การศึกษาทางพยาธิวิทยาของการติดเชื้อระยะเฉียบพลันของ CANINE DISTEMPER VIRUS สายพันธุ์ SNYDER HILL ในหนูถีบจักรที่ตัดแต่งจีน DOG SIGNALING LYMPHOCYTIC ACTIVATION MOLECULE (DogSLAM)

นาย สุชนิทธิ์ งามกาละ

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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PATHOLOGICAL STUDY OF SNYDER HILL STRAIN IN ACUTE CANINE DISTEMPER VIRUS INFECTION IN TRANSGENIC MICE BEARING DOG SIGNALING LYMPHOCYTIC ACTIVATION MOLECULE (DogSLAM)

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สุชนิทธิ์ งามกาละ : การศึกษาทางพยาธิวิทยาของการติดเชื้อระยะเฉียบพลันของ CANINE DISTEMPER VIRUS สายพันธุ์ SNYDER HILL ในหนูถีบจักรที่ดัดแต่งจีน DOG SIGNALING LYMPHOCYTIC ACTIVATION MOLECULE (DogSLAM). (PATHOLOGICAL STUDY OF SNYDER HILL STRAIN IN ACUTE CANINE DISTEMPER VIRUS INFECTION IN TRANSGENIC MICE BEARING DOG SIGNALING LYMPHOCYTIC ACTIVATION MOLECULE (DogSLAM)) อาจารย์ที่ปรึกษาวิทยานิพนธ์: รศ. น.สพ. ดร. อนุเทพ รังสีพิพัฒน์, อาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. น.สพ. ดร. เรียวจิ ยามากูชิ และ รศ. สพ.ญ. ดร. อัจฉริยา ไศละสูต, 66 หน้า, ISBN: 974-53-2474-4

วัตถุประสงค์ของการวิจัยนี้เพื่อทำการศึกษาทางพยาธิวิทยาของการติดเชื้อระยะเฉียบพลัน ของเชื้อไวรัสไข้หัดสุนัข สายพันธุ์ Snyder Hill ซึ่งไวรัสนี้จัดอยู่ในวงศ์ Morbillivirus และเป็นสาเหตุสำคัญที่ก่อให้เกิดอัตราการตายที่สูงในสุนัข โดยทำการศึกษาในหนูถีบจักรที่ดัดแต่งจีน DogSLAM หรือ CD150 ทำการแบ่งกลุ่มของหนูที่ให้ผลบวกต่อการตัดต่อจีนตัวรับ DogSLAM รุ่นที่ 1 ซึ่งมีอายุ 3 และ 12 สัปดาห์ทั้งเพศผู้และเพศเมียออกเป็น 4 กลุ่มดังนี้ กลุ่มที่ 1 (กลุ่มควบคุม) ทำการฉีดอาหารเลี้ยงเชื้อชนิด Dulbecco's modified eagle's medium (DMEM) กลุ่มที่ 2 3 และ 4 ทำการฉีดเชื้อไวรัสไข้หัดสุนัขสายพันธุ์ Snyder Hill จำนวน $1x10^5$ $TCID_{50}$ เข้าจมูก สมองและช่องท้องตามลำดับ สังเกตอาการทางคลินิกในหนูทุกกลุ่มที่เวลา 12 และ 24 ชั่วโมงภายหลังการฉีดเชื้อ และทุกวันติดต่อกัน 14 วันหลังจากฉีดเชื้อ รวมทั้งทำการศึกษาถึงค่าพารามิเตอร์ต่างๆคือ น้ำหนัก การป้ายเซลล์เยื่อบุตาขาวเพื่อตรวจหาอินคลูชั่น บอดี้ของเชื้อไวรัสไข้หัดสุนัข ผลโลหิตวิทยา ชันสูตรรอยโรคทางมหพยาธิวิทยาและจุลพยาธิวิทยา การย้อมอิมมูนโนฮิสโตเคมีเพื่อตรวจหาเชื้อไวรัสไข้หัดสุนัขและส่วนโปรตีนฮีแมกกลูตินิน การตรวจหาเชื้อไวรัสไข้หัดสุนัข โดยวิธีปฏิกิริยาลูกโซ่โพลิเมอเรส (RT-PCR) และการเพาะแยกเชื้อไวรัสจากตัวอย่างอวัยวะภายในต่างๆ

ผลการศึกษาพบว่าทุกพารามิเตอร์ในการศึกษาทางพยาธิวิทยาของการติดเชื้อระยะเฉียบพลัน ของเชื้อไวรัสไข้หัดสุนัขสายพันธุ์ Snyder Hill ในหนูถีบจักรที่ดัดแต่งจีน DogSLAM ให้ผลลบ สรุปได้ว่าการศึกษาโรคไข้หัดสุนัขในหนูถีบจักรที่ดัดแต่งจีนนั้นให้ผลที่ยังไม่ทราบแน่ชัดแตกต่างจากการศึกษาในเซลล์เพาะเลี้ยง แต่อย่างไรก็ตามควรทำการศึกษาข้อมูลเพิ่มเติมเกี่ยวกับปัจจัยที่มีความเกี่ยวข้องกับการแสดงออกของจีนในกลุ่ม SLAM เพื่อประโยชน์ในการศึกษาการติดเชื้อไวรัสไข้หัดสุนัขในสัตว์ทดลองต่อไป

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

ภาควิชา.....พยาธิวิทยา..... .....
สาขาวิชา.....พยาธิชีววิทยาทางสัตวแพทย์.....
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ลายมือชื่อนิสิต สุชนิทธิ์ งามกาละ
ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม Ryoji Yamaguchi
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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SUCHANIT NGAMKALA : PATHOLOGICAL STUDY OF SNYDER HILL STRAIN IN ACUTE CANINE DISTEMPER VIRUS INFECTION IN TRANSGENIC MICE BEARING DOG SIGNALING LYMPHOCYTIC ACTIVATION MOLECULE (DogSLAM). THESIS ADVISOR : ASSOC. PROF. ANUDEP RUNGSIPIPAT, Ph.D., THESIS CO-ADVISOR : ASSOC. PROF. RYOJI YAMAGUCHI, Ph.D., AND ASSOC. PROF. ACHARIYA SAILASUTA, Ph.D., 66 pp. ISBN: 974-53-2474-4

Canine Distemper Virus (CDV), member of Genus Morbilivirus, caused canine distemper disease, the most important viral disease of dogs with high mortality rate. The objective of this study was to verify acute canine distemper virus, Snyder Hill strain, infection in transgenic mice bearing DogSLAM (canine signaling lymphocyte activation molecule, CD150), as a susceptible host for the pathological study of canine distemper *in vivo*. F1 Transgenic mice, positive of DogSLAM tag receptor, 3 and 12 weeks old, both male and female were divided into 4 groups. Group 1 (control group, 4 mice); Dulbecco's modified eagle's medium (DMEM) inoculation. The CDV, Snyder Hill strain, was inoculated at $1x10^5$ $TCID_{50}$ by different routes in each groups as follow, group 2; intranasal inoculation (6 mice), group 3; intracerebral inoculation (6 mice) and group 4; intraperitoneal inoculation (5 mice). Clinical signs of experimental transgenic mice were observed at 12 and 24 hours post infection and daily monitored until 14 days post-infection. Others parameters included weight, conjunctival swab for detection viral inclusion body, blood profile, necropsy and histopathology of various visceral organs, immunohistochemistry for detection of CDV and anti-hemagglutinin (HA) antigen, RT-PCR for detection of CDV and virus isolation, were subsequently performed.

All observed parameters showed negatively results of Snyder Hill strain CDV infection in transgenic mice bearing DogSLAM gene. In conclusion, the results from this study were unable to elucidate for CDV infection in comparison with the tissue culture study. However, further investigation about factors that related to SLAM gene expression for study of CDV infection in animal model should be concerned and investigated.

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จุฬาลงกรณ์มหาวิทยาลัย

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LIST OF ABBREVIATIONS

ad lib	ad libitum
bp	base pair (s)
°C	degree celsius (centrigrade)
CDV	canine distemper virus
cm	centimeter (s)
CPE	cytopathic effect
DogSLAM	signaling lymphocytic activation molecule in dog
d	day (s)
dL	deciliter (s)
et al.	et alii, and others
fl	femtolitre (s)
g	gram (s)
Hb	hemoglobin
Hct	hematocrit, pack cell volume (PCV)
IC	intracerebral inoculation
IN	intranasal inoculation
IP	intraperitoneal inoculation
M	marker
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
ml	milliliter (s)
µL	microlitre (s)
µm	micrometer (s), micron (s)
MV	measles virus
NRL	no remarkable lesion (s)
n	number
P	positive control

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PCR	polymerase chain reaction
pg	picogram (s)
RBCs	red blood cell (s)
RT-PCR	reverse transcriptase polymerase chain reaction
r .p.m.	revolution per minute
%	percentage
SLAM	signaling lymphocytic activation molecule
vero DST cells	vero Dog SLAM tag cells
WBCs	white blood cell (s)
-	negative
+	positive

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CHAPTER I

GENERAL BACKGROUND

1. Introduction

Several strains of canine distemper virus (CDV) had variable virulence and tissue predilection (Shell, 1990) and this phenomena made questionable on CDV receptor in infected animals (Loffler et al., 1997; Schneider-Schaulies, 2000). Previously reports in human virology showed that signaling lymphocytic activation molecule (SLAM) was a cellular receptor for measles virus (MV), closely related virus to CDV, in human and also shown the dogs had the receptor molecules homolog to human SLAM, which act as cellular receptors for CDV (Tatsuo and Yanagi, 2002)

Several advanced techniques have been developed for the introduction of DNA into cultured eukaryotic cells, and allowed an expression of various genes cloned in an expression vector for the study of gene regulation and protein biosynthesis (Niwa et al., 1991). There was a report about transfection by SLAM of dog (DogSLAM), a cellular receptor for CDV into tissue culture (Vero cell), called vero DogSLAM tag (vero DST) cells, use for CDV isolation (Seki et al., 2003; Lan et al., 2005a ; Lan et al., 2005b). On the other hand, there was no report about DogSLAM because of the studies of diseases caused by CDV in dogs have been restricted owing to lack of suitable animal models. Furthermore, the discovery of entered Morbiliiviruses cellular receptors has facilitated the development of transgenic mice that are susceptible to CDV infection. In this study, we attempt to use CDV, Snyder Hill strain, one of the virulence strain of CDV (Appel, 1969) and caused acute encephalomyelitis with predominantly lesions in gray matter of brain and spinal cord (Summers et al., 1984), to study the pathogenesis in acute CDV infection in transgenic mice bearing DogSLAM. The DogSLAM transgenic mouse under development is expected to serve as a useful animal model for CDV infections and get new knowledge of the function of DogSLAM. The objective of this study is to verify acute canine distemper viral, Snyder Hill strain infection in transgenic mice bearing DogSLAM as a susceptible host for the pathological study of canine distemper *in vivo*

2. Literature Reviews

2.1 Canine distemper virus

Morbilivirus are highly contagious pathogens that caused some of the most viral diseases of human and animal worldwide (Griffin and Bellini, 1996). They are members of genus Morbilivirus including measles virus (MV), dolphin morbilivirus, canine distemper virus (CDV), rinderpest virus (RPV), phocine distemper virus and peste des petits ruminants virus. (Appel, 1987; Lamb and Kolakofsky, 1996).

CDV caused canine distemper disease, the most important viral disease of dogs with high mortality rate (Poston and England, 1992). CDV belonged to genus Morbilivirus, family Paramyxoviridae. The genome organizations of CDV consisted of single-stranded negative–sense RNA. The genomic RNA was encapsulated with the nucleocapsid (N) protein served as a template for transcription and replicated by the viral polymerase large (L) protein and its co-factor phosphoprotein (P). The N, P and L proteins together with the viral RNA constituted the ribonucleoprotein (RNP) complex. The viral envelope contained two integral membrane proteins, the fusion (F) and hemagglutinin (H) proteins, and a membrane associated protein (matrix [M] protein). The H glycoprotein mediated the binding of the virus into cell membrane, and the F protein made fusion of two membranes, which enables the entry of the viral RNP into the cytoplasm (Lamb and Kolakofsky, 1996). (Figure 1)

CDV could infect young dogs through several routes such as aerosol or droplet infection and contact upper respiratory tract epithelium. CDV could be able to replicate in macrophages and lymphocytes of the upper respiratory tracts and disseminated to systemic lymph nodes and various target organs including the spleen, thymus, bone marrow and gut associated lymphoid tissue (Appel, 1987). The second day post-infection, viral antigen appeared in mononuclear cells of bronchial lymph nodes and tonsil followed by peripheral blood mononuclear cells at the third day post-infection. Within six days after CDV infection, all lymphatic tissues became infected and the host developed viremic sign. Between six and nine days after exposure, viral antigen spread from lymphatic tissue to epithelial tissue. Within nine to fourteen days after exposure, the dogs developed clinical signs such as fever, anorexia, diarrhea, respiratory problems and/or neurological signs. (Appel, 1987; Shell, 1990). The macroscopic lesions in susceptible host were

hyperkeratosis of foot pads and nose may be presented, conjunctivitis, mucopurulent discharge from eyes and nose, bronchopneumonia and enteritis (Appel, 1987). In microscopic lesions, there were many syncytial cells formation in various target organs (Summers and Appel, 1985). Moreover, the characterization of CDV infection *in vitro* (cell line) was cytopathic effect (CPE) that appeared up to seven days after co-cultivating of primary canine brain cultures with Vero cells. In co-cultures, CPE was characterized by multinucleated giant cells and rounded cells (Alldinger et al., 1993)

There were many methods to make a diagnosis of CDV, such as, conjunctival scraping, pharyngeal washing to detect eosinophilic intracytoplasmic viral inclusion bodies in epithelial cells. Postmortem specimens should include: lungs, tonsils, bronchial lymph nodes, urinary bladder and brain. Direct immunofluorescent technique was used for detection of viral antigen. Serum neutralization test (SN) and enzyme linked-immunosorbent assay (ELISA) were performed for detection of antibody against CDV infection. Furthermore, whole blood, serum or cerebrospinal fluid (CSF) was appropriate specimens for viral isolation (Poston and England, 1992). Vero cells have been widely used for isolation of CDV but several passages of cell line were required for viral isolation and canine pulmonary macrophages were use for isolation without losing virulence of CDV. (Shin et al., 1995; Kai et al., 1993).

