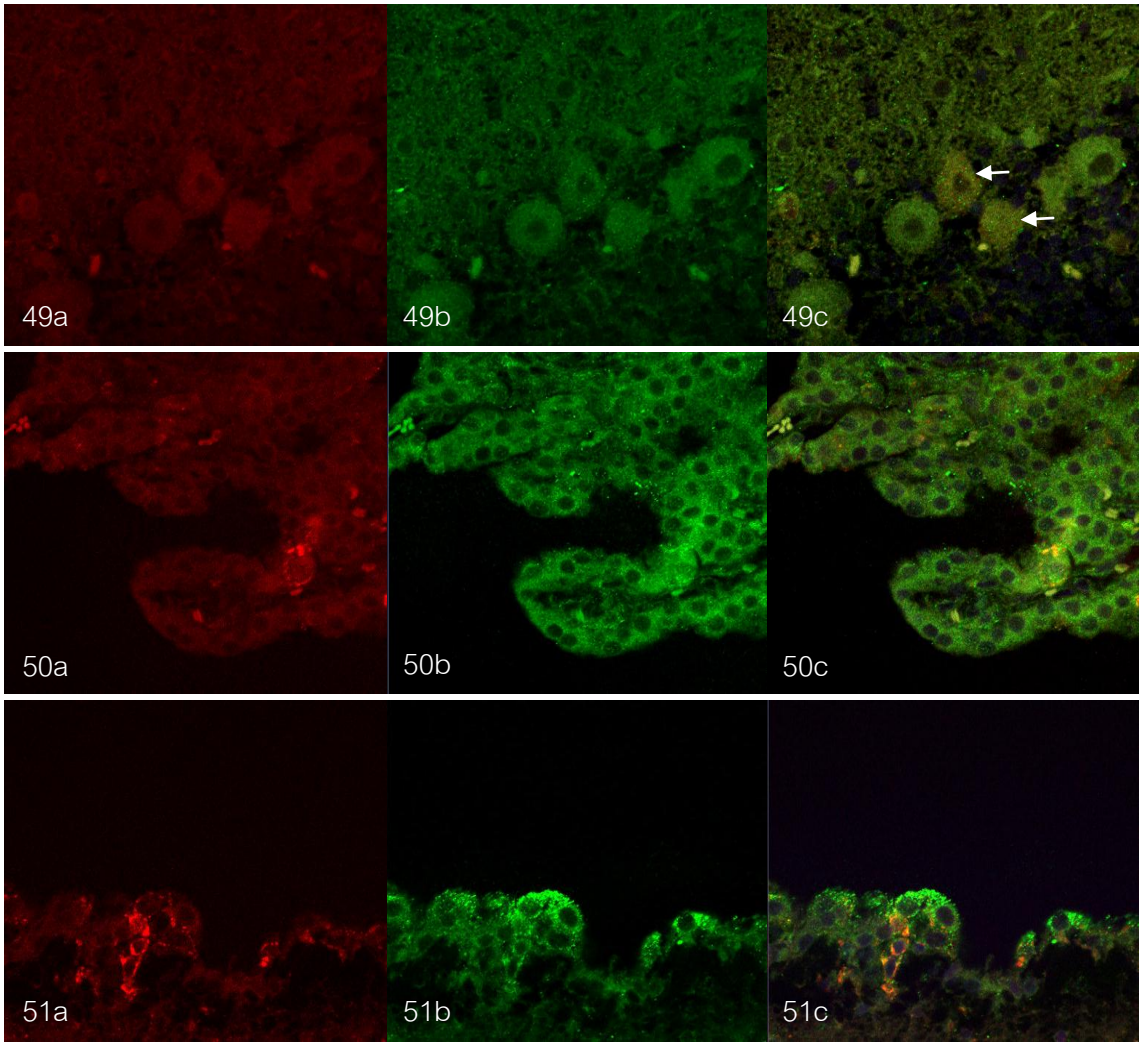


Figure 45: nectin-4 positive in neuron (arrow), cerebrum; negative control (2,000 fold magnification).

Figure 46: nectin-4 positive in Purkinje's cells, cerebellum; negative control (2,000 fold magnification).

Figure 47-48: The double immunofluorescence staining between CDV (red; a panel) and nectin-4 (green; b panel) in neuronal cytoplasm with illustration of overlay (c panel) of co-expression (arrow) and nucleus (blue, counterstained DAPI), cerebrum; TH10/03 (4,000 fold magnification).



