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ที่แยกได้ในประเทศไทย

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MOLECULAR CHARACTERIZATION AND GENOTYPIC LINEAGES

OF CANINE DISTEMPER VIRUS ISOLATES IN THAILAND

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A Dissertation Submitted in Partial Fulfillment of the Requirements

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อารยา รัตนกถิกานนท์ : การจำแนกลักษณะทางพันธุกรรมและการจัดกลุ่มสายพันธุ์ของเชื้อไวรัสไข้หัดสุนัขที่แยกได้ในประเทศไทย. (MOLECULAR CHARACTERIZATION AND GENOTYPIC LINEAGES OF CANINE DISTEMPER VIRUS ISOLATES IN THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.สพญ.ดร. สมพร เตชะงามสุวรรณ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : อ.สพญ. ด ร . จุฑาทิพย์ เขียวเจริญ, 89 หน้า.

เชื้อไวรัสไข้หัดสุนัขนั้นเป็นที่ทราบกันดีว่าก่อให้เกิดอาการป่วยหลายระบบในวงศ์สัตว์กินเนื้อ ถึงแม้ว่าจะมีการใช้วัคซีนเพื่อควบคุมโรคมาเป็นระยะเวลายาวนาน แต่ก็ยังมีรายงานการเกิดโรคในสัตว์ที่ได้รับการฉีดวัคซีนอย่างต่อเนื่อง จุดประสงค์ของการศึกษาคือ เพื่อสำรวจความหลากหลายทางพันธุกรรมและจัดกลุ่มสายพันธุ์ของเชื้อไวรัสไข้หัดสุนัข โดยใช้จีนฟอสโฟโปรตีน, ฮีแมกกลูตินินไกลโคโปรตีน และฟิวชั่นไกลโคโปรตีน ซึ่งมีส่วนสำคัญในกระบวนการก่อโรคของไวรัสไข้หัดสุนัข รวมทั้งพัฒนาเทคนิค Restriction fragment length polymorphism (RFLP) เพื่อใช้ในการแยกแยะการติดเชื้อแต่ละสายพันธุ์ที่มาจากธรรมชาติและสายพันธุ์วัคซีน ตัวอย่างที่ใช้ในการศึกษาประกอบด้วย ผลิตภัณฑ์วัคซีน 4 ตัวอย่าง สารคัดหลั่งจากดวงตา 33 ตัวอย่าง และ ชิ้นเนื้อจากการชันสูตรซาก 13 ตัวอย่าง ซึ่งได้มาจากสุนัขและชะมด ตัวอย่างชิ้นเนื้อจากแต่ละอวัยวะถูกนำมาศึกษารอยโรคทางจุลพยาธิวิทยาและชิ้นเนื้อจากระบบประสาทจะนำมาศึกษาด้วยวิธีอิมมูนโนฮีสโตเคมี การศึกษาทางไวรัสวิทยาทำโดยแยกเชื้อไวรัสในเซลล์เพาะเลี้ยง Vero-DST จากนั้นนำตัวอย่างที่ได้มาทำปฏิกิริยารีเวิสทรานสคริปเตสลูกโซ่โพลิเมอเรส เฉพาะส่วนสารพันธุกรรมที่ได้จากปฏิกิริยาของจีนฟิวชั่นไกลโคโปรตีนจะถูกนำมาศึกษาด้วยเทคนิค RFLP จากนั้นทำการวิเคราะห์ความสัมพันธ์ของลำดับนิวคลีโอไทด์ของทุกจีนเปรียบเทียบกับสายพันธุ์อื่นในธนาคารจีน พบรอยโรคจำเพาะของโรคไข้หัดสุนัขในอวัยวะต่างๆ และผลอิมมูนโนฮีสโตเคมีในระบบประสาทพบความแตกต่างกันของเซลล์ที่ติดเชื้อในแต่ละสายพันธุ์ ผลการวิเคราะห์ลำดับนิวคลีโอไทด์พบว่าไวรัสไข้หัดสุนัขที่แยกได้จากการติดเชื้อในธรรมชาติมีลำดับนิวคลิโอไทด์แตกต่างจากสายพันธุ์วัคซีน และแบ่งออกเป็น 2 กลุ่มคือ กลุ่มที่จัดอยู่ในสายพันธุ์ Asia-1 และกลุ่มที่ไม่สามารถจัดอยู่ในสายพันธุ์ใด ซึ่งจะถูกเรียกว่าสายพันธุ์ new Asia การวิเคราะห์ด้วยเทคนิค RFLP พบว่าสามารถแยกแยะการติดเชื้อสายพันธุ์ Asia-1, new Asia และวัคซีนออกจากกันได้ สอดคล้องกับการวิเคราะห์จากลำดับนิวคลีโอไทด์ เมื่อพิจารณาถึงการศึกษาลำดับนิวคลีโอไทด์ที่มีมาก่อนหน้าพบว่ามีไวรัสไข้หัดสุนัขอย่างน้อย 3 สายพันธุ์ดังกล่าวแพร่ระบาดอยู่ในประเทศไทย ดังนั้นเทคนิค RFLP จึงสามารถใช้แยกแยะการติดเชื้อแต่ละสายพันธุ์ที่มาจากธรรมชาติและสายพันธุ์วัคซีนได้อย่างมีประสิทธิภาพ ซึ่งจะเป็นประโยชน์ในการวินิจฉัยโรคทางคลินิกและการเฝ้าระวังการระบาดของโรคไข้หัดสุนัขในประเทศไทย

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สาขาวิชา : …พยาธิชีววิทยาทางสัตวแพทย์….. ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก…………………

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5375565331 : MAJOR VETERINARY PATHOBIOLOGY
KEYWORDS : CANINE DISTEMPER VIRUS / RESTRICTION FRAGMENT LENGTH POLYMORPHISM / PHYLOGENETIC ANALYSIS / VACCINE / WILD TYPE

ARAYA RADTANAKATIKANON : MOLECULAR CHARACTERIZATION AND GENOTYPIC LINEAGES OF CANINE DISTEMPER VIRUS ISOLATES IN THAILAND. ADVISOR : ASSIST. PROF. SOMPORN TECHANGAMSUWAN, Ph.D., CO - ADVISOR : JUTHATIP KEAWCHAROEN, Ph.D., 89 pp.