2.2 Canine distemper virus receptor

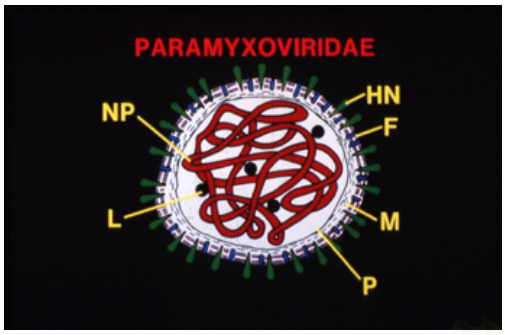
Recently reports showed monoclonal antibody which was able to inhibit CDV infection and bound protein known as CD9 and further experiment supported hypothesis that human CD9 and its homologs in other species were necessary factors for target cells of CDV infection (Loffler et al., 1997). On the other hand, some reports revealed that direct binding of CDV to CD9 could not be demonstrated, suggesting that CD9 was not a susceptible receptor for CDV (Schmid et al., 2000). Recently, many reports focused on human signaling lymphocyte activation molecule (SLAM; also know as CD150), a membrane glycoprotein expressed in some lymphocytes, immature thymocytes and dendritic cells, is a cellular receptor for MV, related closely to CDV (Minagawa et al., 2001; Tatsuo and Yanagi, 2002) (Figure 2). Since the tissue distribution of human SLAM can explain the pathology of Measles, previously reports showed the use of SLAM

as a cellular receptor was a trait common to MV, CDV, and RPV and these Morbiliviruses can use SLAM of non-host species as receptors (Tatsuo et al., 2001; Hahm et al., 2003).

2.3 Transgenic mice

The transgenic mice (Figure 3) are animal that has had DNA introduced into one or more of its cells artificially. This is commonly done in two ways. First of all, DNA can be integrated in a random fashion by microinjecting it into the pronucleus of a fertilized ovum. In this case, the DNA can integrate anywhere in the genome There is no need for homology between the injected DNA and the host genome. Major use for transgenic mice produced by pronuclear injection of DNA is to examine the effects of overexpressing and misexpressing endogenous or foreign genes at specific times and locations in the animal. Second, targeted insertion method is accomplished by introducing the DNA into embryonic stem (ES) cells and selecting for cells in which the DNA has undergone homologous recombination with matching genomic sequences.

In this experiment, we used pronucleus microinjected transgenic mice. Pronuclear injection of DNA is often used to characterize the ability of a promoter to direct tissue-specific gene expression. For example, promoter/enhancer constructs may be used to drive expression of a reporter gene. Many factors influence whether a promoter/transgene construct will express in transgenic mice. The promoters that are used must be known to function appropriately *in vivo*. Transgene constructs may have accumulated mutations during cloning. Perhaps the most important consideration has to do with the trangene's insertion site in the mouse genome. At many chromosomal locations, transgenes will be transcriptionally silent. At others they may express, but with a tissue- and temporal specificity that is not identical to what has previously been seen with the same promoter construct. The intrinsic ability of a promoter construct to drive transgene expression also varies from promoter to promoter (Fielder, 2004). In this experiment, The DogSLAM cDNA, size 1251 bps was subcloned into the eukaryotic expression vector named pCAGDogSLAM tag plasmid and inserted between EcoRI and NotI restriction site. This size of plasmid was 6004 bps that contained CMV-IE (cytomegalovirus-immediate early) and AG (modified chicken β-actin) promoters (Figure 4). We used this plasmid for pronuclear injection in mice. The pCAGDogSLAM tag plasmid was described elsewhere (Niwa et al., 1991).



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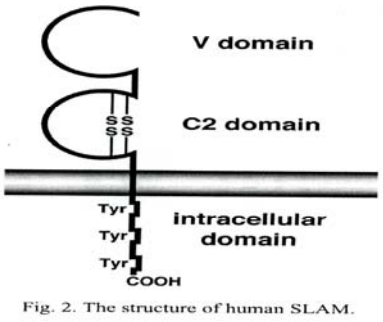

Fig. 2. The structure of human SLAM.

2

Figure 1: The structure of Family Paramyxoviridae; composed of NP, P, HN, L, F and M proteins. (D' Andrea, et al., 2005)

Figure 2: The structure of human SLAM. There were composed of 2 parts; intra and extracellular domain. (Tasuo and Yanagi. 2002)

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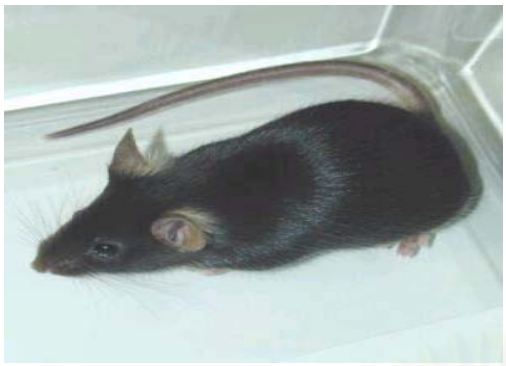

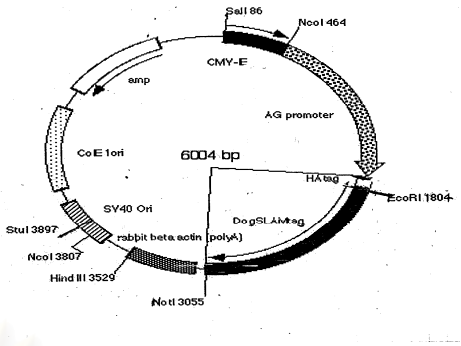

Figure 3: The picture showed 3-week-old, C-57BL/6 strain of transgenic mice (Koike, T. 2006)

Figure 4: The picture showed pCAGDogSLAM tag size 6004 bps. Insert of DogSLAM gene size 1251 bps. [modified from Tatsuo et al., 2001]

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CHAPTER II

MATERIALS AND METHODS

1. CDV viral preparation

Preparation of virus stock and inoculation in cell culture: vero DogSLAM tag (vero DST) cells were generated by Vero cells transfection with pCXN2 and pCAGDogSLAM tag. The cells were grown in DMEM with 7% heat inactivated fetal calf serum, 0.15% sodium carbonate and 0.4 mg of G418 per ml (Seki et al., 2003) until 80% confluent of the Vero.DST cells was observed. The media was aspirated from 80% confluent Vero.DST culture bottle and the cells were washed twice with DMEM 2-3 ml and kept for viral inoculation. 1 ml of 1:100 viral dilution of CDV stock Snyder Hill strain (kindly provided from University of Miyazaki) in DMEM were applied in culture bottle, incubated at 37°C with 4%CO_2 for 60 minutes, added total 9 ml of 10% Tryptose Phosphate Broth (TPB) and DMEM (without FCS) and continue incubated at 37°C with 4%CO_2 for 24 hours. 90% cytopathic effects (CPE) by mean of syncytial cell formation were observed and the virus were harvested and kept at -80°C. Immunofluorescent antibody test (IFA) was applied by mean of CDV viral infection (Figure 5)

The virus stock was melt, resuspended by using pipette and mixed with inoculation cells. The viral suspension was centrifuged at 3,000 rpm, 10 minutes, transferred the supernatant to a new tube and kept in ice box. Viral cell pellets were sonicated with sonicator machine (Biorupture®, Japan), for 7 cycles (each cycle time on for 30 seconds and time off for 1 minute). The viral suspension was transferred and divided about 0.2 ml in small glass bottle and made powder by rapid freeze in the dried-freeze machine (Freeze Zone plus 2.5®, Japan). The other parts were divided in microcentrifuge tube and then kept in -80°C.

2. Animals

50 parent transgenic mice, C-57BL/6 strain that bearing DogSLAM gene, 3-4 month olds, 25 males and 25 female were provided from Kumamoto University and rear in specific pathogen free (SPF) room (authorized by DNA committee) for acclimatization and prepared for the experiment. The mice were fed by sterile food and water and bred by in-house mating. The new

generation of mice (F1) that positive for DogSLAM receptor; were prepared and used for pathological study of Snyder Hill strain of CDV. To confirm F1 that positive for DogSLAM was done by polymerase chain reaction (PCR) (modified from Grunenwald, 2003) as described briefly.

2.1 Preparation of transgenic mice genomic DNA (tail biopsy) (modified from Pearson and Stirling, 2003): A small piece of mice tail (1-2 cm, weight about 100-500 mg) were collected and rapid freezed in liquid nitrogen. The tissue was homogenized in 500 μl of DNA extraction buffer (0.01M Tris-HCl pH 7.6-7.8, 0.01M EDTA, 0.012M sodium citrate and 1% SDS) in microcentrifuge tube, added 25 μl of a 20 mg/ml of proteinase K (Wako Pure Chem®, Japan) and incubated at 37°C overnight with gently shaking. The extraction was centrifuged at 15,000 rpm, 10 minutes, transferred the supernatant to a new tube, added 0.5 ml of phenol : chloroform : isoamyl alcohol (25: 24: 1) and mixed until aqueous phases completely mixed. The mixture were kept for 5 minutes, centrifuged at 15,000 rpm, 5 minutes to separate phases and then transferred supernatant (DNA) to a new tube (repeated until the supernatant was clear). The supernatant was added with 0.5 ml of isopropyl alcohol (IPA), gently mixed, centrifuged at 15,000 rpm, 10 minutes, discard supernatant, added 0.5 ml of 70% ethanol at room temperature and centrifuged at 15,000 rpm, 5 minutes to get pellet DNA. The DNA pellet was vacuum dried and dissolved in 100 μl of Tris-EDTA (TE) (0.01M Tris, 0.001M EDTA, pH 8.0). The contaminated RNA was digested from DNA sample by incubation with 15 μl of standard ribonuclease (RNase) A solution (RNase A 100 mg, 0.1 ml of 1M Tris-HCL (pH 7.5), 30 μl of 5M NaCl, pH 7.5), DW_2 at 37°C for 30 minutes, stop reaction, harvested DNA pellet and dissolved in TE buffer. DNA concentration was determined by spectrophotometry at 260 nm (NanoDrop®, Japan) and then kept in 4°C until used.

2.2 Amplified DNA by polymerase chain reaction: The primers used in this study were DogSLAMtag 1 (forward primer) and DogSLAMtag 6 (reverse primer) (nucleic acid sequence was 5'-GGTACTGCTGCTCTGGGTTC-3' for forward primer and 5'-CCTTCATTTTCCCTCCTGCT-3' for reverse primer, respectively). pCAGDogSLAM tag plasmids were used as a positive control at 210 bp. Master mixed PCR solution (Qiagen®, Germany) composed of 10x PCR buffer 2.0 μl, 25mM $MgCl_2$ 1.5 μl. dNTP 0.5 μl, DogSLAMtag1, 0.4 ml (forward primer), DogSLAMtag 6 0.4 ml (reverse primer), DW_2 12.95 ml. Add Taq polymerase (5 U/μl) (Qiagen®, Germany) 0.25 μl, The total 20 μl of solution composed of 2 μl of DNA and 18 μl of PCR master mix, mixed well, spin

down and entered PCR cycle (PC-802: Astec®, Japan) that composed 3 steps; step 1 (initial PCR activation step) at 95°C for 2 minutes, step 2 PCR reaction had 30 cycles, each cycle composed DNA template denature at 95°C for 1 minute, primer annealing at 50°C for 1 minute and primer extension at 72°C for 1 minute. Step 3 (final extension) at 72°C for 5 minutes. The PCR products were kept at 4°C

2.3 Evaluation of PCR products. The PCR products were applied into 1.2% agarose gel (Invitrogen®, Japan) and electrophoreses in 1x Tris Borate EDTA (TBE), pH 7.6 buffer at electric gradient 100 volts for 30-40 minutes in minigel apparatus (Mupid®-EX, Japan). The gel was stained with 1% ethidium bromide and visualized the PCR products at 210 bps under UV-light. The positive control in this study used pCAGDogSLAM tag plasmid and the PCR products were 427 bps.

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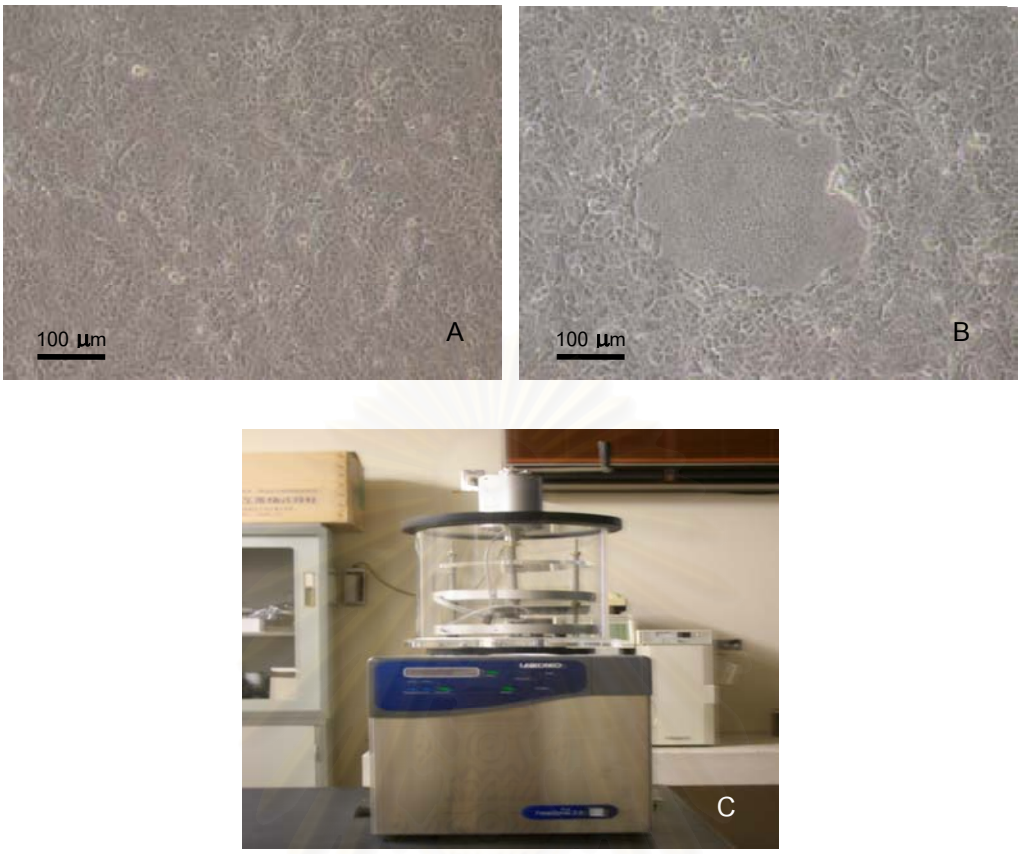

Figure 5: Virus preparation: Phase contrast microscope showed (A) normal vero DST cells; (B) CPE in CDV infected vero DST cells; (C) freezing machine for lyophilization.

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3 Experimental transgenic mice

3.1 Experimental design: according to results of preliminary study, such as, breed line, duration and characteristic of clinical signs, etc. Transgenic mice, positive of DogSLAM receptor (Figure 6) were bred. 3 and 12-week-old, both male and female F1 transgenic mice were divided into 4 groups as follow.

Group 1: Control group composed of 1 transgenic mouse without viral inoculation (12-week-old), 3 transgenic mice (3-week-old) with Dulbecco's modified eagle's medium (DMEM) inoculation, each one was intracerebral, intraperitoneal and intranasal route, respectively.