The double immunofluorescence staining between CDV (red; a panel) and nectin-4 (green; b panel) with illustration of overlay (c panel) and nucleus (blue, counterstained DAPI

Figure 49: The co-expression in Purkinje's cells (arrow), cerebellum; TH11/03 (4,000 fold magnification).

Figure 50: The co-expression in choroid plexus cells, cerebellum; TH10/01 (4,000 fold magnification).

Figure 51: The co-expression in ependymal cells, cerebellum; TH10/01 (4,000 fold magnification).

CHAPTER V

DISCUSSION AND CONCLUSION

Canine distemper is a seriously infectious disease which threaten many mammals particularly canids. Despite usage of commercial live attenuated CDV vaccine for controlling and prevention, it remains predominately viral infected diseases in Thailand (Keawcharoen et al., 2005, Posuwan et al., 2010) and Veitnam (Lan et al., 2009). The clinical manifestations display multisystemic syndrome including anorexia, lethargy, oculonasal discharge, pneumonia, hyperkeratosis of nasal planum and foot pad, skin rash and bloody diarrhea. When disease progresses, the initial symptoms involve nervous signs such as convulsion and myoclonus, and also host immunosuppression (Beineke et al., 2009, Tan et al., 2011). The specimen that restrospectively collected since 2007-2012 from Thailand (13 samples) and Vietnam (7 samples), showed varied symptoms. While, in this study, predominant sign involved nervous system in 75 percentages of occurring, the respiratory signs were primarily detectable symptom. The others were detected as diarrhea. The susceptible age of infection was adolescent through adults. In spite of prior vaccination, dog could be infected by CDV since effectively wild strain and improper vaccine stimulation (Keawcharoen et al., 2005, Lan et al., 2006).

Associated lesions of CDV infection

Following histopathological examination, the naturally infected dog obviously demonstrated distinguishing eosinophilic intracytoplasmic and intranuclear inclusion bodies in diverse organs representing as a pathognomonic lesion of CDV infection. The interplay between individual immune response and opportunistic infection play a role in progressive clinical manifestations and severe pathological changes. The viral agent disseminate

predominately in lymphoid organs, respiratory tract, gastrointestinal tract, urogenital tract and nervous system indicating via immunohistochemical labeling.

Due to primary tropism organ of infection, lymphoid cell depletion was detectably seen in lymphatic tissues, including lymph node, tonsils, gut associated lymphoid tissue (Payer's patch) and spleen. There has been already proved that CDVs induce lymphoid apoptosis leading to lymphopenia and inhibition of lymphoproliferation and resulting in generalized immunosuppression (Kumagui et al., 2004; Schobesberger et al., 2005; Kajita et al., 2006; Yanagi et al., 2009). Moreover necrosis and inflammatory cells influxes also were noticed in many samples. The respiratory tract showed varied microscopic findings with or without suppurative outcome because of acute response to secondary bacterial infection such as *Bordetella brochiseptica* and *Mycoplasma* spp. Moreover, mixture infection by other pneumonia related viruses including canine adenovirus type2 (CAV2) and canine parainfluenza virus type 2 (CpiV2), has been described (Damian et al., 2005). In gastrointestinal tract, positive immunocoloring were abundantly noticed in glandular epithelial cells through lymphocytic cells in lamina propria. It has been reported that existing CDV in gastric tissues shows locally infection neither evidence of nervous signs nor immunohistochemical detection in cerebellum (Kanet and Ortatatli, 2011). The urogenital tract including kidney and urinary bladder is also viral existing organ especially renal pelvis and transitional epithelia.

The central nervous system (CNS) when CDVs actively replicate leading polioencephalitis, meningitis and leukoencephalomalacia (severe demyelination). Conventionally, CDV induced pathological outcomes are acute to chronic demyelinating encephalomyelitis, in addition, alternative varied neuropathological lesions are categorized such as acute encephalopathy, acute encephalitis, polioencephalomalacia, inclusion body encephalitis, old dog encephalitis and post-vaccinal encephalitis (Amude et al., 2010). In this study, we classified the CNS lesions as non-pathological change, acute and chronic group that disassociated other organ. It implies peracute, acute and chronic stage of

infection, respectively. All groups were notably detected in different kinds of cells depending on morphology, such as astrocytes, meningeal cells, ependymal cells, choroid plexus, Purkinje's cells and also neuron as similar as previously investigations of immunohistochemical expression of nucleocapsid protein (Baumgärtner et al., 1989; Vandevelde et al., 1985; Frisk et al., 1999).