Canine distemper virus (CDV) has been known causing multisystemic disease in all families of terrestrial carnivores. Attenuated live vaccines have been used for controlling the disease for many decades, yet a number of CDV infections in vaccinated dog were still observed. The aims of this study were to investigate the genetic diversity of CDV lineages based on phospoprotein (P), hemagglutinin (H) and fusion protein (F) gene that play an important role in viral pathogenesis and to develop the restriction fragment length polymorphism (RFLP) techniques for effective differentiation among individual wild-type and vaccine lineages. Four commercial vaccine products, 23 conjunctival swabs and 13 necropsied tissue from dog and civets were included in the study. Routinely histopathological study of various organs and immunohistochemistry (IHC) staining on brain tissues were performed. Viral isolation was done in Vero-DST cell line for virological study. Reverse-transcription polymerase chain reaction (RT-PCR) on 3 gene regions of specimens and vaccines were carried out, then RFLP analysis upon F-gene amplified fragments was developed. Nucleotide sequence and phylogenetic analysis were compared with other lineages in Genbank. Typical microscopic lesions of CDV were found in various organs. IHC of brain tissues indicated the characteristic cell tropism upon different CDV lineages. Phylogenetic analysis revealed that CDV field isolates were not related to vaccine lineage and could be divided into two clusters; one belonged to Asia-1 lineage and another, not related to any previous recognized lineages was proposed as 'new Asia lineage'. RFLP pattern concordantly to phylogenetic trees was able to differentiate among Asia-1, new Asia and vaccine lineage. Regarding previous CDV studies, there were at least 3 lineages of CDV as mentioned circulating in Thailand. Thus, RFLP technique is able to distinguish individual wild-type from vaccine lineage effectively and this method would be useful for several clinical applications in Thailand.

Department : …..Veterinary Pathology……	Student's Signature…………………………………
Field of Study : …Veterinary Pathobiology….	Advisor's Signature……………………………………
Academic Year : ………….2011…….……..	Co-advisor's Signature………………………………..

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BSA	=	bovine serum albumin
bp	=	base pair (s)
°C	=	degree Celsius (centrigrad)
CDV	=	canine distemper virus
CNS	=	central nervous system
CPE	=	cytopathic effect
DMEM	=	Dulbecco's modified Eagle's medium
DNA	=	deoxyribonucleic acid
DST	=	dog SLAM tag
F	=	fusion protein
g	=	gram (s)
HP	=	histopathology
H	=	hemagglutinin glycoprotein
H&E	=	hematoxylin and eosin staining
IHC	=	immunohistochemistry
min	=	minute (s)
PBS	=	phosphate buffer saline
PCV	=	phocine distemper virus
RFLP	=	restriction fragment length polymorphism
RNA	=	ribonucleic acid
RT-PCR	=	reverse transcription polymerase chain reaction
sec	=	second (s)
SLAM	=	signaling lymphocyte activation molecule
$TCID_{50}$	=	50% tissue culture infectious dose

CHAPTER I

INTRODUCTION

Canine distemper is a worldwide occurring infectious disease caused by a morbillivirus in the family Paramyxoviridae. Canine distemper virus (CDV) infection generally causes a multisystemic disease and severe immunosuppression in numerous families of the order Carnivora such as minks, raccoons, civets, foxes, lions, tigers, bears, and lesser pandas (Deem et al., 2000). This disease has been controlled by the use of attenuated live vaccines for many decades, yet a number of CDV infected dogs have been observed in vaccinated dog populations worldwide. There were previous reports of CDV infected vaccinated dogs in many countries (Lan et al., 2006; Calderon et al., 2007; Simon-Martínez et al., 2008). Vaccination failure might resulted from failure of immunization in animals that were infected prior to vaccination or vaccine strain that are not able to provide protection from the current circulating strains of CDV (Keawcharoen et al., 2005).

In Thailand, canine distemper remains the most life-threatening viral disease in puppies that are naïve or vaccinated as well as the adult vaccinated dogs. This lethal disease causes significant losses in not only domestic dogs, but also wild-life carnivores particularly Asian Palm civets (*Paradoxurus hermaphrodites*) that are worthy in high-priced coffee production. There are limited studies of CDV infected civets in Thailand, whereas CDV infection of wild civets were often reported in Japan and domestic dogs were suggested to be viral carrier (Takayama et al., 2009). To decrease the morbidity and mortality rates as well as the economic loss from vaccine import, the fundamental knowledge of locally circulating CDV strains in Thailand is required. Thus, phylogenetic analysis was studied to investigate the genetic diversity of CDV strains based on phosphoprotein (P), hemagglutinin (H) and fusion protein (F) genes that play an important role in viral pathogenesis.

In addition, attenuated vaccines is used widely in Thailand and the virus strain presented in the vaccine can interfere with polymerase chain reaction (PCR) based diagnostic tests in recently vaccinated animals, so it is crucial to discriminate between wild-type and vaccine strains. Accordingly, a method to specifically detect the wild-type CDV strains is necessary for preventing diagnostically false positive results. The restriction fragment length polymorphism (RFLP) of hemagglutinin (H) and nucleocapsid (N) genes has been established to detect and differentiate wild-type CDV infected dogs from CDV vaccinated dogs (Calderon et al., 2007; Wang et al., 2011). This study concentrated on gene encoding fusion (F) protein which has been demonstrated as a factor in viral pathogenesis and a cause of vaccine failure in canine distemper outbreaks (Lee et al., 2010). The appropriate F gene fragments will be amplified for phylogenetic analysis and the RFLP will be developed not only to differentiate wild-type CDV infected dogs from CDV vaccinated dogs, but also distinguish between CDV wild-type strains that circulate in domestic dogs in Thailand. Taking advantage of different DNA fragments that are digested by proper restriction enzymes, the RFLP analysis would be useful for several clinical applications such as confirmation of nature CDV infection, evaluation of vaccination status and epidemic observation of the circulating viral genotype.

Objectives of Study.