Group 2: Intranasal inoculation group composed of 6 transgenic mice, 3 and 12-week-old, that intranasal viral inoculation at $1x10^5$ $TCID_{50}$ (3-week-old mice : 12-week-old mice, 3:3).

Group 3: Intracerebral inoculation group composed of 6 transgenic mice, 3 and 12-week-old, that intracerebral viral inoculation at $1x10^5$ $TCID_{50}$ (3-week-old mice : 12-week-old mice, 3:3).

Group 4: Intraperitoneal inoculation group composed of 5 transgenic mice, 3 and 12-week-old, that intraperitoneal viral inoculation at $1x10^5$ $TCID_{50}$ (3-week-old mice : 12-week-old mice, 3:2).

Each group was separately kept of each cabinet in SPF room and fed with sterile food and water *ad lib*.

3.2 Mice inoculation: each group of mice was infected by CDV as follow (Figure 7A-7D).

F1 transgenic mice were anesthetized by ether and infected with CDV, Snyder Hill strain, at high titer ($1x10^5$ tissue culture infectious doses; $TCID_{50}$, unpublished data) by different routes, including intranasal (IN), intracerebral (IC) and intraperitoneal (IP). Intranasal and intraperitoneal inoculation was administered viral suspension into both nares and lateral abdominal midline, respectively. Intracerebral inoculation was done along the skull midline using a tuberculin syringe with 27-gauge needle.

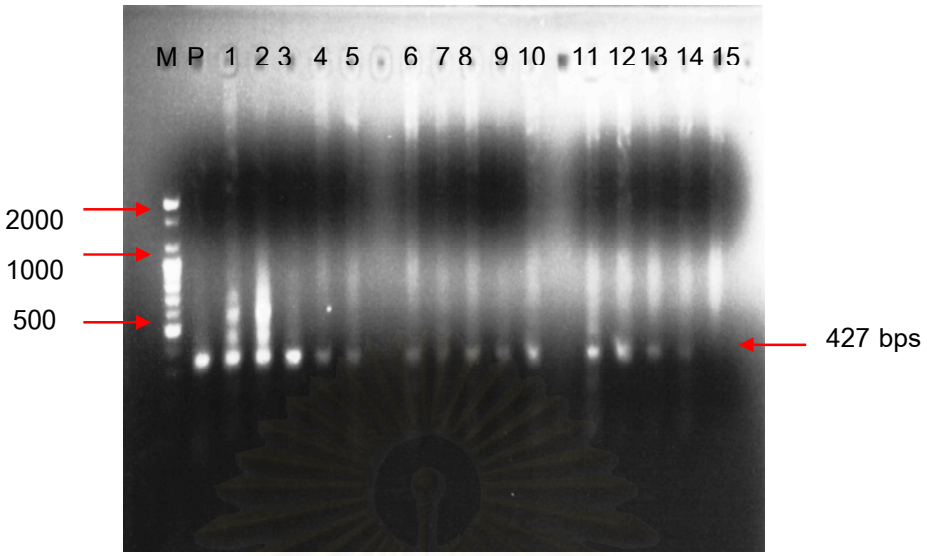

Figure 6: The result of detection of DogSLAM tag gene from DNA of tail biopsy by PCR method. The PCR product showed at position 427 bps.

Lane M: Marker, P: positive control (pCAGDogSLAM tag), Lane 1-15: DNA sample from tail of transgenic mice.

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Figure 7. Anesthesia and euthanasia method, inoculation methods, conjunctival swab technique and, heart blood collection from transgenic mice. The pictures showed (A) anesthesia and euthanasia method by ether. (B), (C) and (D) showed inoculation methods in mice by IN inoculation IC inoculation IP inoculation, respectively. (E) showed conjunctival swab technique and (F) showed blood collection from the heart of transgenic mice.

4 Clinical signs: Clinical signs of inoculated transgenic mice were observed at 12 and 24 hours post infection; including respiratory (ocular and nasal discharge) or gastrointestinal (diarrhea) or nervous system (staggering gate and incoordination). Inoculated transgenic mice were monitored and weighted daily until 14 days post-infection. Moreover, conjunctival swab (Figure 7E) for detection of CDV viral inclusion bodies in conjunctival epithelium, used Dip Quick® and Shorr's stain, was performed every 3 days post inoculation.

5 Necropsy: Inoculated transgenic mice were necropsied after died or euthanized and necropsied at 14 days after infection. The blood was collected from heart (Figure 7F) using EDTA anti-coagulant for complete blood count (CBC) measurement [total red blood cells count (RBCs), hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), thrombocyte count, total white blood cells count (WBCs) and WBC differential cell count]. Gross lesions involving nervous, respiratory and gastrointestinal system were noted. The fresh organs were kept in -80°C for confirmed of CDV viral infection in mice by reverse transcriptase polymerase chain reaction (RT-PCR).

6 Histopathology: Selected specimens (including brain, spinal cord, gastrointestinal (GI) tract, lungs, spleen, lymphoid tissue, thymus, kidney, urinary bladder and prepuce or vagina were preserved in 10% neutral buffered formalin. Routine histological process was performed and the specimens were embedded in paraffin wax, sectioned at 4-6 μm thickness and stained with hematoxylin and eosin (H&E) staining. The microscopic lesions were evaluated under light microscope. The histopathological lesions were scored as NRL: no remarkable lesion, +1: mild degree, +2: moderate degree and +3: severe degree.

Hematoxylin and Eosin (H&E) staining (Lunar, 1968)

After the sections on the slides were dried, the paraffin was removed by xylene for 10 minutes, the same procedure was repeated. The specimen was rehydrated by passing through a gradual series of decreasing concentrations at 100%, 95% and 70% ethyl alcohol, respectively, for 2 minutes with each alcohol concentration. The specimen was washed in running tap water for 5 minutes and stained with Harris hematoxylin solution for 5 minutes. The section was a

bluish-violet color and washed in running tap water for 5 minutes. The section was removed the excess hematoxylin in 1% acid alcohol 1 dip, and washed the excess acid in running tap water for 5 minutes. The section was then neutralized by dipping into saturated lithium carbonate for 4 dips and washed in running tap water for 5 minutes. Counterstain the section with eosin working solution for 45 seconds to produce a pink or red color. After stained, the specimen was dehydrated by passing through a gradual series of increasing concentrations of 95% ethyl alcohol 3 dips and 100% ethyl alcohol twice for 2 minutes of each. The specimen was cleared (made transparent) with xylene twice for 5 minutes. Permanent mounting prepared by covered the specimen with a DePex® mounting medium [contained xylene (mixture of isomer), Dibutyl phthalate] and topped with the cover slip.

Shorr's staining (Page green method) (Lunar, 1968)

After the section on the slide was dried, the paraffin was removed with xylene for 10 minutes, the same procedure was repeated. The specimen was rehydrated by passing through a gradual series of decreasing concentrations at 100%, 95% and 70% ethyl alcohol, respectively, for 2 minutes with each alcohol concentration. The specimen was washed in running tap water for 5 minutes and stained with Harris hematoxylin solution for 5 minutes. The section was a bluish-violet color and washed in running tap water for 5 minutes. The section was removed the excess hematoxylin in 1% acid alcohol 1 dip, and washed the excess acid in running tap water for 5 minutes. The section was then neutralized by dipping into saturated lithium carbonate for 4 dips and washed in running tap water for 5 minutes. Counterstain the section with Shorr's solution for 1 minute to produce inclusion body staining. Then removed excess Shorr's solution in 95% ethyl alcohol. After stained, the specimen was dehydrated by passing through a gradual series of increasing concentrations of 95% ethyl alcohol 3 dips and 100% ethyl alcohol twice for 2 minutes of each. The specimen was cleared (made transparent) with xylene twice for 5 minutes. Permanent mounting prepared by covered the specimen with a DePex® mounting medium and topped with the cover slip.

7. Immunohistochemistry for detected CDV antigen: A labeled streptavidin–biotin (LSAB) was performed for detection of viral antigen in various target organs included the brain and spinal cord, stomach, intestine, lungs, spleen, lymph nodes, thymus, kidney and urinary bladder. The paraffin-embedded specimens were cut 4-6 μm thickness and deparaffinized in xylene for 30 minutes. Antigen retrieval method was performed by autoclave at 121°C, 5 minutes in PBS. Non specific endogenous peroxidase reaction was discarded by add 0.3% hydrogen peroxide (H_2O_2) in methanol at room temperature for 10 minutes. The sections were washed with phosphate buffer saline (PBS) for 5 minutes, 3 times. Primary antibody, monoclonal mouse anti-CDV antibody (Monotope Verostat®) at dilution 1:10 were applied onto section and incubated at 37°C for 60 minutes (or 4°C overnight). The sections were incubated with secondary biotinylated anti-mouse IgG antibody and envision polymer (Envision Polymer DAKO®, Denmark) at 37°C for 30 minutes. The sections were stained with 3,3-diaminobenzidine tetrahydrochloride (DAB) 2 minutes and counterstained with Mayer's hematoxylin. Positive controls used in this study were infected vero DogSLAM tag (vero DST) cells or dog brain tissue of clinical CDV infection whereas negative control was non-infected vero DogSLAM tag (vero DST) cells or normal mouse brain tissue.

8. Immunohistochemistry for detected DogSLAM antigen: It was performed by using monoclonal mouse anti-HA antibody at dilution 1:200 as primary antibody. Tissue used for detection of DogSLAM antigen were the brain and spinal cord, stomach, intestine, lungs, spleen, lymph nodes, thymus, kidney and urinary bladder. Other steps were as same as previous immunohistochemical method for detection of CDV antigen. Positive controls used in this study were vero DogSLAM tag (vero DST) cells whereas negative control was normal mouse brain tissue.

The results of both Ag immunohistochemistry was observed under light microscope and the immunohistochemical positive cells were scored as (Iwatsuki et al., 1995): -: negative, +1: low number of positive cells (<10%), +2: moderate number of positive cells (20-50%) and +3: high number of positive cells (>50%).

9. RT-PCR for confirmed of viral infection in mice (modified from Bartlett and Stirling, 2003): The viral RNA was extracted from the brain, lung, spleen, and/or peripheral blood mononuclear cells (PBMC) by acid phenol guanidine isothiocyanate method (Trizol®, GibcoBRL™, USA). Briefly, 200-300 μl supernatant of homogenized tissue samples was lysed with 300 μl Trizol®, incubated at -80°C overnight, add 60 μl of phenol: chloroform: isoamyl alcohol (25: 24: 1), mixed until aqueous phases completely mixed and centrifuged at 13,000 rpm for 15 minutes at 2 to 8°C. The supernatant was transferred into new tube, added 20 mg/ml glycogen 0.5 μl, spin down, added 150 μl of isopropyl alcohol, stored at room temperature for 15 minutes and centrifuged at 10.000 rpm for 15 minutes. The RNA pellet was wash with 70% ethanol 2 ml, centrifuged at 13,000 rpm for 15 minutes, discarded supernatant, dried at room temperature for 20-30 minutes and dissolved in 100 μl deionized water without shaking and then kept in -20°C until used.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR): RT-PCR was used for CDV RNA amplification of NP gene. The PCR mix was 25 μl in volume containing 5 μl template RNA, 5 μl solution A (one step RT-PCR kit, Invitrogen®) and 10 μl solution B (invitrogen®) composed of 5x reverse transcriptase buffer 4 μl, 0.1M dithiothreitol (DTT) 2 μl, 10mM dNTP 1 μl, DEPC 2.5 μl and 200 U/μl reverse transcriptase 0.5 μl, 1 μl of forward primer, 1 μl of reverse primer and 3 μl of RNA free water. The primers in this study were UPP1, 5'-ATGTTTATGATCACAGCGGT-3' (forward primer) and UPP2, 5'-ATTGGGTTGCACCACTTGTC-3' (reverse primer).

The PCR mix was placed in thermoregulator PC-802 (Astec®, Japan) and the PCR condition as cDNA synthesis at 70°C for 30 minutes and initial PCR activation step at 95°C for 2 minutes. The PCR reaction had 30 cycles, each cycle composed DNA template denaturation at 95°C for 1 minute, primer annealing at 50°C for 1 minute, primer extension at 72°C for 1 minute and final extension at 72°C for 5 minutes. The PCR products were kept at 4°C. The PCR products were applied into 1.2% agarose gel (Invitrogen®, Japan) and electrophoreses in 1x Tris Borate EDTA (TBE), pH 7.6 buffer at electric gradient 100 volts for 30-40 minutes in mini gel apparatus (Mupid®-EX, Japan). The gel was stained with 1% ethidium bromide and visualized the PCR products under UV-light. The positive control in this study was CDV Snyder Hill strain that give 429 bps PCR product.

10. CDV viral isolation (modified from Seki et al., 2003): Single cell suspension was prepared from spleen (about 0.5 g each) of CDV inoculated mice, sonicated in 10 ml of DMEM medium with 10% antibiotic. The cell suspension was clarified by centrifugation and the supernatant was collected. vero.DST cells were prepared as previous method and plated in 24-well plates and infected with sonicated spleen samples. The infected cells were incubated at 37°C with 5%CO_2 incubator for 24 hours and observed of CPE by mean of CDV infection every day.

11. Statistic analysis: The statistical values were averaged and expressed as the mean and standard error of mean. The statistical evaluations were performed by one-way analysis of variance (ANOVA). The results were considered significant different at $p < 0.05$; equal variances assumed using Latin square design (LSD).

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CHAPTER III

RESULTS

1. Mice bearing DogSLAM gene: There were evidence from transgenic mice tail DNA that DogSLAM transgene was inserted in mice DNA and detected by PCR method. Figure 6 showed PCR product at position 427 bps.

2. Weight: An average mean and standard deviation values of weight gain in each group (control, intranasal, intracerebral and intraperitoneal inoculation group) were divided based on the age of mice as followed: 3-week-old transgenic mice were 13.65 ± 2.60, 14.52 ± 3.07, 15.32 ± 3.67 and 12.45 ± 2.43 grams, respectively. 12-week-old transgenic mice were 26.65 ± 0.87, 25.45 ± 1.01, 23.59 ± 1.17 and 25.81 ± 1.92 grams, respectively. There were statistical significant between groups both 3 and 12 week olds transgenic mice ($p < 0.05$) (Table 1, Figure 8 and 9). But the results in all groups and ages were within normal weight value.

3. Clinical sign: No any significant clinical signs were observed in any groups and ages. The mice had normal appetite and stool was normal texture (Figure 10).

4. Conjunctival swab for detection of viral inclusion body: No any viral inclusion bodies were observed in conjunctival epithelium both Dip Quick® and Shorr's staining in any groups **(Figure 11)**

5. Blood profiles: An average mean and standard deviation values of complete blood count (CBC) measurement [total red blood cells count (RBCs), hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), thrombocyte count, total white blood cells count (WBCs) and WBC differential cell count] of each experimental group were demonstrated in Table 2. There was no statistical significant between groups, both 3 and 12-week-old transgenic mice ($p > 0.05$), as shown in Table 2 except RBC values between control and intraperitoneal inoculation group.