Following our dual immunohistochemical procedure, we distinctly showed the co-visualization of GFAP and CDV antigen, which as same as CDV and NeuN, that clarified target CDV invaded cells. Moreover, it was predominately revealed positivity in astrocytes. We suggested that the disseminated route of CDV infection is via hematogenous, hence the scatter astrocytes, gial cells surrounding blood vessels, are the first cell barrier and initial viral infection. When brain is infected resulting in acute encephalitis, the immense elevating number of astrocytes is recognized, via GFAP positive staining (Headley et al., 2001). The vimentin positive astrocytes have been published in prolong aggressive demyelinated canine brain, while acute infected lesion, GFAP positive astrocytes with co-habiting CDV antigen were recognized (Seehusen et al., 2007). Astrocytes are not only the preliminary affected cells in the brain, but also involve in viral persistence (Wyss-Fluehmann et al., 2010). Likewise hematogenous route of infection via astrocyte, the choroid plexus and ependymal cells, which immunohistochemically showed the infection of CDV, were also associated with viral invasion throughout CNS and spread to cerebrospinal fluid (CSF) (Rudd et al., 2006). The viral RNA of CDV could be detected in CSF in affected dog by using reverse transcription-PCR (Frisk et al., 1999).

Several studies of CDV infected difference cell type of CNS have been documented. For instance, the increasing number and function of microglia following CDV *ex vivo* experiment has been described by using flow cytometric and immunophenotyping methods. Besides, CDV-persisting microglial elevation in chronic affected samples revealed marked demyelination (Stein et al., 2004). Our study was also demonstrated the same result, CDV infected microglia, immunological co-localized Iba-1 and CDV, were noticed.

While a restricted CDV infection in oligodendrogial cells has been explored *in vitro*. In addition, morphological comparison of infected brain with demyelination between in situ hybridization (ISH) and immunohistochemistry revealed the greater expression of viral RNA than viral protein. These findings indicate that oligodendrocytes able to be infected by CDV, nevertheless rarely detected the expression of viral protein (Zurbriggen et al., 1998). Although different CDV strains infect dissimilar cell types has been reported (Vandevelde and Zurbriggen, 2005), just certain part of samples were genomic categorized by sequencing. The correlation between CDV lineage and particular cell type infection remains unclear.

Alternative cellular receptor of CDV

To understand the pathogenesis, the cellular receptor facilitating viral uptake should be comprehended. As well-known acceptance, dog SLAM, restrictedly expressed on canine immune system cells such as dendritic cells, macrophages, immature thymocytes and lymphoid tissues, is the crucial receptor for CDV influencing virulent evidence (Minagawa et al., 2001; Tatsuo et al., 2001; Seki et al., 2003; Sidorenko and Clark, 2003). In several organs of dogs including lung, stomach, intestine and transitional epithelial cell of the urinary tract, the expression and distribution of SLAM positive cells resembling lymphocyte and macrophage are identified by using immunohistochemistry. In brain, SLAM positive cells are located in blood vessel wall. Interestingly, the up-regulation of SLAM receptors in many organs is shown while acute infection of CDV indicating progression of viral amplification, eventhough that in human are down regulation (Techangamsuwan et al., 2009[2]; Wenzlow et al., 2007). Whereas, *in vivo*, epithelial and nervous cells are silencing SLAM, those infected cells still reveal viral inclusion bodies by histopathological findings.

Backward to the previous viral researches, Vero cells and Madin-Darby canine kidney (MDCK) cells are conventionally mediated for *in vitro* mimic CDV infection, although there are lacking SLAM. Nowadays, dog SLAM expressed Vero cells with tag, called Vero-

DST cells, have been developed for wild type CDV isolation (Seki et al., 2003; Lan et al., 2005). The Vero-DST lines effectively initiate cytopathic effect (CPE) post various strains of CDV inoculationcharacterized by scattered small round cells and syncytial formation (Radtanakatikanon, 2011). Interestingly, the blind SLAM CDV which effectively infects primary ferret epithelial cells comparison with wild type strain has been generated for researches (von Messling et al., 2006). Hence, there is probably other cellular receptor that virus can use for cell entry.