1. To develop the reverse transcription - polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP) techniques for effective diagnosis and differentiation between individual wild-type and vaccine strains in CDV infected dogs in Thailand

2. To investigate the genetic diversity of currently circulating CDV strains in Thailand comparing with the previous reports in GenBank by phylogenetic analysis

CHAPTER II

LITERATURE REVIEW

Canine distemper virus (CDV) belonged to family *Paramyxoviridae*, genus *Morbillivirus* and closely related to measles virus (MV), rinderpest virus (RPV), peste des petites ruminant virus (PPRV) and phocine distemper. CDV infection causes a high morbidity and mortality rate in broad host range. Numerous families of the order *Carnivora* are affected including Canidae (dogs, foxes), Felidae (cats, lions), Mustelidae (ferret, mink, badger), Procyonidae (raccoons, kinkajous, lesser panda) and Ursidae (Bears) (Deem et al., 2000). Domestic dogs have been suspected as a reservoir for free-ranging wildlife infection because of asymptomatic clinical signs (Beineke et al, 2009).

The CDV virion is a spherical structure of about 150-300 nm, it contains approximately 15,600-kb genome surrounded by a lipid envelope. CDV has unsegmented single-strand RNA encoding six structural proteins; the hemagglutinin (H), the fusion (F), the nucleocapsid (N), the phospho- (P), the large (L) and the matrix (M) protein, and two other non-structural proteins termed C and V protein (Lamb and Kolakofsky, 2001). The lipid envelop is integrated with H and F surface glycoproteins which facilitated viral attachment and membrane fusion. Surrounded by the envelop, helical nucleocapsid core including P, L and N protein are essential for initial viral replication in host cells. The M protein which connected between the envelop glycoproteins and nucleocapsid core is important during viral maturation and assembly (Beineke et al, 2009).

H protein displays the most genetic variation comparing to other structural proteins. The amino acid sequence of H protein shows about 10% variability among different CDV strains, while the F protein sequence varies about 4% which is approximate to the variability of the other CDV proteins (Beineke et al, 2009). However, these two envelop glycoproteins evidently play important roles in host immunity. Both H and F glycoproteins function concomitantly to mediate membrane fusion leading to the entry and exit of viral particles from the susceptible host cells. The H glycoprotein, a type II integral membrane protein binding to the specific cellular receptor, mediates viral attachment to host cell membrane in the first step of infection. Not only a CDV tropism, but also cytopathogenicity and fusion efficiency in susceptible cell line are contributed by H protein. The F glycoprotein is a type I integral membrane protein which is necessary for operating extracellular viral particle and host membrane fusion (von Messling et al., 2001).

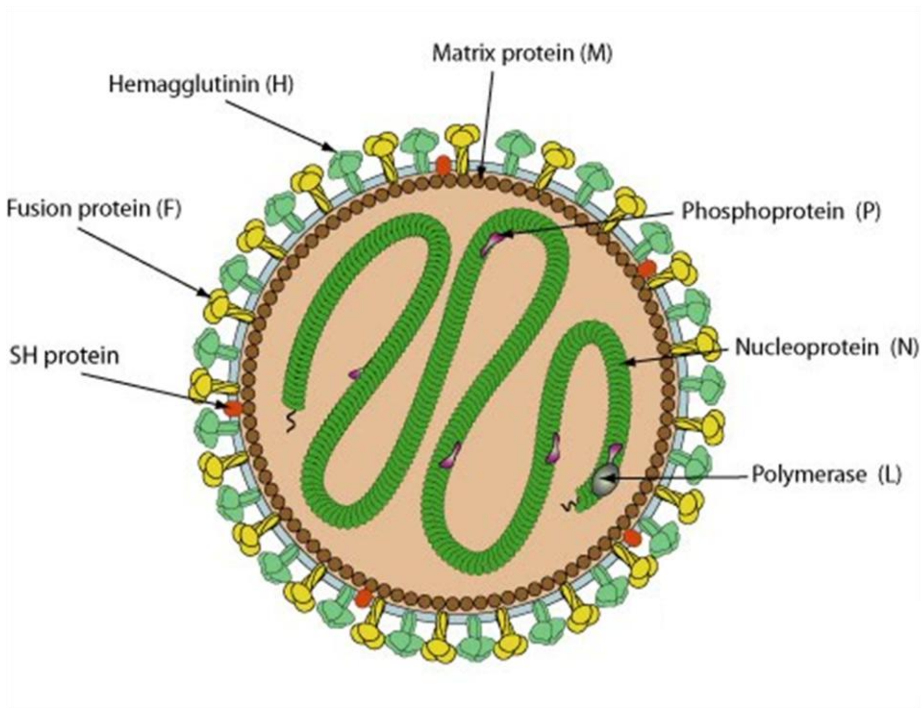


Figure 1 Morphology of canine distemper virus

(Available from: http://viralzone.expasy.org/viralzone/all_by_species/86.html)

After binding to cellular receptor, the attachment H protein changes its tri-dimensional conformation which consequently modifies the structure of F protein. As a result, the hydrophobic part of fusion peptide penetrates into host cells leading to membrane merging. Finally, the viral nucleocapsid core is delivered into the cellular cytoplasm in where CDV replication occurs. The progeny viruses are formed and released from the host plasma membrane by budding with an inactive precursor form of the fusion protein (F_0). The F_0 has to be activated by furin, a host cellular protease, resulting in the generation of the two subunits F_1 and F_2. The cleavage of F_0 is postulated to be a key factor that influences both infectivity and pathogenicity of paramyxoviruses causing direct cell to cell spread via fusogenic activity (Plattet et al., 2005). The infected cells that express H and F proteins on their surfaces can fuse with the receptor-containing neighbour cells displaying a characteristic feature of morbillivirus infection; the presence of multinucleated syncytial cells and cellular detachment in cell line (Meertens et al., 2003).

After the entering of virus, the incubation period takes 1 to 4 weeks depending on individual host's immunity. Primary virus replication takes place in lymphoid tissues of respiratory tract. Early clinical signs might be observed such as anorexia, weight loss and oculonasal discharge. During the first viremia phase, the progeny of virus present in macrophages and monocytes leading to viral dissemination to the distant hematopoietic tissues via hematogenous and lymphatic routes. Viral multiplications in various lymphoid tissues including thymus, spleen, gut-associated lymphoid tissue (GALT) and hepatic Kupffer cells lead to lymphopenia and further severe immunosuppression (Schobesberger et al., 2005; Vandevelde and Zurbriggen, 2005). Several days later, the second viremic phase occurs associated with the spreading of virus via lymphocytes, platelets as well as free-viral particles. Finally, the virus disseminates to various target epithelial tissues and the CNS. Systemic clinical signs for instance footpad hyperkeratosis, vomiting and diarrhea depend on the predominantly affected organs. CDV invades the CNS resulted in various nervous signs including myoclonus, chewing movement, ataxia, incoordination, circling, tetraparesis, nystagmus and convulsion.