6. Gross lesions: There were no remarkable lesions in all experimental groups compared to control group (Table 3, Figure 10). Macroscopic of all organs were normal in texture and position.

7. Histopathology: The histopathological lesions, haemorrhage and congestion, were mainly observed in lung in every group as follow,

Control and intranasal inoculation group showed mainly lesions of the lung that had atelectasis, mild to moderate lung haemorrhage and congestion. Intracerebral inoculation group showed lesion in both the respiratory and lymphatic system, mild to moderate lung haemorrhage and congestion and congestion of lymph node. Moreover, congestion at corticomedullary junction of the adrenal gland and congestion of the brain were noted. In intraperitoneal inoculation group, mainly lesions in the respiratory system were observed, included lung atelectasis, mild to moderate lung haemorrhage and congestion, focal vasculitis in the lung and increased pulmonary alveolar macrophages (PAMs). Congestion and extramedullary erythropoiesis in the spleen and congestion of the kidney were observed.

Other lesions in various organs were shown in Table 4, Figures 12, 13, 14, 15 and 16.

8. Immunohistochemistry for detection of anti-CDV antigen: The immunohistochemically positive cells for CDV antigen stained bright brown to dark brown in the nucleus of neuron of infected brain dog as shown in Figure 17. There was no positive staining for detection of both CDV antigen in all ages and tissues of every group as shown in Table 5.

9. Immunohistochemistry for detection of anti-HA antigen (DogSLAM): A positive staining of anti-HA antibody was bright brown to dark brown in cytoplasmic membrane of positive control (vero DST cells) as shown in Figure 18. There was no positive staining for detection of both CDV antigen and DogSLAM protein in all groups, age and tissues of all groups as shown in Table 6.

Group	Weight (g) (Mean ± standard deviation)		*Normal weight of 4 to 8-week-old mice (g) (Mean ± standard deviation)	
	3-week-old (n)	12-week-old (n)	male	female
1. Control group	$13.65 \pm 2.60^{a.a}$ (n=3)	$26.65 \pm 0.87^{b.a}$ (n=1)		
2. Intranasal inoculation group	$14.52 \pm 3.07^{a.a}$ (n=3)	$25.45 \pm 1.01^{a.b}$ (n=3)	19.272 ± 1.686	16.03 ± 1.252
3. Intracerebral inoculation group	$15.32 \pm 3.67^{a,b}$ (n=3)	$23.59 \pm 1.17^{a,a}$ (n=3)		
4. Intraperitoneal inoculation group	$12.45 \pm 2.43^{b.a}$ (n=3)	$25.81 \pm 1.92^{a,b}$ (n=2)		

TABLE 1: Showed average of weight in 3 and 12-week-old transgenic mice (mean ± standard deviation)

*Normal value of weight (grams) in mice strain c57BL/6 (available from http://jaxmice.jax.org/info/weight/000664)

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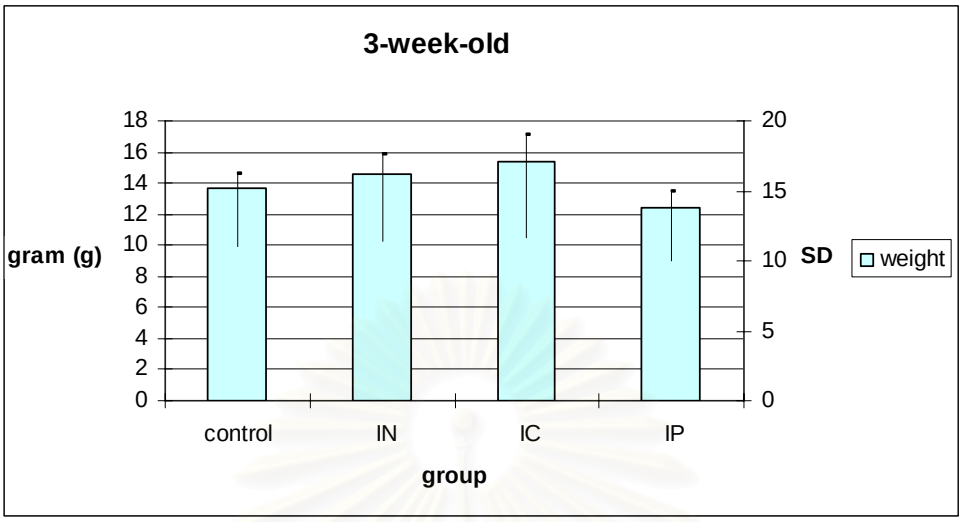

Figure 8: Weight in 3-week-old transgenic mice in control group (n=3), IN inoculation group (n=3), IC inoculation group (n=3) and IP inoculation group (n=3), respectively. (mean ± standard deviation)

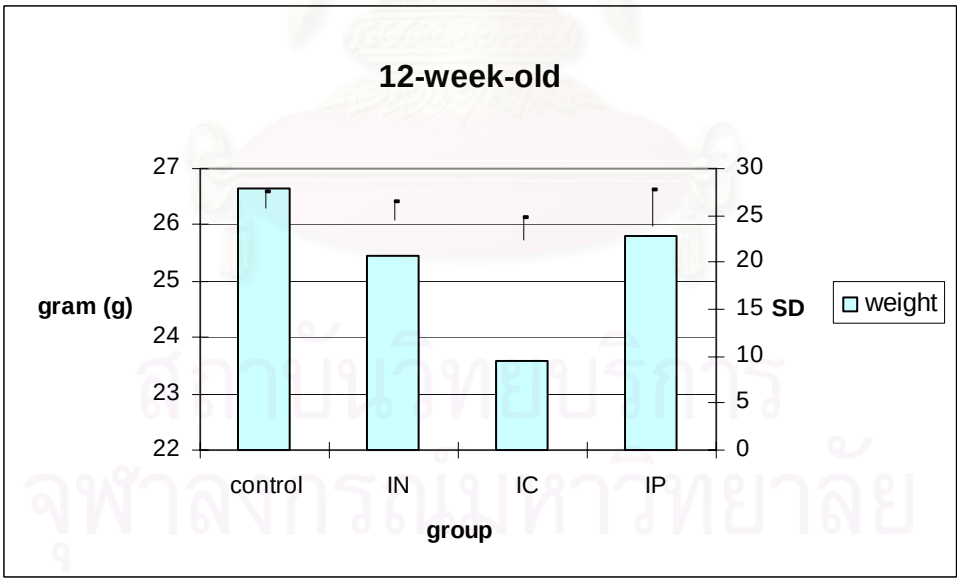

Figure 9: Weight in 12-week-old transgenic mice in control group (n=1), IN inoculation group (n=3), IC inoculation group (n=3) and IP inoculation group (n=2), respectively. (mean ± standard deviation).

	RBC (10^6/μL)	Hb (g/dL)	Hct [%]	MCV [fL]	MCH [pg]	MCHC [g/dL]	Platelet [10^4 /μL]	WBC [10^3 /μL]	Neutrophil [%]	Lymphocyte [%]	Monocyte [%]	Eosinophil [%]	Basophil [%]
1. Control group (n=4)	6.80 ± 1.93[a]	12.05 ± 1.71[a]	37.70 ± 11.30[a]	55.15 ± 1.79[a]	18.85 ± 6.43[a]	34.55 ± 13.12[a]	0.02 ± 0.01[a]	4.35 ± 1.26[a]	0.25 ± 0.10[a]	3.92 ± 1.29[a]	0.17 ± 0.11[a]	0 ± 0[a]	0 ± 0[a]
2. Intranasal inoculation group (n=6)	8.24 ± 0.63[a]	13.15 ± 0.57[a]	45.01 ± 1.86[a]	54.73 ± 2.50[a]	15.96 ± 0.70[a]	29.21 ± 0.59[a]	0.14 ± 0.31[a]	4.58 ± 1.95[a]	0.31 ± 0.52[a]	4.19 ± 1.96[a]	0.06 ± 0.05[a]	0.008 ± 0.02[a]	0 ± 0[a]
3. Intracerebral inoculation group (n=6)	7.31 ± 1.26[a]	12.28 ± 1.80[a]	40.25 ± 6.68[a]	55.13 ± 1.95[a]	17.2 ± 4.18[a]	31.08 ± 6.74[a]	0.04 ± 0.05[a]	3.50 ± 1.59[a]	0.14 ± 0.13[a]	3.22 ± 1.51[a]	0.12 ± 0.07[a]	0 ± 0[a]	0 ± 0[a]
4. intraperitoneal inoculation group (n=5)	8.47 ± 0.74[b]	12.50 ± 1.30[a]	45.70 ± 2.81[a]	54.04 ± 1.94[a]	14.74± 0.62[a]	27.28 ± 1.42[a]	0.15 ± 0.09[a]	3.80 ± 1.01[a]	0.31 ± 0.07[a]	3.33 ± 1.03[a]	0.14 ± 0.02[a]	0 ± 0[a]	0 ± 0[a]
Normal value*	8.3 [6.5-10.1]	13.1 [1.1-16.1]	40.4 [32.8-48.0]	49.1 [42.3-55.9]	15.9 [13.7-18.1]	32.3 [29.5-35.1]	1.16 [0.78-1.54]	6.33 [2.61-10.05]	1.20 [0.4-2.0]	4.86 [1.27-8.44]	0.14 [0-0.29]	0.08 [0-0.17]	0 [0-0.02]

TABLE 2: Showed average mean and standard deviation of blood profile in 3 and 12 week olds transgenic mice and normal value (Campbell, 2004)

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Organs	Control (n=4)	Intranasal inoculation group (n=6)	Intracerebral inoculation group (n=6)	Intraperitoneal inoculation group (n=5)
Brain	NRL	NRL	NRL	NRL
Spinal cord	NRL	NRL	NRL	NRL
Lung	NRL	NRL	NRL	NRL
Heart	NRL	NRL	NRL	NRL
Spleen	NRL	NRL	NRL	NRL
Lymph node	NRL	NRL	NRL	NRL
Adrenal gland	NRL	NRL	NRL	NRL
Liver	NRL	NRL	NRL	NRL
Pancreas	NRL	NRL	NRL	NRL
Stomach	NRL	NRL	NRL	NRL
Small intestine	NRL	NRL	NRL	NRL
Large intestine	NRL	NRL	NRL	NRL
Kidney	NRL	NRL	NRL	NRL
Urinary bladder	NRL	NRL	NRL	NRL
Vagina/ prepuce	NRL	NRL	NRL	NRL

TABLE 3: Gross lesions of various organs in each group of transgenic mice

NRL: No remarkable lesion, +1: mild degree, +2: moderate degree and +3: severe lesion

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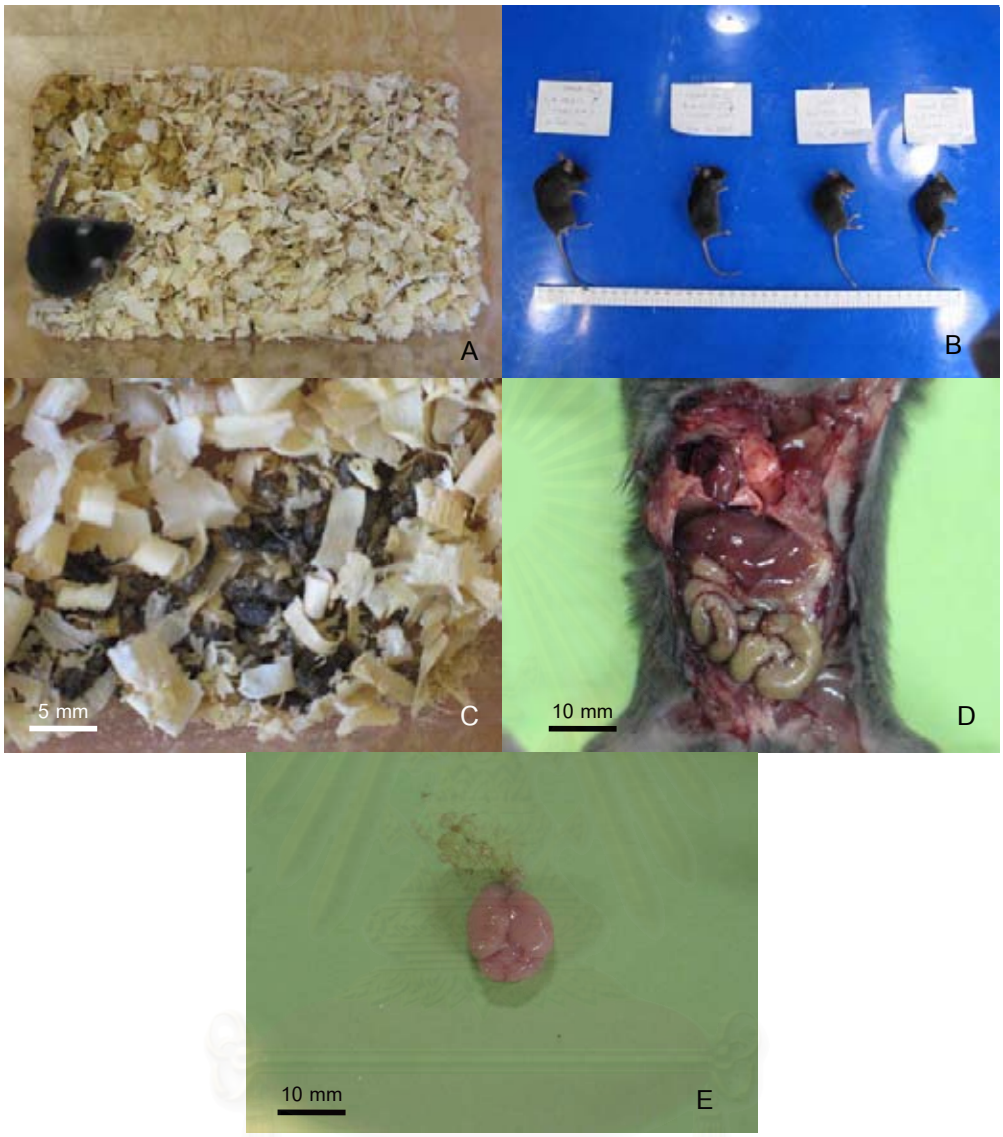

Figure 10: Clinical signs and gross lesion in inoculated transgenic mice. The pictures showed (A) the mice did not show any clinical signs post inoculation, day 14 and (B) the size of mice in each group in same age at day 14. (C) Stool of control group post inoculation. (D) Gross findings of visceral organs *in situ*, day 14. (E) Gross findings of brain in intracerebral inoculation group, day 14.