Several alternative cell receptors have been paid attentions as related Morbillivirus receptors. The conventional CD46, a membrane cofactor protein, is rather Measles virus (MV) vaccine strain tropism receptors than CDV (Galbraith et al., 1998; Erlenhofer et al., 2002). However, only the neoplastic lymphoid cells of dog have been exhibited CD46 *in vitro* (Suter et al., 2005), whereas it is broadly expressed on nucleated cells, lymphocytes, endothelia and epithelia in human. In addition, the down regulation of CD46 is observed in human brain following MV infection (McQuaid. and Cosby, 2002). Heparin sulfate (HS) molecule has been revealed as receptor and involved in CDV infection, despite it expresses only in human 293 cell line (Fujita et al., 2007). CD9, a tetraspan transmembrane protein (TM4), is associated with CDV induced cell-to-cell fusion and also syncytial formation but it is not precisely CDV specific receptor (Schmid et al., 2000). Recent study exhibits the other unidentified receptor of CDV in chicken embryo fibroblast and Vero cells, which unlike CD9, as 57-kDa and the 42-kDa protein, respectively (Singethan et al., 2006; Chen et al., 2011). In neuron, the neurokinin-1 (NK-1) acting as a receptor for trans-synaptic spread of MV in transgenic mice models, have been explained eventhough it is not a direct receptor for MV (Makhortova et al., 2007). Additionally, N protein of MV (MV-N)incorporate with a transmembrane glycoprotein of target cells, called CD147 or extracellular matrix metalloproteinase inducer (EMMPRIN), via mostly cyclophilin B rendering invasion through cell (Watanabe et al., 2010). However, both NK-1 and CD147 have not been investigated in CDV research yet.

Besides SLAM receptor, elusive epithelial receptor (EpR) for Morbillivirus has been recently identified. The wild type MV produced syncytia in the independent SLAM human epithelial lung cells which have tight junction formation (Leonard et al., 2008). Until nowadays epithelial junction receptor, nectin-4, has been precisely reported for MV infection (Noyce et al., 2011; Mühlebach et al., 2011). It might be act as an epithelial cellular receptor for CDV in a similar manner of MV infection. Recently, the dog nectin-4 expressed Vero cell line was generated by transfecting Vero cells with pCXN2-dNectin-4. Following CDV inoculation *in vitro*, the Vero/dogNectin-4 cells revealed syncytia formation accordingly with Vero-DST cells infected with wild CDV strain (Pratakpiriya et al., 2012). Though, CD147 (MV related receptor, expressed in epithelial, endothelial, and neuronal cells) is used as an entry of morbillivirus *in vitro* (Yurchenko et al., 2005; Watanabe et al., 2010). Also Human Immunodeficiency Virus type1 (HIV-1) is associated with interaction between cyclophilin A and CD147 prior penetration into target cells (Pushkarsky et al., 2001). However, those findings have not been postulated *in vivo* yet. On the other hand, this present study directly revealed the nectin-4 as a cellular receptor in canine tissues. Despite the fact that the binding affinity of MV-N to CD147 has been documented, it is not a predominant protein involving natural infectivity. In addition, the anti-CD147 antibody partially inhibited 40% of MV infection in HEK293 cells (Watanabe et al., 2010), whereas anti-nectin-4 antibody completely blocked wild strain CDV infection in Vero/dogNectin-4 cells (Pratakpiriya et al., 2012).

Distribution of nectin-4 expression in canine tissues

Nectin-4 is normally expressed in human epithelial cells, placental trophoblastic cells, hair and skin by using immunohistochemistry (Reymond et al., 2001; Brancati et al., 2010). Moreover, Human Protein Atlas Project (www.proteinatlas.org) revealed the strength of nectin-4 protein expression as weak immunoreactivity in many tissues including neuronal cells, respiratory epithelial cells in bronchus and glandular epithelial cells of several organs

such as uterus, prostate gland, epididymis. Strong to moderate positive glandular epithelial cells of gastrointestinal tract and tubular epithelial cells of kidney are also exhibited. However, the controversial finding is reported that nectin-4 is hardly found in normal human tissues, for instance heart, lung, liver, kidney and trachea but abundantly expressed in various neoplastic cells (Takano et al., 2009). Through our study, we demonstrated the expression of nectin-4 by using immunohistochemistry technique in a variety of canine tissues that clinical healthy and natural CDV infection. Based on sequence data available in Genbank, the homology of amino acids sequences between human and dog nectin-4 is 94% identity. Therefore, the specificity of human nectin-4 antibody was interspecies cross reaction with positive results in mouse brain as positive control.