Therefore, the severity and clinical signs of infected dogs are influenced by age, individual immune status and strain of virus. The symptoms are often exacerbated by secondary bacterial infections in respiratory and gastrointestinal systems that cause high mortality rate in young infected dogs (Koutinas et al., 2002; Schobesberger et al., 2005; Beineke et al, 2009).

Pathological findings of CDV infection can be found both in non-nervous and nervous tissues, frequently associated with characteristic intracytoplasmic and intranuclear inclusion bodies. Respiratory lesions include serous to mucopurulent rhinitis, interstitial pneumonia, necrotizing bronchiolitis which is often complicated by secondary bacterial pneumonia. Gastrointestinal manifestations result in gastroenteritis associated with the depletion of Peyer´s patches. Generalized lymphocytic depletion of lymphoid organs is commonly found and is associated with an impairment of the immune response. Skin infection displays variable features, including pustular dermatitis of the thighs and ventral abdomen, and hyperkeratosis of the footpads and nasal planum. Furthermore, CDV-associated bone lesions have been shown in young dogs with systemic distemper infection. Metaphyseal osteosclerosis develops due to persistence of the primary spongiosa and atrophy as well as necrosis of osteoclasts and bone marrow cells (Beineke et al, 2009).

The severity of neurological lesions depends on host immune status, age of infection and strain of virus. Generally, there are two types of CNS lesion which are characterized by pathogenesis and different distribution pattern of lesions; polioencephalitis and leukoencephalitis. Polioencephalitis, a rarely recognized type, is identified by an acute inflammation of the brain's gray matter including mononuclear perivascular cuffing, neuronophagia and inclusion bodies in the neuron and astrocytes. Leukoencephalitis is more commonly found identified by inflammation of the white matter of cerebellum including gliosis and demyelination. CDV gains access to the CNS by different pathways (Amude et al., 2010). The hematogenous route is a typical transmitting pathway carrying infected peripheral blood mononuclear cells which penetrate the blood brain barrier into the CNS. In addition, cell-free viruses are circulating in the cerebrospinal fluid and fuse with the ependymal lining of the ventricles. The olfactory route represents an alternative pathway allowing CDV transmission and transneuronal spreading along the olfactory axons into the nervous system (Rudd et al., 2006). CDV antigen is detected in CNS capillary endothelial cells as well as in perivascular cuffing lymphocytes in 6-8 days post infection. CDV antigens in choroid plexus indicate the release of virus along cerebrospinal fluid resulting in the spreading of infection to ependymal cell and subependymal white matter. Several studies show that there is the impairment of viral protein translation in gray matter; CDV RNA is frequently observed with low viral protein expression. These are the result from antigenic modulation of virus escaping from host's producing viral protein specific antibody leading to persistent infection (Koutinas et al., 2002; Baumgärtner and Alldinger, 2005).

Oligodendrocytes are focused as the main CDV tropism due to the predominantly demyelinating lesion occurring in almost CDV infected cases. However, many *in vivo* and *in vitro* studies failed to explain oligodendrocytes infection. Massive myelin damage is proved to be a result from the overwhelming immune response in chronic phase of infection, while the direct effect of CDV on oligidendrocytes infection remains unclear. Additionally, viral RNA expression is found, yet the protein expression in oligodendrocytes is significantly low indicating the restriction infection in oligodendrocytes (Vandevelde and Zurbriggen, 2005). The restriction infection is also explained the CDV infected neurons that RNA is evidently noted with the absence of neuron containing viral protein. On the other hand, astrocytes are represented as the major affected population especially in early phase of infection. Moreover, immature astrocytes seem to act as latent cells containing the pathogen during the advance stage of demyelination (Seehusen et al., 2007).

CDV and Measle virus (MV) are well known for their ability to cause a chronic demyelinating disease of the CNS in their natural hosts, dogs and humans, respectively. Therefore, dogs infected with CDV have been considered a naturally-occurring translational model to investigate the pathogenesis of virus-triggered and immune-mediated demyelination in human diseases including multiple sclerosis (MS)

(Baumgärtner and Alldinger, 2005). Many mechanisms that virus takes advantages for evading from host immunity and persisting in CNS contributed to the persistent infection. Viral persistence in CNS is proved to be important for neuropathogenesis in advanced lesion. The persistence of CDV associated with the restriction infection and non-cytolytic viral cell-to-cell spread are proposed.

Regarding the restriction infection, CDVs infect oligodendrocytes and also neurons with transcription of totally viral genome but limited translation of viral protein. By this process, viral harboring cells gain effective way to escape from immunosurveillance and the reactive virus is able to consequently generate the new cycle of infection (Vandevelde and Zurbriggen, 2005). Another factor influencing on viral persistence is the lack of cell-to-cell fusion. Virulent CDV strains with ability to induce persistent infection can reduce the exposure to host immunity by exhibiting only a few cytolytic syncytial formations and limiting the extracellular viral production. Ondersterpoort strain representing as CDV non-persistent strain produces extensive cell fusion and central cytolysis of large cytolytic syncytium on monolayer Vero cells accompaning with large amount of viral particles that are detected in the supernatant. In contrary, A75/17 strain standing for CDV virulent strain displays heterogenous infection pattern comprised of a single infected cell among uninfected ones and absence of cell fusion associated with a little production of infectious virus in the supernatant of cell culture (Meertens et al., 2003). In CNS, this viral transfer mechanism is observed in astrocytes using their foot processes to deliver infectious particles to the other distant astroglial network (Wyss-Fluehmann et al., 2010). Furthermore, the coexpression of H and F glycoprotein on infected cell surface is a key factor for inducing cellular fusogenicity. CDV persistent strain reveals a limited colocalization of both envelop glycoprotein, while that in the attenuated strain always coexpresses (Meertens et al., 2003). The signal pre-peptide domain of F protein from persistent strain is demonstrated to control cell surface protein expression by reducing total protein expression and indirectly induce a limited cell to cell fusion activity (Meertens et al., 2003; Platet et al., 2005, 2007). Either lack of cytolysis, syncytium or little extracellular viral shedding in the

CNS lead to a limited stimulation of the local immunosurveillance, resulting in ultimately favoring viral persistence.