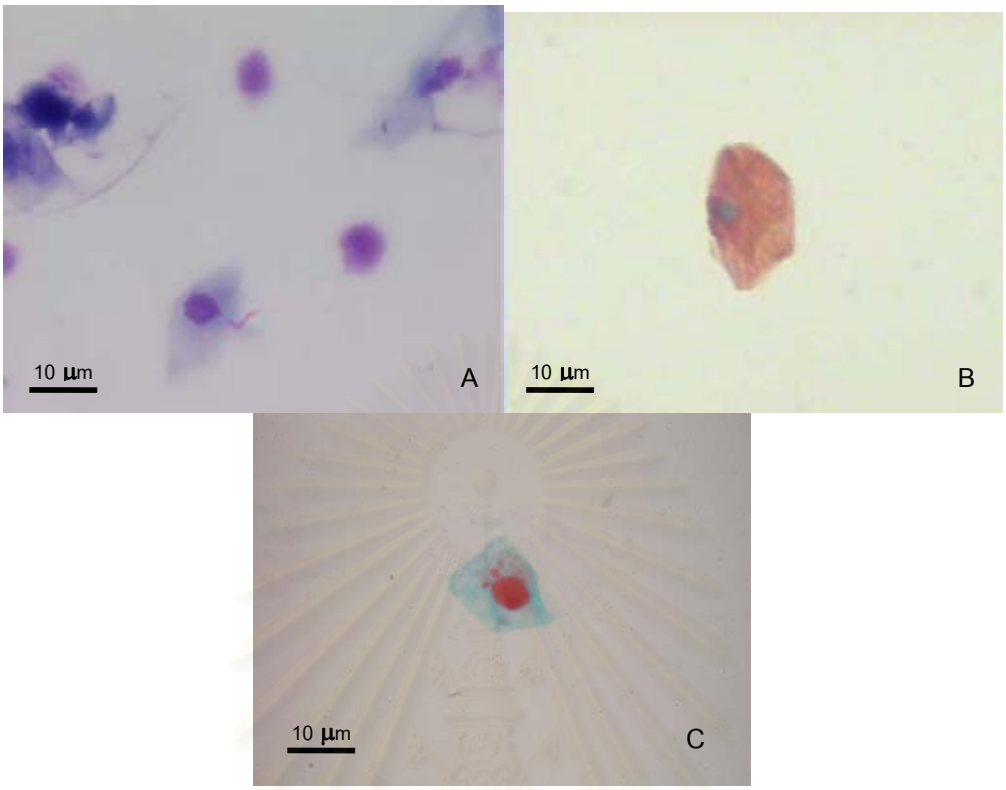

Figure 11: Detection of CDV inclusion bodies from conjunctival swab that showed negative result in conjunctival epithelium. (A) DipQuick® and (B) Shorr's staining. (C) showed positive staining in conjunctival epithelium cell from CDV infected dog by Shorr's staining.

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Organs	Control (n=4)	Intranasal (n=6)	Intracerebral (n=6)	Intraperitoneal (n=5)
Brain				
- congestion	+1 (1/4)	NRL (6/6)	+1 (1/6)	NRL (5/5)
Spinal cord	NRL (4/4)	NRL (6/6)	NRL (6/6)	NRL (5/5)
Lung				
- Edema	-	-	+1 (1/6)	-
- Congestion	-	-	+1 (3/6)	+1 (1/5)
- Hemorrhage	+2 (1/4)	+1 (1/6), +2 (1/6)	+2 (1/6)	+1(1/5); +2 (1/5); +3 (1/5)
- Atelectalsis	+1 (1/4)	+1 (1/6)	-	+1 (2/5)
- Emphysema	-	-	-	+1 (1/5)
- Focal vasculitis	-	-	-	+1 (1/5)
- Increase PAM	-	-	-	+1 (1/5)
- Interstitial pneumonia	-	-	+1 (1/6)	-
Heart	NRL (4/4)	NRL (6/6)	NRL (6/6)	NRL (5/5)
Spleen	NRL (4/4)	NRL (6/6)		
- Hemosiderosis	-	-	+1 (1/6)	+1 (2/5)
- Congestion	-	-	-	+2 (1/5)
Lymph node				
- Congestion	NRL (4/4)	NRL (6/6)	+2 (1/6)	NRL (5/5)
Adrenal gland				
- Congestion at CM junction	NRL (4/4)	NRL (6/6)	+2 (1/6)	NRL (5/5)
Liver	NRL (4/4)	NRL (6/6)	NRL (6/6)	NRL (5/5)
Pancreas	NRL (4/4)	NRL (6/6)	NRL (6/6)	NRL (5/5)
Stomach	NRL (4/4)	NRL (6/6)	NRL (6/6)	NRL (5/5)
Small intestine	NRL (4/4)	NRL (6/6)	NRL (6/6)	NRL (5/5)
Large intestine	NRL (4/4)	NRL (6/6)	NRL (6/6)	NRL (5/5)
Kidney				
- congestion	NRL (4/4)	NRL (6/6)	+1 (1/6)	+1 (1/5)
Urinary bladder	NRL (4/4)	NRL (6/6)	NRL (6/6)	NRL (5/5)
Vagina/ prepuce	NRL (4/4)	NRL (6/6)	NRL (6/6)	NRL (5/5)

TABLE 4: The histopathological lesions of various organs in each group of transgenic mice. - : negative, NRL: No remarkable lesion, +1: mild degree, +2: moderate and +3: severe degree.

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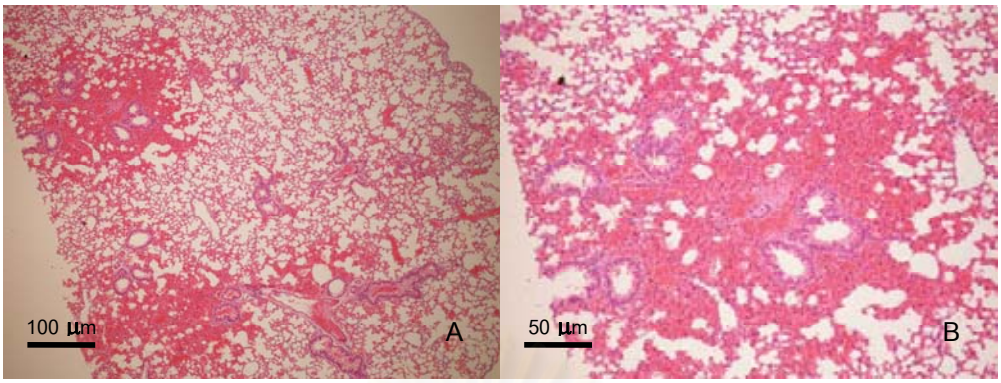

Figure 12: The histopathological fiindings in control group. (A) and (B) showed mild lung haemorhage, H&E

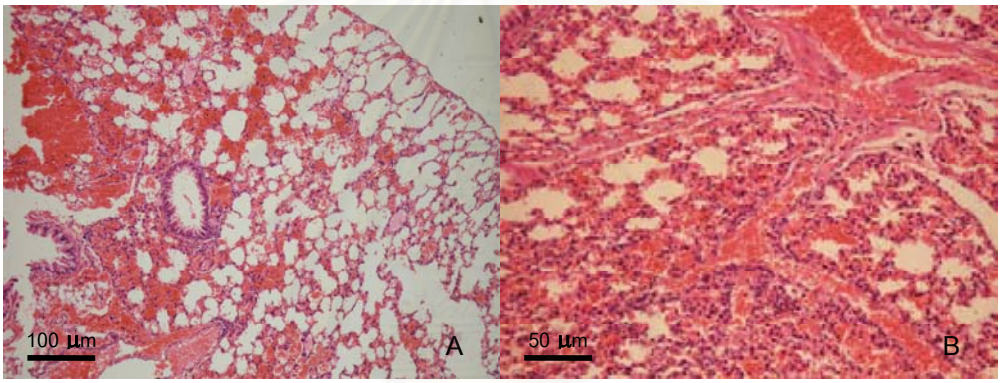

Figure 13: The histopathological findings in IN inoculation group. (A) and (B) showed moderate lung haemorhage, H&E

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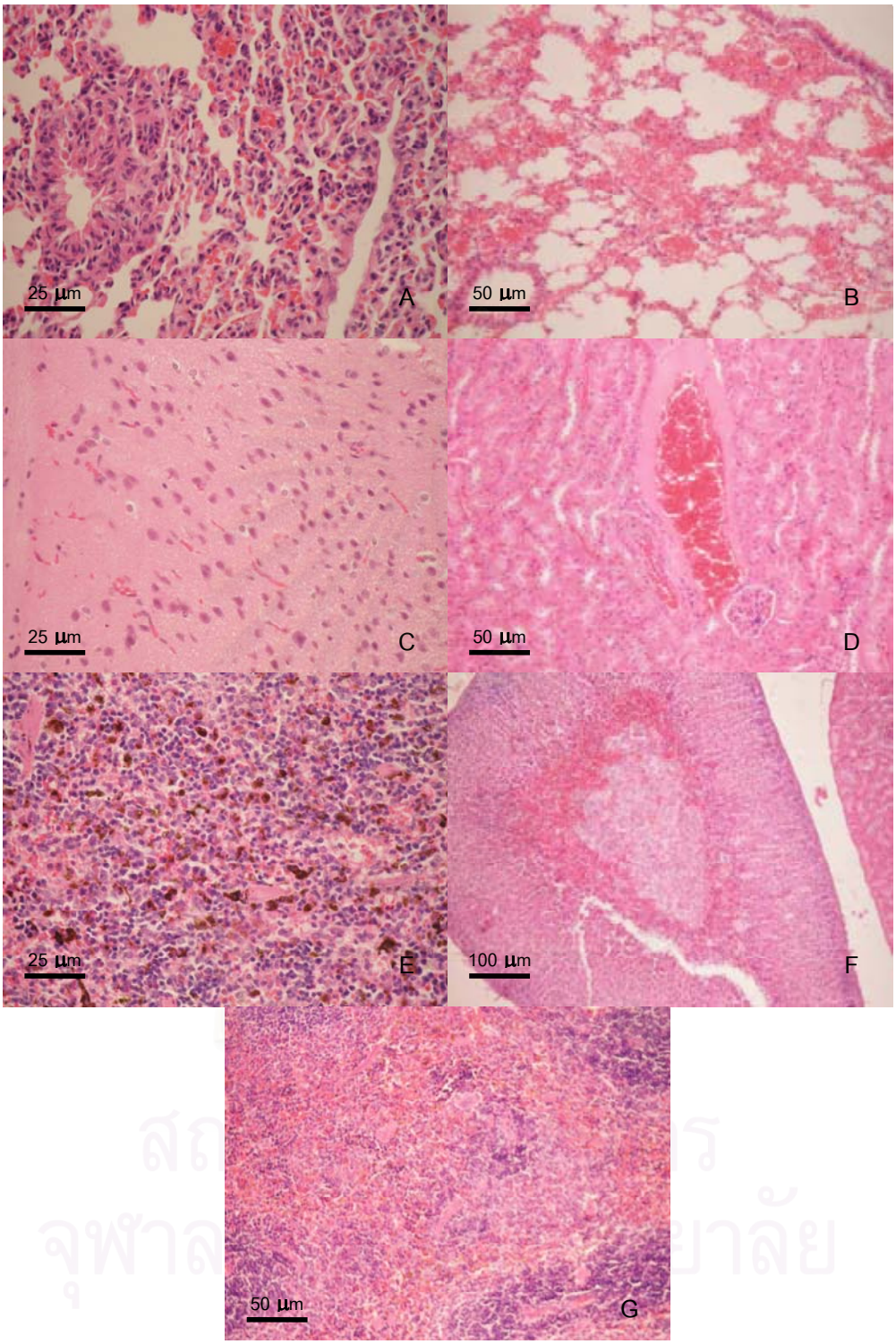

Figure 14: The histopathological findings in IC inoculation group. (A) and (B) showed mild interstitial pnumonia and moderate lung haemorrhage, respectively. (C) showed mild brain congestion. (D) showed mild congestion of kidney. (E) showed mild hemosiderosis of spleen. (F) showed moderate corticomedullary congestion of adrenal gland. (G) showed moderate congestion of lymph node, H&E

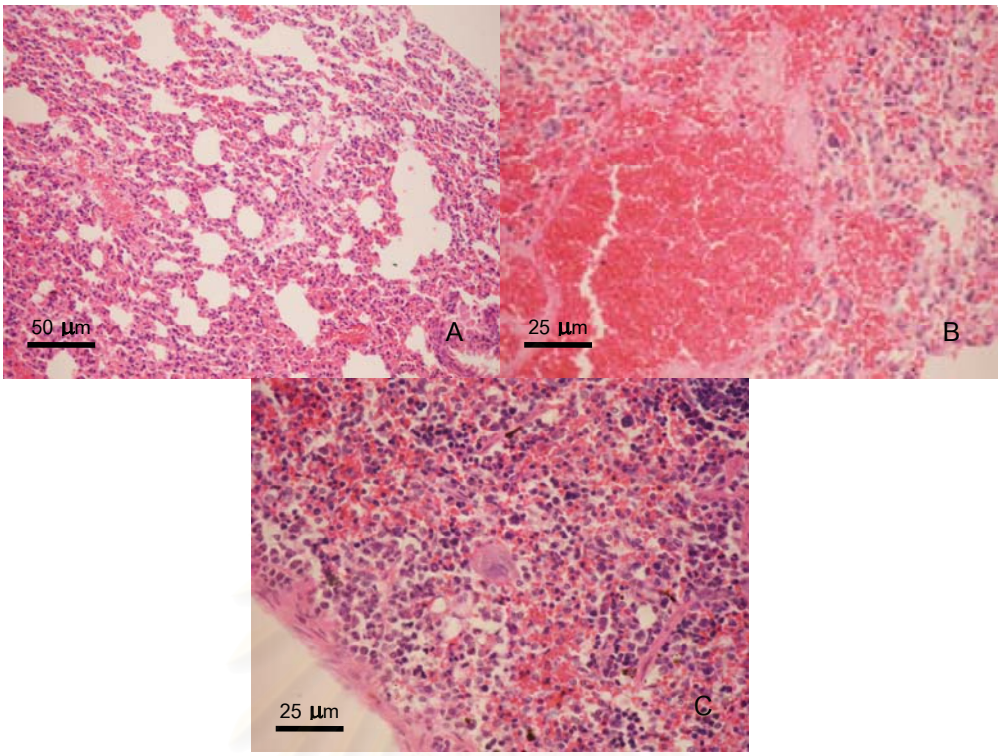

Figure 15: The histopathological findings in IP inoculation group, H&E. (A) lung atelectasis with moderate lung haemorrhage. (B) showed focal vasculitis with severe haemorrhagic pneumonia and (C) showed extramedullary hemopoiesis of spleen, H&E