We demonstrated that the expression of nectin-4 in canine tissues was similar as that in humans. Independence of CDV infection, nectin-4 expressed in various canine tissues including neuron, Purkinje's cells, glandular epithelial cells, bronchial epithelium, renal tubular epithelial cells and renal pelvis epithelium. This is in agreement with Measles virus experimental infection in primates (Mühlebach et al., 2011). They showed nectin-4 immunofluorescent positivity in the tracheal epithelium, however, they did not mention regarding nectin-4 expression in mock-infected control. Recently, *in vitro* study also demonstrated the down-regulation of nectin-4 expression following Measles virus infection (Noyce et al., 2011). In agreement with a previous study, nectin-4 receptors are massively expressed by immunofluorescence as already shown in bronchial, bronchiolar, gastric and intestinal glandular epithelial cells, transitional epithelial cells, renal pelvis epithelia, epithelium of tonsil and keratinocytes of epidermis.

In order to identify the cell type that expressed nectin-4 in brain, the dual immunohistochemistry labeling nectin-4 and NeuN has been performed. It exhibited the coincidence of both nectin-4 and NeuN in cytoplasm. In contrary, double staining of nectin-4 with GFAP and nectin-4 with Iba-1 were separately presented in each cell. We suggested that nectin-4 was markedly expressed in neurons, not astrocytes or microglia, of infected

and non-infected host. In cerebellum of negative sample and also CDV infected group, Purkinje's cells were also immunohistochemically expressed nectin-4 as weak positive and precisely revealed by immunofluorescent staining.

In spite of nectin-4 expression in neuronal cells is noticed in infected and non-infected brain, the intensity and distribution of nectin-4 are remained unclear. Our study showed only the sporadic expression especially at cortico-medullary junction in cerebral cortex and neurons which positive NeuN. Surprisingly, each histopathological group of brain is demonstrated varied detectable intensity of nectin-4. The non-infected brain is occasionally seen as similar as chronic group, while acute and no pathological lesion group were abundantly detected. It might be up-regulation of nectin-4 when peracute to acute stage of CDV infection and down-regulation when chronic lesion is occurred.

For the nectin-4 expression in epithelial cells, the intensive staining is observed in suprabasal to granular layer of epidermis of human skin as well as cultured keratinocytes (Brancati et al., 2010). In this study, the expression of nectin-4 in each one sample of canine tonsil's epithelia and also epidermis was noticed by immunohistochemistry and immunofluorescence methods. The investigation of nectin-4 expression in canine foot pad has not been done yet because of lacking samples and specimens were not evidenced of signs hence foot pad were not collected to pathological identification.

Nectin-4 is a specific receptor for canine distemper virus

Morphologically, the cohabiting appearance of double immuno-positivity of CDV and nectin-4 were seen in many organs including bronchoepithelial cell, renal pelvis epithelium and glandular epithelial cells of gastrointestinal tract, transitional epithelial cells and epidermis. In brain, some cohabiting positivity in neuron was also occasionally observed. Therefore this finding is suggested that the nectin-4 might play a role as an alternative cellular receptor for CDV. At first, CDV utilize dog SLAM for primary infection on immune cells. Subsequently, they act as a Trojan horse by carrying infective virus to

peripheral tissues such as epithelial-lining organs or brain. Finally, it enters through those dog SLAM$^-$/nectin-4$^+$ cells.

Recently, there have been reported that the human nectin-4-blind CDV infected experimental ferrets showed no clinical signs except leukopenia because those blind viruses could infect and immensely spread to immune cells which are SLAM$^+$(Sawatsky et al., 2012). Besides, recombinant SLAM blind CDV is exactly avirulent in experimental ferret (von Messling et al., 2006). Hence, that supports our suggestion of infection pathways. On the other hand, blind EpR-MV, mutagenized H protein residuals, infected experimental primates showed rash and anorexia (Leonard et al., 2008). In that case, we suspected that the created MV might remain an affinity to nectin-4 leading minor clinical symptom of primates and the role of nectin-4 as morbillivirus receptor remained poor understand at that time. Moreover, CDV contains a stronger affinity with epithelial receptors than MV based on the finding that MV possesses a low ability to epithelial infection in primate model. Hence, the difference of viral ability might be interfered comparison between MV and CDV (Ludlow et al., 2009). In addition, *in vitro*, nectin-4 blind CDV inoculated dog and ferret cell lines did not showed CPE especially syncytia formation, conversely, inoculated Vero-DST cells notably revealed (Sawatsky et al., 2012). Based on the fact that that epithelial receptor, nectin-4, are abundant in basolateral area of epithelial cells, thus MV infects cells through that surface and shed by cell-cell via apical side (Noyce et al., 2011).