Because of CDV typical symptoms displaying highly immunosuppression characterize in lymphopenia, lymphoid depletion and increase susceptibility to opportunistic infection, several studies concentrate on the tropism of CDV on lymphocytes. Signaling lymphocyte activation molecule (SLAM; CD150), a membrane glycoprotein molecule acting as known *Morbilliviruses* receptor, expresses on immune cell of human, dogs, mice and cattle. Since SLAM is evident on immature thymocytes, peripheral T and B lymphocytes, mature monocytes and dendritic cells, the selective damage of receptor expressed cells might lead to immunosuppression induced by CDV. In contrast, CDV could infect epithelial cells including neuronal cells, pneumocytes, intestinal mucosa and transitional cells of urinary bladder which are SLAM-negative cells indicating other unrecognized cellular receptors might involve. CD9, a tetraspan transmembrane-protein is found associated with cell-to-cell fusion, but not virus-to-cell fusion. This membrane protein takes part in the regulation of H protein binding to the unknown cellular receptor. So CD9 is mentioned as a cofactor for CDV induced syncytial cell formation (Singethan et al., 2006).

Virus isolation in cell culture is one of the techniques that widely use to study of CDV. The different CDV strains grow efficiently in cell cultures from many species suggesting that CDV has a broad cell tropism *in vitro* as well. Mitogen-stimulated canine lymphocytes and peritoneal macrophages from ferrets are used for CDV isolation. Due to their difficulty to detect CPE and inconvenience to prepare lymphocytes from healthy dogs, marmoset lymphoid (B95a) cells and Madin-Darby canine epithelial kidney (MDCK) cells are used instead (Lan et al., 2005). SLAM expressing cells of the immune system is recently found to be a receptor for CDV. Therefore, Vero cell expressing canine SLAM (Vero-DogSLAMTag; Vero-DST) is developed for efficient virus isolation. Nowadays, Vero-DST is the most effective cell line for CDV isolation due to its ability to early detect CPE with least mutation of nucleotide sequences (Seki et al., 2003).

Since CDV infection occurs in broad host range, domestic dogs have been suspected as a reservoir for free-ranging wildlife infection because of asymptomatic clinical signs (Beineke et al, 2009). The phylogenetic analysis of CDV has been performed to investigate the genetic relationship in CDV susceptible species and to identify genetic variation among CDV wild-type and vaccine strains. Based on nucleotide alignment of the H gene, the most variable part of the genome and mostly reliable classification for genetic diversity, CDV has been categorized into 7 major lineages including Asia-1, Asia-2, Europe, European wild-life, vaccine (America-1), America 2 and Arctic-like (Woma et al., 2010). The P gene that highly conserved between CDV isolates also has been chosen for genetic comparison and supported the H gene alignment that CDV genotypes are classified according to geographic distribution rather than host species. Recently, Zhao et al. (2010) propose a novel genotype, Asia 3, which are isolated from foxes in China and suggest that this strain is evolving divergently in this geographical area. In Thailand, there is a report of 2 CDV lineages displaying nucleotide homology to Asia-1 and vaccine (Onderstepoort) lineages based on N gene (Kaewcharoen et al., 2005). In addition, Charoenvisal (2008) proposes a new lineage genetically different from the others reported in GenBank, indicating that there are at least 3 strains of CDV circulating in Thailand.

CHAPTER III

MATERIALS AND METHODS

1. Specimens

A total number of thirty-six specimens were obtained from dogs and zoo animals suspected of canine distemper virus infection by showing clinical signs of respiratory, gastrointestinal and nervous system or positive result of rapid CDV testkit. Fourteen conjunctival swabs of the dogs were collected from private animal hospitals in Bangkok. Nine carcasses of the dogs diagnosed as CDV infection were submitted to Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University. All zoo animal specimens were obtained from private civet coffee farm at Kanchanaburi province where CDV outbreak occurred. The carcasses from three Asian palm civets (*Paradoxurus hermaphroditus*) and one Small indian civet (*Viverricula indica*) that died following clinical signs suggesting CDV infection were submitted for routine necropsy. Conjunctival swabs were also taken from eight Asian palm civets and one Masked palm civet (*Paguma larvata*) living in the same farm. All of the specimens were collected from December 2009 to December 2011 following the approval of Chulalongkorn University Animal Care and Use Committee (No. 11310088).

General signalments, vaccination history and clinical signs of the specimens were recorded. The cotton swabs from conjunctiva were maintained in sterile Phosphate buffered saline (PBS) solution. The carcasses were routinely performed a necropsy then tissue samples including brain, lung, small intestine, spleen, mesenteric lymph nodes and third eyelids were collected either fresh or fixed in 10% neutral buffered formalin. Fresh necropsied tissues and conjunctival swabs in PBS were kept at -80ºC for virus isolation. The commercial vaccine products composing of Vanguard® HTLP5/L (Pfizer, Thailand), Quantum® DHA2PPv (Schering-Plough, US), Canigen® DHA2PPi (Virbac, Thailand) and Tetradog® (Merial, Thailand) were included in this study.

2. Histopathological examination

1) Histopathological study

Variously visceral organs fixing in 10% neutral buffered formalin were routinely histologically processed. Tissue samples were embedded in paraffin wax, cut into four micron thickness and stained with Hematoxylin and Eosin (H&E) for observing under light microscope. For non-nervous tissues, histopathological discussion was observed for areas of affected organs and severity of lesions. Nervous tissues including the cerebrum, cerebellum, brain stem and spinal cord were categorized into 3 groups as follows; group I (no remarkable lesion), group II (acute lesion, i.e. intranuclear or intracytoplasmic inclusion body, focal vacuolization, mild gliosis and neuronal death) and group III (chronic lesion, i.e. prominent perivascular cuffing of mononuclear cells, intranuclear or intracytoplasmic inclusion body, gliosis with activated astroglia and microglia, malacia concurring demyelination). Samples in group I were observed comparing with negative brain which had neither nervous sign nor CDV positive PCR result (adapted from Bregano et al., 2011).