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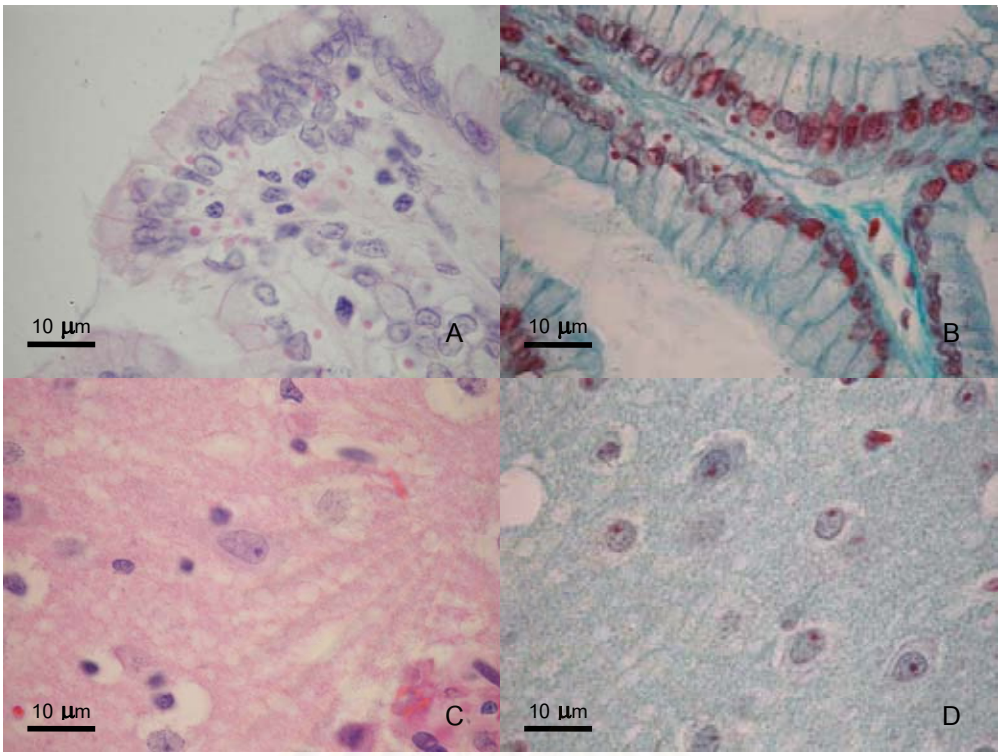

Figure 16: The histopathological pictures showed tissue of CDV naturally infected dog: (A) and (B) showed eosinophilic intracytoplasmic inclusion bodies in epithelial cells of urinary bladder, stained by H&E and Shorr's solution, respectively. (C) and (D) showed eosinophilic intranuclear inclusion bodies in glia cells, H&E and Shorr's staining, respectively.

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Organs	Control group (n=4)	Intranasal inoculation group (n=6)	Intracerebral inoculation group(n=6)	Intraperitoneal inoculation group (n=5)
Brain	-	-	-	-
Spinal cord	-	-	-	-
Lung	-	-	-	-
Heart	-	-	-	-
Spleen	-	-	-	-
Lymph node	-	-	-	-
Adrenal gland	-	-	-	-
Liver	-	-	-	-
Pancreas	-	-	-	-
Stomach	-	-	-	-
Small intestine	-	-	-	-
Large intestine	-	-	-	-
Kidney	-	-	-	-
Urinary bladder	-	-	-	-
Vagina/ prepuce	-	-	-	-

Table 5: Immunohistochemistry result for detection of CDV antigen by monoclonal mouse anti-CDV antibody (Monotope Verostat®) at dilution 1:10 of various organ in each group of transgenic mice.

- : negative, + : positive

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Organs	Control group (n=4)	Intranasal inoculation group (n=6)	Intracerebral inoculation group (n=6)	Intraperitoneal inoculation group (n=5)
Brain	-	-	-	-
Spinal cord	-	-	-	-
Lung	-	-	-	-
Heart	-	-	-	-
Spleen	-	-	-	-
Lymph node	-	-	-	-
Adrenal gland	-	-	-	-
Liver	-	-	-	-
Pancreas	-	-	-	-
Stomach	-	-	-	-
Small intestine	-	-	-	-
Large intestine	-	-	-	-
Kidney	-	-	-	-
Urinary bladder	-	-	-	-
Vagina/ prepuce	-	-	-	-
Others	-	-	-	-

Table 6: Immunohistochemistry result for detection of DogSLAM protein by monoclonal mouse anti-HA antibody at dilution 1:200 of various organ in each group of transgenic mice.

- : negative, + : positive

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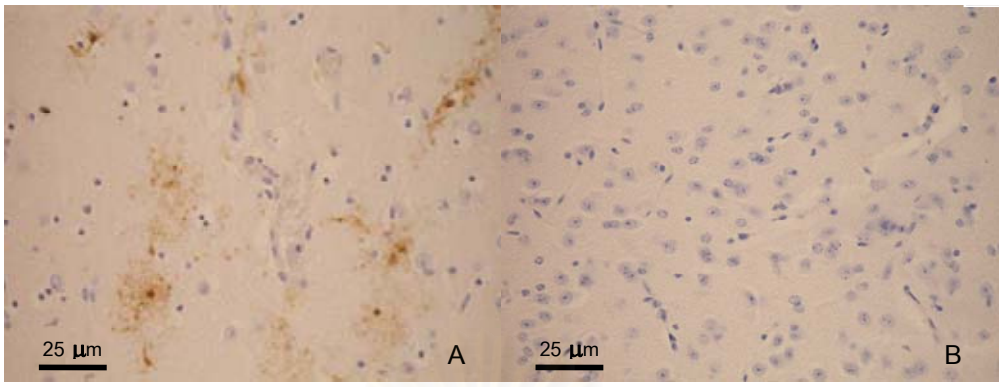

Figure 17: Immunohistochemistry, stained with DAB, for detection of CDV protein in transgenic mice. (A) Positive inclusion bodies staining located in cytoplasm of neuronal cells from dog naturally infected CDV. (B) Negative staining of cerebrum from transgenic mice.

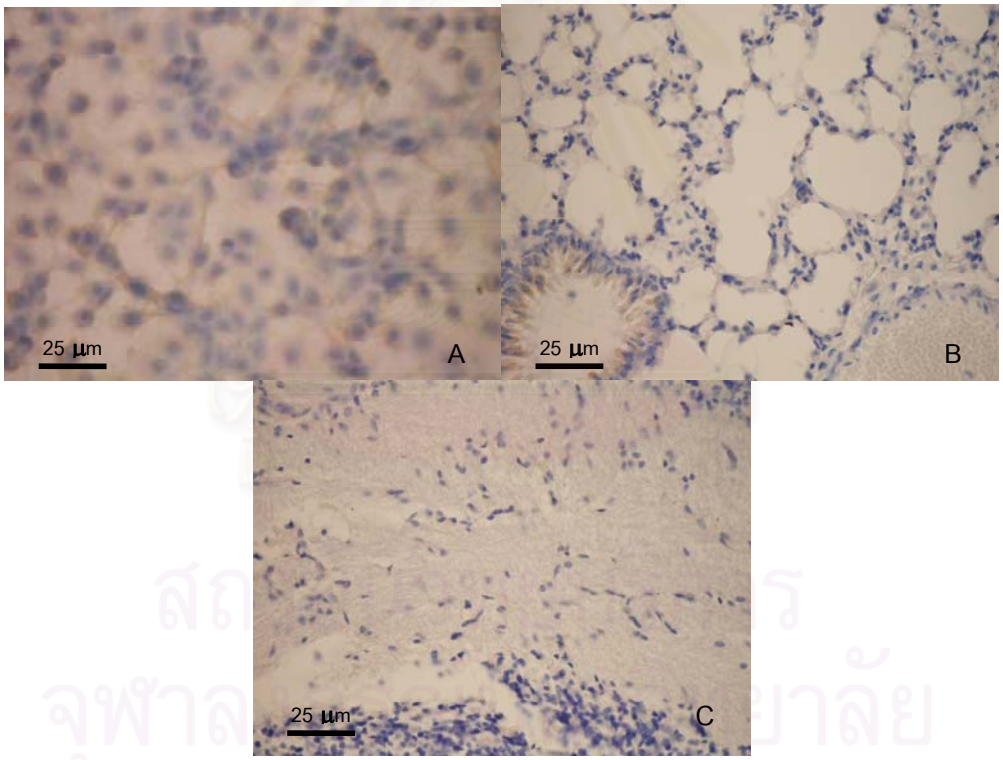

Figure 18: Immunohistochemistry, stained with DAB, for detection of DogSLAM protein in transgenic mice. (A) Positive staining located in cytoplasmic membrane of vero DogSLAM tag (vero DST) cells. (B) Negative staining of lung and (C) negative staining of cerebellum.

10. RT-PCR method for CDV detection: There was no positive RT-PCR product from tissues samples (brain, blood, lung and spleen) in all groups. The positive control was RNA of CDV, Snyder Hill strain that showed positive band of PCR product at 429 bps (Figure 19). For internal control, we used pairs of primer for detection of β-actin protein (house keeping gene) (data not show)

11. Viral isolation: There was no CPE formation in vero DST cells from brain, lung and spleen samples from every group of transgenic mice as shown in Figure 20.

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Figure 19: The result of detection of CDV antigen from brain, blood, lung and spleen RNA by RT-PCR method. The PCR product showed at position 429 bps.
Lane M: Marker, P: positive control (RNA of CDV, Snyder Hill strain), Lane 1-12: RNA sample from transgenic mice.

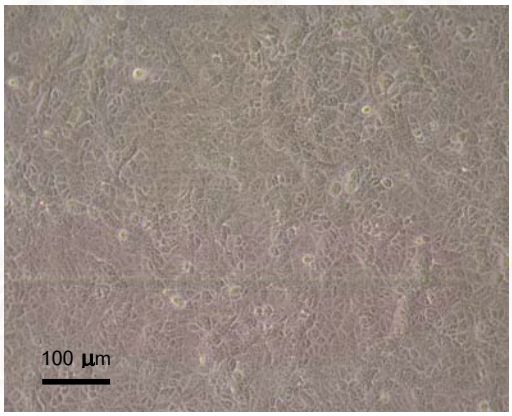

Figure 20: Viral isolation in vero DST cells. Phase contrast microscope showed no CPE formation.

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CHAPTER IV

DISCUSSION AND CONCLUSION

Discussion

Clinical sign and weight: Transgenic mice in this study did not show any clinical signs and evidence of weight loss due to anorexia and diarrhea. Even the weight in both 3 and 12-week-old transgenic mice were in normal range but significantly different among each group. It was not related with CDV infection. Moreover, the transgenic mice did not develop clinical signs due to the failure of virus to induce infection in DogSLAM bearing transgenic mice. Naturally, the mice were not the susceptible host for CDV infection as same as canine host and there has been no report of CDV both naturally and experimentally infection in mice yet. Compare with experimentally induced Snyder Hill strain infected dog, it showed significant clinical signs of mild depression, and subsequent signs of mucopurulent occulonasal discharge, dyspnea and gastrointestinal signs. In some cases developed nervous signs, such as, incoordination and staggering. Moreover, the earliest onset of illness leading to a moribund state occurred consistently and was first seen 14 days post infection. In addition, experimental infection with Snyder Hill strain of CDV was consistently acute; dog either succumbed 14 to 19 days post infection or recovered (Summers et al., 1984; Appel, 1987).

Conjunctival swab for detection of viral inclusion body: From the experiment, inclusion bodies could not be detected in conjunctival epithelium, an inappropriate duration of conjunctival swab should be rule out when comparison with CDV infected dog. Moreover, the failure of virus to induce infection in DogSLAM bearing transgenic mice should be concerned. Compare with Snyder Hil strain infected dog, viral antigen may be demonstrated in conjunctival imprints by eosinophilic intracytoplasmic inclusion body in epithelial cells staining with Dip Quick®. The page-green method for detection of viral inclusion bodies (Shorr's staining) showed brilliant red inclusion body (Luna, 1968) Moreover, the test is available and useful in acute case approximately 7-14 days post infection, depend on virus strain virus spreads to the surface epithelium of the alimentary, respiratory and urogenital tract, endocrine gland, exocrine gland and CNS (Appel, 1987).

In addition to chronic case, virus gradually disappears from most organs except CNS and eyes (Appel, 1987).

Blood profiles: All blood profile parameters were in normal range, but RBCs in control and intraperitoneal inoculation group was significantly different. Compare with platelet count, in control group was less than intraperitoneal group, too. Platelets decreased due to some blood clot during collected time that cause decreasing of RBCs (Duncan and Prasse. 1986). Compare with Snyder Hill strain infected dog, hematology yields non-specific results. In acute cases, lymphopenia and thrombocytopenia could be detected. The number of monocytes may increased (Appel, 1987).

Gross lesions: There was no any gross lesions that induce the infection in DogSLAM bearing transgenic mice. Compare with Snyder Hil strain infected dog, the only consistent postmortem finding in uncomplicated CDV is thymus atrophy. Lungs do not collapse completely and may contain areas of consolidation. Moreover, there were mucopurulent occulonasal discharge, enteritis and sometimes haemorrhage (Appel, 1987).

Histopathology: There were some histopathological lesions, mainly lung haemorrhage in all groups but the histopathological findings in this report did not relate with CDV due to no transgene (DogSLAM) expression from detection of DogSLAM protein by immunohistochemistry. The lesion might cause due to trauma during blood collection procedure in 1 mouse of each group. Compare with CDV infected dog, in lymphatic tissues, lymphoid depletion are commonly seen in acute CDV. Inclusion bodies and occasional syncytia can be seen in lymphocytes, bronchial epithelium and hematopoietic precursor cells. Moreover, interstitial pneumonia was presented in all cases of acute CDV, including haemorrhage and congestion. Lesion of central nervous system may be with or without inflammatory cells invasion, and with or without demyelination (Appel, 1987). There were multifocal and occurred in both grey and white matter, especially in Snyder Hill strain infected dog, however, lesions in grey matter were more severe than white matter. Perivascular cuffing by lymphocytes, plasma cells and histiocytic cells were noted (Summers et al., 1984).

Immunohistochemistry for anti-CDV antibody and anti-HA antibody (DogSLAM): The immunohistochemistry showed negative result maybe due to viral receptor gene expression problem (Alberts et al., 2002), so the inoculating virus could not infect and propagate in any cells (Schwimmbeck et al., 1990).

RT-PCR method for CDV detection: There was no CDV antigen in all samples maybe due to viral receptor gene expression problem, so the inoculating virus could not infect and disappear from all tissues. Compare with PCR product in positive control from Snyder Hill strain that showed at position 429 base pairs (Figure 9). There were several reports revealed that RT-PCR to detect viral NP gene provides a fast, sensitive, and supplementary method for the diagnosis of CDV infection in dog (Shin et al., 1995).