Whereas comparing CDV immunohistochemically stained neuron in the same areas, the number of CDV and nectin-4 colocalized cells were lesser. In strongly labeling CDV positive cell, the nectin-4 reactivity could not morphologically detected while surrounding neurons abundantly expressed nectin-4. However, the precise evidence of co-localization was enhanced by using immunofluorescence staining. We hypothesized that nectin-4 is an initial receptor for viral attachment, and then, it might be down-regulated when viral fusion and propagation in affected neurons. Furthermore, CDV might induce not only the up-regulation and expression of nectin-4 in naïve surrounding neurons during peracute to

acute stage of infection, but also the down-regulation of this epithelial receptor when progressive lesion in chronic infection.

The CDV infected Purkinje's cells have been reported (Vandevelde et al., 1985; Frisk et al., 1999), whereas the receptor remained unclear. There has been published that Purkinje's cells in cerebellum of spontaneous CDV infection revealed a marked synaptosomal-associated protein (SNAP-25) expression especially in aggressive demyelination. SNAP-25 is a membrane protein which associates with neurotransmitter secretory function, although its function as a receptor for viral fusion is not yet exactly known (Bregano et al., 2011). Nevertheless, in this study, we could show the expression of nectin-4 in Purkinje's cells and also co-habiting with CDV. Moreover, the ependymal cells of choroid plexus and ventricles expressed nectin-4 with co-localizied CDV antigens. Taken together, we suggested that nectin-4 is one of the crucial receptor for viral infection in those cells when CDV infection occurs through hematogenous route.

While CDV intracerebral injected weanling mice showed hind-limb paralysis (Gilden et al., 1981) and its antigens were also detectable in brain by using immunofluorescene method (Bernard, et al 1983), the CDV inoculated Dog SLAM transgenic C57BL/6 mice showed neither nervous signs nor CDV antigens detected by RT-PCR and viral isolation (Techangamsuwan et al., 2010). These investigations leading us to assume that an abundant expressing nectin-4 in mice brain might be involved in CDV-induced pathogenesis in nervous system. This assumption needs to be confirmed in a future study.

The nectin-4 abundantly expresses in various adenocarcinoma cell lines from lung, breast and ovarian, which are susceptible to MV inoculation and show cytopathic effect. MDCK cells, originated from canine kidney epithelial cells, demonstrate nectin-4 protein on cellular surface (Noyce et al., 2011) and commonly apply to viral isolation for CDV. Despite the CDV isolation from MDCK cells is effective, CPE inadequately occur and prolong detectable viral antigen (Lednicky et al., 2004). Nevertheless, Tan and colleagues (2011) demonstrated the CDV induced CPE in inoculated MDCK are occurred as syncytial

formation, and viral isolation after 6 passages still remained virulent capacity to do infect the new host. This evidence was not true for MV infection with MDCK cells, which only expressed nectin-4 on their surface. The investigator speculated that dog's and human's nectin-4 were different and led to the differential outcomes (Noyce et al., 2011). Resulting from numerous previous research, several cell lines, such as Vero cells, B95a B-cell and hamster (HmLu, BHK) cells, have been implicated as a mimic infection of CDV *in vitro* (Seki et al., 2003; Sultan et al., 2009). The primary brain cell cultures were also infectable in various cell types including neuron, astrocyte, oligodendrocyte and microglia (Zurbriggen et al., 1998). In addition, olfactory primary cells and Schwann cells are CDV tropism (Techangamsuwan et al., 2009[1]). Nevertheless, all of above mentioned cells, except MDCK, that effective for CDV isolation, have not yet investigated the expression of nectin-4.

As mentioned, keratinocytes *in vivo* and *vitro* in human research significantly express nectin-4 (Brancati et al., 2010). While CDV related dermal manifestations are skin rash, hyperkeratosis of footpad or nasal planum and pustular dermatitis, additionally pathognomonic intranuclear and/or intracytoplasmic inclusion bodies are observed in keratinocytes of foot pad. Even though CDV has been immensely detected in keratinocytes of infected foot pad (Grone et al., 2004), the expression of SLAM in foot pad of both normal and CDV infected dogs could not demonstrated by immunohistochemistry method (Wenzlow et al., 2007). Despite, we are able to show evidence of nectin-4 on keratinocytes in epidermis of CDV infected dogs, more supporting investigation of revealing nectin-4 on keratinocytes in foot pad should be done to ensure.