2) Immunohistochemical study

Nervous tissues were used for detecting CDV antigen. Immunohistochemical staining was performed using chain polymer-conjugated method. Four micron paraffin section of selected organs were deparaffinized in xylene for 15 minutes, rehydrated in graded alcohols for 10 minutes and rinsed with distilled water. The antigen was retrieved by autoclave at 121°C for 5 minutes in distilled water and followed by blocking endogenous peroxidase activity with 3% hydrogen peroxide (H_2O_2) in methanol for 5 minutes. The slides were washed in phosphate buffer saline (PBS) for 5 minutes, 3 times before incubation with 1:200 dilution of mouse monoclonal anti-envelope CDV antibody (Monotope Virostat®, USA) as primary antibody at 37°C for 60 minutes and following by Dako REAL™ EnVision™ Detection System (Dako®, Denmark) at 37°C for 60 minutes. After 3 cycles of washing in PBS for 5 minutes, positive antigen-antibody reaction was observed by labeling with 3, 3-diaminobenzidine tetrahydrochloride (DAB) for 2 minutes and counterstained with Mayer’s hematoxylin. Positive control was a brain tissue from necropsy case previously diagnosed as CDV infection (Charoenvisal, 2008). Immunohistological results of nervous tissues were discussed in descriptive analysis based on distribution of CDV antigen in different cell types and histopathological category from H&E staining.

3. Virus isolation

1) Specimen preparation

Fresh necropsied tissues were collected from the CDV diagnosed carcasses by using sterile technique. Various organs from each case were pooled and homogenized with of Dulbecco's modified Eagle's medium (DMEM) (0.1 g tissue : 1 ml DMEM) by using TissueRupture (Qiagen®, Thailand). The homogenates were sonicated and centrifuged at 2,500 rpm, 4°C for 10 minutes then the supernatant was collected and filtrated through 0.2 μm Millipore filters (Corning Inc., USA). The conjunctival swabs were also centrifuged at 2,500 rpm, 4°C for 10 minutes to obtain viral suspensions. For commercial vaccine products, lyophilized powders were dissolved into 1 ml sterile distill water for performing virus isolation.

2) Virus isolation

Supernatant of homogenized samples, viral suspensions from conjunctival swabs and reconstituted vaccines were applied on monolayer African monkey kidney cell line (Vero cells) expressing canine signaling lymphocyte activation molecules (Vero-DST) (kindly provided by Prof. Dr. Ryoji Yamaguchi, University of Miyazaki, Japan) in a 24-well plate and further incubated at 37ºC for 1 hour. Then, DMEM and Geneticin® (G418, 0.4 mg/ml, Invitrogen, USA) were added up to 1 ml into each well, CDV-infected cells were incubated at 37ºC in 5% CO_2 incubator for 4-5 days. Each isolated sample was observed daily by an inverted microscope for cytopathogenic effect (CPE) characterized by multinucleated syncytial formation and cellular detachment. If CPE showed more than 70%, the infected-cell suspension was collected and kept at -80 ºC until used.

4. Reverse - transcription polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP)

Total RNA was extracted from conjunctival swabs, tissue homogenates and CDV-infected Vero-DST cells by using NucleoSpin Extract Viral RNA Kit (Macherey-Nagel, Düren, Germany). Oligonucleotide primers were specific to the region on P, H and F gene (Table 1). For amplification of 390 bp-fragment of P gene, the forward primer was Upp1 and the reverse primer was Upp2. For amplification of 1,824 bp-fragment of H gene, the forward primer was CDV-HS1 and the reverse primer was CDV-HS2 (Charoenvisal, 2008). For amplification of 1,031 bp-fragments of F gene, the primer pair was designed based on the genomic sequences of CDV strains published in GenBank with PrimerSelect software (DNASTAR Inc., USA). The RT-PCR reaction was done using a one-step RT-PCR system kit (Access Quick™, Promega, USA). Amplification steps were optimized as follows; reverse transcription at 50°C for 30 min, denatured reverse-transcription at 94°C for 2 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 53°C for 30 sec and extension at 72°C for 1 min for P gene, 4 min for H gene and 1 min 10 sec for F gene followed by a 10 min final extension step at 72°C, using the thermoregulator ATC 401 (NYX Technik Inc., USA). The PCR products were visualized by 1.5% agarose gel electrophoresis in Tris-borate-EDTA (TBE) and stained with 10% ethidium bromide for observation under an UV illuminator.

For RFLP analysis, the F gene sequences from PCR product were aligned (genetic sequencing step will be described subsequently). The appropriate restriction enzyme was selected using NEBcutter Version 2.0 program (New England BioLabs Inc., USA; http://tools.neb. com/NEBcutter2). Based on predictable program, sequences digested by *Taq$^{\alpha}$I* (New England BioLabs Inc., USA) were divided into 3 groups as follows; sequences from group 1 showed 2 fragments of 393 and 638 bp length, sequences from group 2 generated 3 fragments of 60, 279 and 692 bp length, whereas sequences from group 3 were undigested and gave intact 1,031 bp products. The RFLP reaction was performed according to the manufacturer's instructions. One µl *Taq$^{\alpha}$I*, 3 µl 10X buffer, 3 µl 10X BSA, 2 µg PCR product and nuclease-free water were added up to

30 µl of total reaction volume. The reaction was then incubated at 65°C for 1.5 hour using the thermoregulator ATC 401(NYX Technik Inc., USA). The different cleaved fragments were used to determine strains of CDV after monitoring on 2% agarose gel electrophoresis.

Table 1 Primers for RT-PCR of CDV P, H and F gene

Gene	Primer	Sequence (5'-3')
P	UPP1	ATGTTTATGATCACAGCGCGGT
P	UPP2	ATTGGGTTGCACCACTTGTC
H	CDV-HS1	AACTTAGGGCTCAGGTAGTCC
H	CDV-HS2	ATGCTGGAGATGGTTTAATTCAATCG
F	CDVF-Fo1031	CCTCAATGCTCAAGCAATCC
F	CDVF-Re1031	CAAGGATCTGGTTAGAGGAG

5. Sequence analyses

The amplified products were purified by using NucleoSpin Extract II (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions and submitted for genetic sequencing (1st BASE Pte Ltd, Singapore). The nucleotide sequences of P, H and F gene were aligned with other submitted CDV strains available in GenBank. Using Clustal W analysis program within MEGA 5 software package, the percentage of homologous nucleotides was analyzed.

The nucleotide accession numbers of P gene sequence of reference strain are: Onderstepoort (AF378705), Synder Hill (AY286481), Rockborn (AF181446), 00-2601/raccoon/USA (AY443350), A75/17 (AF164967), 007Lm/dog /JP (AB474397), 009L/dog/JP (AB252714), Hamamatsu/JP (AB028915), Th3/dog/TH (AB299191), Th14/dog/TH (AB299193), Th270Br/dog/TH (AB301064), Th290Br/dog/TH (AB299202), VcX/HUN (EU072201).