Viral isolation: There was no CDV antigen in all samples maybe due to viral receptor gene expression problem, so the inoculating virus could not infect and disappear from all tissues. Moreover, maybe the method and time for virus isolation was not appropriate. By approximately 7 days post infection, CDV can be isolated from all lymphatic tissues and from blood lymphocytes in dog. In addition, virus isolation from spleen is usually positive from acute or subacute case. (Appel, 1987)

Signaling lymphocytic activation molecule (SLAM, CD150) was identified to be the suitable morbillivirus receptor (Tatsuo et al., 2001). Many reports revealed that Measles virus (MV) can use human signaling lymphocyte activation molecule (SLAM) as a cellular receptor (Erlenhoefer et al., 2001; Hahm et al., 2003) In this study, we examined whether canine distemper, closely related MV also use DogSLAM as cellular receptors in transgenic mice that contained the fragment containing the leader sequence, HA tag, and DogSLAM was further subcloned into plasmid called pCAGDogSLAM tag, which was expected to direct the expression of canine SLAM with the HA tag on eukaryotic cells. (Tatsuo et al., 2001).

The result of all procedures in this study (RT-PCR, virus isolation, histopathology and immunohistochemistry) showed negative result except detection of transgenic DogSLAM gene from tail biopsy by PCR method. It means DogSLAM transgene was successfully inserted into mice DNA but no gene expression. The amount of proteins that cell express maybe depend upon many related factors, internal and external factor.

There are many internal factors, First, the generation of transgenic mice might involved the expression of protein, in this study we used F1 mice that may cause of unstable and non-functional transgene may occurring during on next generation. There were many reports that used F2, F3 or else generated transgenic mice (Mrkic et al., 1998), sometimes at least 20 generation to make homozygous mice line. (available from http://www.aceanimals.com2c57bl6.htm).

Second, some intracellular host factors, such as, introns or type of cells possibly associated with the translation of protein. (Horvat et al., 1996). Because some natural genes were too large to manipulate conveniently and many transgene based on cDNA expression vectors lack of introns. There were some reports showed the lack of introns in some genes effected on a decrease transcriptional efficiency in the transgenic mice (Brinster et al., 1988) Moreover, using unsuitable promoter and vector did not show protein expression. Previously study on measles virus infection used *lck* proximal promoter that specific to express gene in T cells (Hahm et al., 2003) or use another vector, neuron-specific expression vector (NSE) that express inserted gene in neuron (Rall et al., 1997). So in CDV case, these promoters and vectors concerned in pathogenesis of CDV in nervous system because of CD150 is expressed on B cells, may activate dendritic cells and mainly memory T cells in humans. Positional effects can also affect promoters.

Third, when DNA construction was injected into a nucleus, they will incoorporate into the genome in a random way. As a result the construct may be in a position in which it is repressed. Perhaps the most important consideration has to do with the transgene's insertion site in the mouse genome. At many chromosomal locations, transgenes will be transcriptionally silent. Moreover, concentration, form and size of DNA for injection were affected on this phenomena (Brinster et al., 1985). An epitope tagging used in this study was the influenza hemagglutinin protein (HA), YPYDVPDYA sequence, derived from the human influenza haemagglutinin protein. HA could be easily inserted at the desired position within the protein coding sequence. In particular, anti-HA antibody represented a powerful antibody for detection of HA tagged proteins due to its high affinity and did not interfere the binding of H protein of CDV (Tatsuo et al., 2001; Seki et al., 2003). However, it was possible that an insertion of multiple copies of the epitope tag may interfere with protein function. A large and complex expression vector also was difficult to work with in transgenic animals. Furthermore, DogSLAM gene may work by itself or need other

stimulated molecules (called costimulatory molecules) or receptors or some chemicals to induce protein expression (Tatsuo and Yanagi, 2002).

To analysis the *in vitro* MV infection, Previously report showed that the stimulate T lymphocytes could be done before infected with MV by using phorbol myristate acetate (PMA) or ionomycin to induce *in vitro* expression (Hahm et al., 2003) that differ from *in vivo* expression. An alternative splicing produced a soluble form (lacking transmembrane domain) and a variant membrane form, although *in vivo* relevance of these forms is unknown (Punnonen, 1997). Even no expression *in vivo*, there were some reports showed Morbilivirus could also infect SLAM-negative cells with very low efficiency that support our suggestion that alternative or other receptors (costimulatory molecules) may need to work. (Tatsuo and Yanagi, 2002).

Normally, mouse CD150 receptor mapped to chromosome 1, band 1H2.2-2.3 but less was known about the distribution of CD150 expression in a mouse. For human CD150, it located on chromosome 1 q22. The complete mouse CD150 gene showed highly homolog to human SLAM in terms of nucleotide sequences and intron-exon organization that differ from the DogSLAM (Tatsuo and Yanagi. 2002). For this reason, the mouse SLAM may interfere with expression of the DogSLAM transgene. Previous reports revealed an amino acid residues at position 60, 61 and 63 were also important for the function of SLAM as a receptor for CDV (Ohno et al., 2003). So, the substitution of some amino acids of mice SLAM may use to study pathogenesis of CDV infection in transgenic mice and we suggested that knock out mice in mouse SLAM gene could showed us the mouse and dog SLAM function in transgenic mice.

For external factor, because of the nature of RNA genomes that easily adapt itself or high mutation rate of virus may cause virus to use alternative receptor. (Tatsuo and Yanagi, 2002). In addition, virus strain not adaptive strain, compare with MV, rodents have been use as models for MV infection but not only with neuroadapted strain. (Rall et al, 1997). So, Snyder Hill strain should be adapted first because cellular receptor for virus linked to cell trophism (Schneider-Schaulies, 2000). Moreover, the infectivity dose in transgenic mice may be higher than use in this experiment. Because of the infectivity dose in cell culture ($TCID_{50}$) did not relate with infectivity dose in animal model. Finally, the duration to detect CDV infection may influence (Appel, 1987). So, for further investigation and detection of CDV infection in peracute or acute phase in transgenic mice should be investigated and encountered.

Conclusion

The results from this study were unable to elucidate for CDV infection in comparison with the tissue culture study. However, further investigation about factors that related to SLAM gene expression for study of CDV infection in animal model should be concerned and investigated.

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APPENDICES

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX A

Reagents

1. 10% neutral buffer formalin solution. In 1 L of solution composed of 100 ml formalin (approximately 40% formaldehyde gas in water called formalin), distilled water 900 ml, sodium phosphate diabasic (anhydrous) 6.5 g (Lunar, 1968).

2. Harris hematoxylin stain solution

Harris hematoxylin solution was prepared by dissolving 5 g of hematoxylin crystal in absolute alcohol 50 ml. Aluminium potassium sulfate dodecahydrate 100 g was added in distilled water 100 ml and heated to dissolve. The two solutions were mixed and boiled as rapidly as possible less than 1 minute boiled with continuously stirred, removed from heat and added 2.5 g of mercuric oxide (red) slowly, reheated to a simmer and removed from heat immediately after the color of solution became dark purple and the solution was cool in a basin of cold water. In 100 ml of the solution 2-4 ml of glacial acetic acid was added to increase the precision of the nuclear stain. The solution was filtered before use.

3. Eosin stain solution

3.1 Eosin stock solution was prepared by dissolving 1 g of eosin Y in 20 ml distilled water, and adding 80 ml 95% alcohol

3.2 Eosin working solution was prepared by mixing eosin stock solution 1 part and 80% alcohol 3 parts. To 100 ml of the stain solution added glacial acetic acid 0.5 ml and stirred.

Tail digestion buffer 500 ml included of:

1. **20x Saline Sodium Citrate (SCC)**
    a. NaCl (sodium chloride) 43.8 g
    b. $C_6H_5O_7Na_3.2H_2O$ (sodium citrate) 22.1 g
    c. Distilled water (DW) 200 ml

    Adjust pH to 7 by high concentration HCl and then add DW up to 250 ml
2. **2M Tris HCl** (pH 7.6-7.8)
    a. Tris 60.6 g
    b. DW 200 ml

    Adjust pH to 7.6-7.8 by high concentration HCl and then add DW up to 250 ml
3. **0.5M EDTA**
4. **10% Sodium Dodecyl Sulfate (SDS)**
    a. Sodium Dodecyl Sulfate 10 g
    b. DW 100 ml

To make Tail digestion buffer, added 20x SCC 20 ml, 2M Tris HCl 2.5 ml, 0.5M EDTA 1 ml and added DW up to 450 ml. After that, the solution bring to autoclave at 121°C for 20 minutes. Cooled down and added 10% SDS 50 ml.

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APPENDIX B

The procedure for preparation of the tissue processing

Objective	Reagent	Time (mins)
Dehydration	80% ethyl alcohol	30
	80% ethyl alcohol	30
	95% ethyl alcohol	30
	95% ethyl alcohol	30
	100% ethyl alcohol	40
	100% ethyl alcohol	40
Clearing	xylene	30
	Xylene	30
Infiltration	Melted paraffin	30
	Melted paraffin	30

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX C

Statistical analysis of weight in 3-week-old transgenic mice.

Oneway

Descriptives

WEIGHTT (3-WEEK-OLD)

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	51	13.6569	2.60641	.36497	12.9238	14.3899	8.64	18.48
2	51	14.5298	3.07679	.43084	13.6644	15.3952	7.75	19.94
3	51	15.3265	3.67997	.51530	14.2915	16.3615	7.92	22.07
4	51	12.4535	2.43285	.34067	11.7693	13.1378	7.02	16.01
Total	204	13.9917	3.15297	.22075	13.5564	14.4269	7.02	22.07

ANOVA

WEIGHTT (3-WEEK-OLD)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	232.012	3	77.337	8.660	.000
Within Groups	1786.050	200	8.930		
Total	2018.062	203			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: : WEIGHTT (3-WEEK-OLD)

	(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LSD	1	2	-.8729	.59178	.142	-2.0399	.2940
		3	-1.6696 *	.59178	.005	-2.8365	-.5027
		4	1.2033 *	.59178	.043	.0364	2.3703
	2	1	.8729	.59178	.142	-.2940	2.0399
		3	-.7967	.59178	.180	-1.9636	.3703
		4	2.0763 *	.59178	.001	.9093	3.2432
	3	1	1.6696 *	.59178	.005	.5027	2.8365
		2	.7967	.59178	.180	-.3703	1.9636
		4	2.8729 *	.59178	.000	1.7060	4.0399
	4	1	-1.2033 *	.59178	.043	-2.3703	-.0364
		2	-2.0763 *	.59178	.001	-3.2432	-.9093
		3	-2.8729 *	.59178	.000	-4.0399	-1.7060

*. The mean difference is significant at the .05 level.

Statistical analysis of weight in 12-week-old transgenic mice.

Oneway

Descriptives

WEIGHTT (12-WEEK-OLD)

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	17	26.6594	.87111	.21127	26.2115	27.1073	25.30	27.93
2	51	25.4508	1.01289	.14183	25.1659	25.7357	23.85	27.55
3	51	23.5953	1.17594	.16466	23.2646	23.9260	21.20	25.26
4	34	25.8112	1.92081	.32942	25.1410	26.4814	22.45	29.01
Total	153	25.0467	1.69011	.13664	24.7767	25.3166	21.20	29.01

ANOVA

WEIGHTT (12-WEEK-OLD)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	179.852	3	59.951	35.122	.000
Within Groups	254.334	149	1.707		
Total	434.185	152			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: WEIGHTT (12-WEEK-OLD)

LSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	1.2086 *	.36589	.001	.4856	1.9316
	3	3.0641 *	.36589	.000	2.3411	3.7871
	4	.8482 *	.38809	.030	.0813	1.6150
2	1	-1.2086 *	.36589	.001	-1.9316	-.4856
	3	1.8555 *	.25873	.000	1.3443	2.3668
	4	-.3604	.28926	.215	-.9320	.2112
3	1	-3.0641 *	.36589	.000	-3.7871	-2.3411
	2	-1.8555 *	.25873	.000	-2.3668	-1.3443
	4	-2.2159 *	.28926	.000	-2.7875	-1.6444
4	1	-.8482 *	.38809	.030	-1.6150	-.0813
	2	.3604	.28926	.215	-.2112	.9320
	3	2.2159 *	.28926	.000	1.6444	2.7875

*. The mean difference is significant at the .05 level.

Statistical analysis of blood profile in 3-and 12-week-old transgenic mice.

Oneway

Descriptives

RED BLOOD CELL

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	4	6.80500	1.933744	.966872	3.72798	9.88202	4.280	8.790
2	6	8.24667	.634497	.259032	7.58080	8.91253	7.270	8.690
3	6	7.31333	1.268569	.517891	5.98205	8.64461	5.320	8.960
4	5	8.47400	.741033	.331400	7.55389	9.39411	7.660	9.330
Total	21	7.75952	1.270612	.277270	7.18115	8.33790	4.280	9.330

ANOVA

RED BLOOD CELL

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.815	3	2.938	2.128	.134
Within Groups	23.474	17	1.381		
Total	32.289	20			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: RED BLOOD CELL

LSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-1.44167	.758512	.074	-3.04199	.15865
	3	-.50833	.758512	.512	-2.10865	1.09199
	4	-1.66900 *	.788269	.049	-3.33210	-.00590
2	1	1.44167	.758512	.074	-.15865	3.04199
	3	.93333	.678434	.187	-.49804	2.36470
	4	-.22733	.711547	.753	-1.72857	1.27390
3	1	.50833	.758512	.512	-1.09199	2.10865
	2	-.93333	.678434	.187	-2.36470	.49804
	4	-1.16067	.711547	.121	-2.66190	.34057
4	1	1.66900 *	.788269	.049	.00590	3.33210
	2	.22733	.711547	.753	-1.27390	1.72857
	3	1.16067	.711547	.121	-.34057	2.66190

*. The mean difference is significant at the .05 level.