As well documented, tonsil is counted as a preliminary target of CDV infection via SLAM-positive dendritic cells or lymphocyte. Towards our observation, canine tonsil epithelium contained nectin-4 expression which may facilitate the portal of SLAM-independent CDV attachment. This finding was also evident in transitional epithelial cells of urogenital tract (Wenzlow et al., 2007). Which and when those receptors play a role should be investigated subsequently. Interestingly, Mühlebach and colleagues (2011) found that H

protein of MV bind with nectin-4 displays the strongest affinity by determining kinetic parameters, whereas the H-MV bind with SLAM more efficiently than that with nectin-4 in generated cells.

In human oncology researches, increasing level of nectin-4 in serum or tissues might apply for particular tumor marker or stage of metastasis. Using ELISA technique, an overexpression of nectin-4 due to its cleaving by metalloproteinase ADAM17 (A Disintegrin And Metalloproteinase 17) in serum and tissue of breast and ovarian cancer-bearing patients is recognized (Fabre-Lafay et al., 2007; DeRycke et al., 2010). This shed some light on the veterinary community because the detection of nectin-4 and metalloproteinase ADAM17 in canine serum as well as tumor research are not yet reported, though it deserves for further investigation in dog. Probably it might be comprehensive pathway to protect epithelial cells or neurons from CDV uptake via nectin-4 receptor.

CDV shed the infective particles via a various kinds of discharges (oculonasal discharge, saliva, feces and urine) and able to be detected by using reverse transcriptase polymerase chain reaction (RT-PCR) in naturally 3-30 days post infected dogs (Saito et al., 2006). According to experimental primates, there was no viral shedding cross airway epithelium in epithelial receptor blind MV inoculated model (Leonard et al., 2008). Moreover, in ferret model, the viral shedding from blind-nectin-4 generated CDV infected specimens were not detected through urine and throat swab (Sawatsky et al., 2012). These observations maybe supported by and in agreement with our results. We notably revealed the presence of nectin-4 in respiratory airway, epithelia of tonsils and renal pelvis epithelium with CDV co-expression. We assumed that it might be the pathway of viral shedding through urine and oral discharge as similar as previous reports.

In spite of the infectivity of CDV in various brain cells *in vivo* and in primary dog brain cell culture (DBCCs) which predominately comprised of astrocytes, there are lack of SLAM expression in both systems. Upon the variant time of post CDV inoculation in DBCCs *in vitro*, they did not express or up-regulate SLAM level (Wyss-Fluehmann et al., 2010).

Nevertheless it has been reported the partial SLAM expression in pathological areas of brain by immunohistochemistry, but the specific SLAM positive cell type did not declare (Wenzlow et al., 2007). Due to the neurological related CDV is a model of MV which involves in multiple sclerosis in humans, this finding might be a part of progression of neuropathogenesis.

By the way, there remain many questions about nectin-4 and CDV infection. How conformational changes of CDV and nectin-4 binding on host cell? What role of nectin-4 in aspect of viral shedding? Does nectin-4 spread viral progeny via only cell-to-cell or virus-to-cell or both? What is a specific astrocytic receptor for CDV when astrocytes are modified both silencing SLAM and nectin-4 receptor? All of them draw us to pay attention in future study

Conclusion

CDV is lymphotropic and epitheliotropic virus which predominately infect lymphoid organs and distinctly effect central nervous system and epithelia of diverse organs. The nectin-4 is lately assured as MV epithelial receptor. Following our study, nectin-4 are obviously expressed in various canine tissues as same as that in human. In addition, we suggested that CDV also utilize the nectin-4 as an alternative potential cellular receptor through host cells especially various epithelial cells and neuronal cells. These findings might play a key role to elucidate the pathogenesis of canine distemper during viremia and spread to multiple organs. Furthermore, nectin-4 distribution of whole body and the consequence in target cells after viral attachment via nectin-4 receptor are still elusive. The astrocytic specific receptor for CDV entry is also unclear. Hence further investigation should be attempted to find out the pathogenesis of CDV infection in depth.

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