The nucleotide accession numbers of H gene sequence of reference strain are: Onderstepoort (AF378705), Convac (Z35493), Synder Hill (AF259552), GR88/dog/GND (Z47760), H05Bp7F/dog/HUG (DQ889183), Liud/dog/CHN (AF172411), 00-

2601/raccoon/USA (AY443350), giant-panda/CHN (AF178038), A75/17 (AF164967), KDK-1/ dog/JP (AB025271), Yanaka/dog/JP (D85755), HLJ2-07/dog/CHN (EU593894), 007Lm/dog/JP (AB474397), Seoul/dog/KOR (EU252148), HLJ1/dog/CHN (EU743934), Th270BR/dog/TH (AB301065), DK91/dog/DNK (Z47761), 2544/dog/GER (Z77672), DK86/mink/DNK (Z47759), 207-00/fox/ITA (DQ228166), , VacX/HUN (EF095750).

The nucleotide accession numbers of F gene sequence of reference strain are: Onderstepoort (AF378705), 00-2601/raccoon/USA (AY443350), A75/17 (AF164967), 007Lm/dog/JP (AB474397), Ac961/dog/JP (AB512286), GN/tanuki/CHN (EF596900), VacX /HUN (EU072198).

6. Phylogenetic analyses

Phylogenetic trees of investigated genes (P, H and F) were constructed based on nucleotide sequences from this study and those of the reference strains from GenBank (as mentioned in sequence analysis) using MEGA 5 software package. The Maximum Likelihood algorithm was use to constructed the tree. Standard errors were calculated by the bootstrap method using 1000 replicates.

7. Data analysis

Histopathological results are discussed in descriptive analysis included the areas of affected organs, severity of lesions, distribution of CDV antigen and cell trophism of CDV infected organs comparing between nervous and non-nervous groups. The results from RFLP technique is verified by genetic sequencing and evaluated the accuracy of this technique.

CHAPTER IV

RESULTS

1. Specimens

Total thirty-six clinical cases suspected of canine distemper virus infection were included in this study. Conjunctival swab samples were collected from eight Asian Palm civets, one Masked Palm civet and fourteen dogs. Necropsied tissues composed of lung, intestine, lymphoid organ, urinary bladder, brain and spinal cord were obtained from three Asian Palm civets, one Small Indian civet and nine dogs. The ages of affected dogs ranged from 1 month to 5 years and those of civets ranged from 4 months to 2 years. Six dogs had previously been vaccinated against CDV (26.0%), all thirteen civets and four dogs had not, while thirteen dogs had obscure vaccination history. Other general signalments and clinical signs were described in Table 2.

Respiratory symptoms such as purulent oculonasal discharge, cough, dyspnea and crackle lung sounds were predominantly observed in fourteen CDV infected dogs and four civets (58.0%). Neurological signs including convulsion, myoclonus, chewing gum and ataxia were noted in thirteen dogs and four civets (54.8%). Six civets and six dogs suffered from gastrointestinal signs as vomiting and diarrhea (38.7%). Severe dehydration and hyperkeratosis of foot pads and nasal planum were displayed in all CDV infected civets. Other clinical signs such as conjunctivitis, uveitis and pustular dermatitis were seen in some dogs.

Table 2 General signalments and clinical signs of clinically CDV suspected animals

Case No.	Breed	Age	Sex	VH	CDV specific clinical signs	Samples
BKK01/09	Mongrel	1 m	F	MD	purulent oculonasal discharge, convulsion, conjuctivitis	CS
BKK02/09	Mongrel	MD	M	MD	purulent oculonasal discharge, convulsion	CS
BKK03/09	Mongrel	>1 y	F	MD	Purulent nasal discharge, myoclonus, foot pad hyperkeratosis	CS
BKK04/09	Mongrel	MD	MD	MD	Purulent oculonasal discharge, convulsion	CS
BKK05/09	Rottweiler	3 m	M	MD	Convulsion, myoclonus, presence of inclusion body in red and white blood cells	NT
BKK01/10	Shih-Tzu	2 m	M	Yes	Lung edema, diarrhea	CS
BKK02/10	Mongrel	>1 y	M	No	Muscular atrophy, chewing gum	CS
BKK03/10	Mongrel	MD	F	MD	MD	NT
BKK04/10	Golden retriever	2 m	F	No	Purulent oculonasal discharge, convulsion, conjunctivitis,	NT
BKK05/10	Chi hua hua	MD	M	MD	MD	NT
BKK06/10	Pomeranian	2 m	F	Yes	Serous nasal discharge, dyspnea, lung edema, diarrhea	NT
BKK01/11	Miniature pincher	5 y	F	Yes	Oculonasal discharge, increased lung sound, ataxia, uveitis, pustular dermatitis	NT

Case No.	Breed	Age	Sex	VH	Clinical signs	Samples
BKK02/11	Golden retriever	2 m	M	No	Purulent nasal discharge, decreased lung sound, productive cough, diarrhea	CS
BKK03/11	Mongrel	3 m	M	No	Purulent oculonasal discharge, interstitial lung pattern, convulsion, vomit, diarrhea	CS
BKK04/11	Golden retriever	2 m	MD	MD	MD	NT
BKK05/11	Mongrel	MD	MD	MD	MD	CS
BKK06/11	Mongrel	2 m	F	MD	Purulent oculonasal discharge, ataxia, diarrhea	CS
BKK07/11	Saint Bernard	1 y	M	MD	Dyspnea, convulsion, vomit, chronic dermatitis	NT
BKK08/11	Poodle	2 m	MD	Yes	Cough, dyspnea, convulsion, chewing gum	CS
BKK09/11	Shih-Tzu	3 m	M	Yes	Convulsion, exposure to CDV infected dogs	CS
BKK10/11	Mongrel	M	MD	MD	Oculonasal discharge (dog living in civet farm)	CS
BKK11/11	Mongrel	M	MD	MD	Oculonasal discharge, diarrhea	CS
BKK12/11	Dachshund	1 y 5 m	MD	Yes	Lung edema, dyspnea, diarrhea	NT
BKKZ01/11	Asian palm civet	4 m	M	No	Hyperkeratosis foot pads and nasal planum, serous nasal discharge, ataxia, dehydration	NT
BKKZ02/11	Asian palm civet	4 m	F	No	Hyperkeratosis foot pads and nasal planum, serous nasal discharge, ataxia, dehydration	NT