Oneway

Descriptives

Hb

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	4	12.0500	1.71756	.85878	9.3170	14.7830	9.90	14.10
2	6	13.1500	.57879	.23629	12.5426	13.7574	12.30	13.90
3	6	12.2833	1.80379	.73640	10.3904	14.1763	8.70	13.30
4	5	12.5000	1.30576	.58395	10.8787	14.1213	10.70	13.80
Total	21	12.5381	1.36399	.29765	11.9172	13.1590	8.70	14.10

ANOVA

Hb

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.596	3	1.199	.606	.620
Within Groups	33.613	17	1.977		
Total	37.210	20			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Hb

LSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-1.1000	.90767	.242	-3.0150	.8150
	3	-.2333	.90767	.800	-2.1483	1.6817
	4	-.4500	.94327	.639	-2.4401	1.5401
2	1	1.1000	.90767	.242	-.8150	3.0150
	3	.8667	.81184	.301	-.8462	2.5795
	4	.6500	.85147	.456	-1.1464	2.4464
3	1	.2333	.90767	.800	-1.6817	2.1483
	2	-.8667	.81184	.301	-2.5795	.8462
	4	-.2167	.85147	.802	-2.0131	1.5798
4	1	.4500	.94327	.639	-1.5401	2.4401
	2	-.6500	.85147	.456	-2.4464	1.1464
	3	.2167	.85147	.802	-1.5798	2.0131

Oneway

Descriptives

HCT

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	4	37.7000	11.30310	5.65155	19.7143	55.6857	22.50	48.50
2	6	45.0167	1.86699	.76220	43.0574	46.9760	42.40	47.40
3	6	40.2500	6.68154	2.72773	33.2382	47.2618	30.30	47.60
4	5	45.7000	2.81336	1.25817	42.2068	49.1932	41.60	48.90
Total	21	42.4238	6.58482	1.43693	39.4264	45.4212	22.50	48.90

ANOVA

HCT

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	211.615	3	70.538	1.829	.180
Within Groups	655.583	17	38.564		
Total	867.198	20			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: HCT

LSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-7.3167	4.00852	.086	-15.7739	1.1406
	3	-2.5500	4.00852	.533	-11.0072	5.9072
	4	-8.0000	4.16577	.072	-16.7890	.7890
2	1	7.3167	4.00852	.086	-1.1406	15.7739
	3	4.7667	3.58533	.201	-2.7977	12.3310
	4	-.6833	3.76032	.858	-8.6169	7.2503
3	1	2.5500	4.00852	.533	-5.9072	11.0072
	2	-4.7667	3.58533	.201	-12.3310	2.7977
	4	-5.4500	3.76032	.165	-13.3836	2.4836
4	1	8.0000	4.16577	.072	-.7890	16.7890
	2	.6833	3.76032	.858	-7.2503	8.6169
	3	5.4500	3.76032	.165	-2.4836	13.3836

Oneway

Descriptives

MCV

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	4	55.1500	1.79907	.89954	52.2873	58.0127	52.60	56.60
2	6	54.7333	2.50493	1.02263	52.1046	57.3621	52.10	58.30
3	6	55.1333	1.95516	.79819	53.0815	57.1852	52.40	57.00
4	5	54.0400	1.94756	.87098	51.6218	56.4582	52.40	57.30
Total	21	54.7619	1.99261	.43482	53.8549	55.6689	52.10	58.30

ANOVA

MCV

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.041	3	1.347	.304	.822
Within Groups	75.369	17	4.433		
Total	79.410	20			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: MCV

LSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.4167	1.35914	.763	-2.4509	3.2842
	3	.0167	1.35914	.990	-2.8509	2.8842
	4	1.1100	1.41246	.443	-1.8700	4.0900
2	1	-.4167	1.35914	.763	-3.2842	2.4509
	3	-.4000	1.21565	.746	-2.9648	2.1648
	4	.6933	1.27499	.594	-1.9967	3.3833
3	1	-.0167	1.35914	.990	-2.8842	2.8509
	2	.4000	1.21565	.746	-2.1648	2.9648
	4	1.0933	1.27499	.403	-1.5967	3.7833
4	1	-1.1100	1.41246	.443	-4.0900	1.8700
	2	-.6933	1.27499	.594	-3.3833	1.9967
	3	-1.0933	1.27499	.403	-3.7833	1.5967

Oneway

Descriptives

MCH

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	4	18.8500	6.43972	3.21986	8.6030	29.0970	15.30	28.50
2	6	15.9667	.70899	.28944	15.2226	16.7107	15.10	16.90
3	6	17.2000	4.18569	1.70880	12.8074	21.5926	12.80	25.00
4	5	14.7400	.62290	.27857	13.9666	15.5134	14.00	15.60
Total	21	16.5762	3.59290	.78403	14.9407	18.2117	12.80	28.50

ANOVA

MCH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	42.103	3	14.034	1.104	.375
Within Groups	216.075	17	12.710		
Total	258.178	20			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: MCH

LSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	2.8833	2.30130	.227	-1.9720	7.7386
	3	1.6500	2.30130	.483	-3.2053	6.5053
	4	4.1100	2.39158	.104	-.9358	9.1558
2	1	-2.8833	2.30130	.227	-7.7386	1.9720
	3	-1.2333	2.05834	.557	-5.5761	3.1094
	4	1.2267	2.15881	.577	-3.3280	5.7814
3	1	-1.6500	2.30130	.483	-6.5053	3.2053
	2	1.2333	2.05834	.557	-3.1094	5.5761
	4	2.4600	2.15881	.270	-2.0947	7.0147
4	1	-4.1100	2.39158	.104	-9.1558	.9358
	2	-1.2267	2.15881	.577	-5.7814	3.3280
	3	-2.4600	2.15881	.270	-7.0147	2.0947

Oneway

Descriptives

MCHC

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	4	34.5500	13.12364	6.56182	13.6674	55.4326	27.30	54.20
2	6	29.2167	.59133	.24141	28.5961	29.8372	28.30	29.90
3	6	31.0833	6.74727	2.75456	24.0025	38.1642	24.40	43.90
4	5	27.2800	1.42021	.63514	25.5166	29.0434	25.70	29.40
Total	21	30.3048	6.64345	1.44972	27.2807	33.3288	24.40	54.20

ANOVA

MCHC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	128.575	3	42.858	.966	.431
Within Groups	754.135	17	44.361		
Total	882.710	20			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: MCHC

LSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	5.3333	4.29927	.232	-3.7373	14.4040
	3	3.4667	4.29927	.431	-5.6040	12.5373
	4	7.2700	4.46793	.122	-2.1565	16.6965
2	1	-5.3333	4.29927	.232	-14.4040	3.7373
	3	-1.8667	3.84538	.634	-9.9797	6.2464
	4	1.9367	4.03307	.637	-6.5724	10.4457
3	1	-3.4667	4.29927	.431	-12.5373	5.6040
	2	1.8667	3.84538	.634	-6.2464	9.9797
	4	3.8033	4.03307	.359	-4.7057	12.3124
4	1	-7.2700	4.46793	.122	-16.6965	2.1565
	2	-1.9367	4.03307	.637	-10.4457	6.5724
	3	-3.8033	4.03307	.359	-12.3124	4.7057

Oneway

Descriptives

PLATELET

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	4	.02650	.015022	.007511	.00260	.05040	.013	.048
2	6	.14600	.317981	.129815	-.18770	.47970	.009	.795
3	6	.04533	.057656	.023538	-.01517	.10584	.001	.159
4	5	.15352	.099165	.044348	.03039	.27665	.045	.290
Total	21	.09627	.177265	.038682	.01558	.17696	.001	.795

ANOVA

PLATELET

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.066	3	.022	.668	.583
Within Groups	.562	17	.033		
Total	.628	20			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: PLATELET

LSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-.11950	.117385	.323	-.36716	.12816
	3	-.01883	.117385	.874	-.26649	.22883
	4	-.12702	.121990	.312	-.38440	.13036
2	1	.11950	.117385	.323	-.12816	.36716
	3	.10067	.104992	.351	-.12085	.32218
	4	-.00752	.110117	.946	-.23985	.22481
3	1	.01883	.117385	.874	-.22883	.26649
	2	-.10067	.104992	.351	-.32218	.12085
	4	-.10819	.110117	.340	-.34051	.12414
4	1	.12702	.121990	.312	-.13036	.38440
	2	.00752	.110117	.946	-.22481	.23985
	3	.10819	.110117	.340	-.12414	.34051

Oneway

Descriptives

WHITE BLOOD CELL

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	4	4.3500	1.26095	.63048	2.3435	6.3565	3.10	6.00
2	6	4.5833	1.95286	.79725	2.5339	6.6327	2.60	8.20
3	6	3.5000	1.59750	.65218	1.8235	5.1765	1.70	6.10
4	5	3.8000	1.00995	.45166	2.5460	5.0540	2.90	5.50
Total	21	4.0429	1.49786	.32686	3.3610	4.7247	1.70	8.20

ANOVA

WHITE BLOOD CELL

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.193	3	1.398	.584	.634
Within Groups	40.678	17	2.393		
Total	44.871	20			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: WHITE BLOOD CELL

LSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-.2333	.99851	.818	-2.3400	1.8733
	3	.8500	.99851	.406	-1.2567	2.9567
	4	.5500	1.03768	.603	-1.6393	2.7393
2	1	.2333	.99851	.818	-1.8733	2.3400
	3	1.0833	.89309	.242	-.8009	2.9676
	4	.7833	.93668	.415	-1.1929	2.7596
3	1	-.8500	.99851	.406	-2.9567	1.2567
	2	-1.0833	.89309	.242	-2.9676	.8009
	4	-.3000	.93668	.753	-2.2762	1.6762
4	1	-.5500	1.03768	.603	-2.7393	1.6393
	2	-.7833	.93668	.415	-2.7596	1.1929
	3	.3000	.93668	.753	-1.6762	2.2762

Oneway

Descriptives

NEUTROPHIL

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	4	.25475	.106174	.053087	.08580	.42370	.180	.407
2	6	.31550	.521292	.212817	−.23156	.86256	.070	1.377
3	6	.14750	.136727	.055819	.00401	.29099	.017	.366
4	5	.31700	.075147	.033607	.22369	.41031	.185	.370
Total	21	.25629	.284525	.062088	.12677	.38580	.017	1.377

ANOVA

NEUTROPHIL

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.110	3	.037	.415	.744
Within Groups	1.509	17	.089		
Total	1.619	20			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: NEUTROPHIL

LSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	−.06075	.192290	.756	−.46645	.34495
	3	.10725	.192290	.584	−.29845	.51295
	4	−.06225	.199834	.759	−.48386	.35936
2	1	.06075	.192290	.756	−.34495	.46645
	3	.16800	.171990	.342	−.19487	.53087
	4	−.00150	.180384	.993	−.38208	.37908
3	1	−.10725	.192290	.584	−.51295	.29845
	2	−.16800	.171990	.342	−.53087	.19487
	4	−.16950	.180384	.361	−.55008	.21108
4	1	.06225	.199834	.759	−.35936	.48386
	2	.00150	.180384	.993	−.37908	.38208
	3	.16950	.180384	.361	−.21108	.55008

Oneway

Descriptives

LYMPHOCYTE

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	4	3.92275	1.297127	.648563	1.85873	5.98677	2.790	5.580
2	6	4.19117	1.968888	.803795	2.12495	6.25739	2.366	8.036
3	6	3.22733	1.510952	.616844	1.64169	4.81298	1.615	5.673
4	5	3.33240	1.030913	.461038	2.05235	4.61245	2.407	5.060
Total	21	3.66019	1.478597	.322656	2.98714	4.33324	1.615	8.036

ANOVA

LYMPHOCYTE

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.629	3	1.210	.513	.679
Within Groups	40.096	17	2.359		
Total	43.725	20			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: LYMPHOCYTE

LSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-.26842	.991338	.790	-2.35996	1.82312
	3	.69542	.991338	.492	-1.39612	2.78696
	4	.59035	1.030228	.574	-1.58324	2.76394
2	1	.26842	.991338	.790	-1.82312	2.35996
	3	.96383	.886679	.292	-.90690	2.83456
	4	.85877	.929957	.369	-1.10327	2.82080
3	1	-.69542	.991338	.492	-2.78696	1.39612
	2	-.96383	.886679	.292	-2.83456	.90690
	4	-.10507	.929957	.911	-2.06710	1.85697
4	1	-.59035	1.030228	.574	-2.76394	1.58324
	2	-.85877	.929957	.369	-2.82080	1.10327
	3	.10507	.929957	.911	-1.85697	2.06710

Oneway

Descriptives

MONOCYTE

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	4	.17250	.113283	.056641	-.00776	.35276	.062	.296
2	6	.05933	.051333	.020957	.00546	.11320	.000	.153
3	6	.12517	.073295	.029923	.04825	.20209	.061	.220
4	5	.14320	.027869	.012464	.10860	.17780	.110	.185
Total	21	.11967	.076681	.016733	.08476	.15457	.000	.296

ANOVA

MONOCYTE

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.036	3	.012	2.496	.095
Within Groups	.082	17	.005		
Total	.118	20			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: MONOCYTE

LSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.11317 *	.044733	.022	.01879	.20754
	3	.04733	.044733	.305	-.04704	.14171
	4	.02930	.046488	.537	-.06878	.12738
2	1	-.11317 *	.044733	.022	-.20754	-.01879
	3	-.06583	.040010	.118	-.15025	.01858
	4	-.08387	.041963	.062	-.17240	.00467
3	1	-.04733	.044733	.305	-.14171	.04704
	2	.06583	.040010	.118	-.01858	.15025
	4	-.01803	.041963	.673	-.10657	.07050
4	1	-.02930	.046488	.537	-.12738	.06878
	2	.08387	.041963	.062	-.00467	.17240
	3	.01803	.041963	.673	-.07050	.10657

*. The mean difference is significant at the .05 level.

Oneway

Descriptives

EOSINOPHIL

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	4	.00000	.000000	.000000	.00000	.00000	.000	.000
2	6	.00867	.021229	.008667	-.01361	.03095	.000	.052
3	6	.00000	.000000	.000000	.00000	.00000	.000	.000
4	5	.00000	.000000	.000000	.00000	.00000	.000	.000
Total	21	.00248	.011347	.002476	-.00269	.00764	.000	.052

ANOVA

EOSINOPHIL

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	3	.000	.810	.506
Within Groups	.002	17	.000		
Total	.003	20			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: EOSINOPHIL

LSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-.00867	.007432	.260	-.02435	.00701
	3	.00000	.007432	1.000	-.01568	.01568
	4	.00000	.007723	1.000	-.01629	.01629
2	1	.00867	.007432	.260	-.00701	.02435
	3	.00867	.006647	.210	-.00536	.02269
	4	.00867	.006971	.231	-.00604	.02338
3	1	.00000	.007432	1.000	-.01568	.01568
	2	-.00867	.006647	.210	-.02269	.00536
	4	.00000	.006971	1.000	-.01471	.01471
4	1	.00000	.007723	1.000	-.01629	.01629
	2	-.00867	.006971	.231	-.02338	.00604
	3	.00000	.006971	1.000	-.01471	.01471

Oneway

Descriptives

BASOPHIL

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	4	.00000	.000000	.000000	.00000	.00000	.000	.000
2	6	.00000	.000000	.000000	.00000	.00000	.000	.000
3	6	.00000	.000000	.000000	.00000	.00000	.000	.000
4	5	.00000	.000000	.000000	.00000	.00000	.000	.000
Total	21	.00000	.000000	.000000	.00000	.00000	.000	.000

ANOVA

BASOPHIL

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	3	.000	.	.
Within Groups	.000	17	.000		
Total	.000	20			

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

VITA

Mr. Suchanit Ngamkala was born on January 12, 1979 in Bangkok, Thailand. He graduated with Bachelor Degree of Veterinary Sciences (DVM) in academic year 2003 from the Faculty of Veterinary Sciences, Chulalongkorn University. He was a candidate of graduated program of Veterinary Pathobiology, Department of Pathology, Chulalongkorn University. He appointed the scholarship as an exchange student for 1 year (April 2004 – April 2005) from the Association of International Exchange Student Program of Japan (AIEJ) to study and done research work in Department of Pathology, Faculty of Agriculture, University of Miyazaki, Japan.

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