Case No.	Breed	Age	Sex	VH	Clinical signs	Samples
BKKZ03/11	Asian palm civet	4 m	M	No	Hyperkeratosis foot pads and nasal planum, serous nasal discharge, ataxia, dehydration	NT
BKKZ04/11	Small indian civet	4 m	F	No	Hyperkeratosis foot pads and nasal planum, serous nasal discharge, ataxia, diarrhea	NT
BKKZ05/11	Asian palm civet	1 y	F	No	Ulceration of tongue and hyperkeratosis of foot pads, dehydration	CS
BKKZ06/11	Asian palm civet	1 y 8 m	F	No	Hyperkeratosis of foot pads, dehydration	CS
BKKZ07/11	Asian palm civet	8 m	MD	No	Hyperkeratosis of foot pads, dehydration	CS
BKKZ08/11	Masked palm civet	2 y	F	No	Hyperkeratosis of foot pads, dehydration	CS
BKKZ09/11	Asian palm civet	1 y 6 m	M	No	Hyperkeratosis of foot pads, dehydration	CS
BKKZ10/11	Asian palm civet	2 y	M	No	Hyperkeratosis of foot pads, dehydration	CS
BKKZ11/11	Asian palm civet	1 y 5 m	M	No	Hyperkeratosis of foot pads, diarrhea, dehydration	CS
BKKZ12/11	Asian palm civet	2 y	M	No	Hyperkeratosis of foot pads, dehydration	CS
BKKZ13/11	Asian palm civet	1 y 5 m	M	No	Hyperkeratosis of foot pads, diarrhea, dehydration	CS

Note: VH: vaccination history (Yes - vaccinated at least 1 time, No - never vaccinated), MD: missing data, NT: necropsied tissues, CS: conjunctival swabs, M: male, F: female, y: year, m: month.

2. Histopathological examination

1) Histopathological study

After histological processing, various organs from necropsied cases were observed under light microscope for histopathological changes comparing with gross lesions (Table 3, 4).

Gross lesion of nervous system showed cerebral congestion in some specimens. Microscopically, brain lesions were classified into 3 groups for descriptive analysis comparing with immunohistochemical results. Group I: three dogs (BKK05/10, BKK06/10 and BKK04/11) and three civets (BKKZ01/11, BKKZ02/11 and BKK03/11) had no histopathological change comparing with negative control (Figure 7a). Group II: four dogs (BKK05/09, BKK04/10, BKK07/11 and BKK12/11) and one civet (BKKZ04/11) displayed acute stage of distemper encephalitis characterized by mild focal vacuolization, mild gliosis, non-suppurative meningitis, eosinophillic intranuclear and intracytoplasmic inclusion bodies in neuron and glia cells (Figure 7b). Group III: two dogs (BKK03/10 and BKK01/11) were suffering from chronic stage of distemper encephalitis identified by eosinophillic intranuclear and intracytoplasmic inclusion bodies, mononuclear perivascular cuffing, severe spongiosis and encephalomalacia, active gemistocytes and gitter cells, syncytial formation of neural cells (Figure 7c-f).

Macroscopic lesions of lung from all specimens showed pneumonia associated with pulmonary edema, congestion and hemorrhage in different degrees. For histopathology, five in thirteen specimens showed suppurative bronchointerstitial pneumonia (BKK05/09, BKK06/10, BKK01/11, BKK04/11 and BKK12/11) and eight in thirteen specimens revealed interstitial pneumonia (BKK03/10, BKK04/10, BKK05/10, BKK07/11, BKKZ01/11, BKKZ02/11, BKKZ03/11 and BKKZ04/11). Syncytial formation of bronchiolar epithelium (BKK03/10 and BKK04/11), proliferation of PAMs (BKK05/09, BKK06/10, BKK01/11 and BKK12/11) and bronchiolar epithelium sloughing (BKK12/11) were also noted. Eosinophillic intracytoplasmic and intranuclear inclusion bodies in bronchiolar epithelium, pneumocytes and pulmonary alveolar macrophages (PAMs) were found in all specimens (Figure 2a-d).

Gross lesions of gastrointestinal system from all specimens showed catarrhal gastroenteritis. Other lesions such as thickening of intestinal mucosa (BKKZ01/11), gas containing stomach (BKKZ03/11), gastric ulcer (BKKZ04/11) and hepatic congestion (BKK03/10) were also seen. Histopathologically, five in thirteen specimens showed lymphoplasmacytic enteritis (BKK03/10, BKK05/10, BKK01/11, BKK12/11 and BKKZ04/11) and catarrhal enteritis (BKK05/09, BKK05/10, BKK04/11 BKKZ01/11 and BKKZ02/11). Three in thirteen specimens revealed necrotic gastroenteritis (BKKZ01/11, BKKZ03/11 and BKKZ04/11). Eosinophilic intracytoplasmic and intranuclear inclusion bodies in mucosal epithelium and were evidenced in all cases. (Figure 4a-d).

Gross findings of hematopoietic organs such as lymph nodes and spleen often showed no remarkable lesion. Microscopically, severe lymphoid depletion and eosinophilic intranuclear inclusion bodies were noticed in lymphocytic cells in all cases. Lymph node sections of six from thirteen specimens presented histiocytic infiltration with occasional hemosiderosis (BKK05/09, BKK03/10, BKK05/10, BKK06/10, BKK01/11 and BKKZ04/11). Necrotic splenitis which characterized by the necrosis of lymphocytic cells in spenic white pulps was also observed in four specimens (BKK05/09, BKK07/11, BKK12/11 and BKKZ02/11) (Figure 3a-b).

Gross lesions from civet cases showed marked thickening of foot pads and nasal planum correlating with microscopic finding that presented hyperkeratosis of stratum corneum and eosinophilic intranuclear inclusion bodies in squamous epithelial cells (BKKZ02/11 and BKKZ03/11) (Figure 5). In the other hand, urinary bladder which displayed no remarkable lesion in gross finding distinctly revealed eosinophillic intracytoplasmic and intranuclear inclusion bodies in transitional epithelium (BKK05/09, BKK06/10, BKK04/11, BKKZ02/11 and BKKZ03/11) and lymphocytic infiltration (BKK01/11) (Figure 6).

Table 3 Histopathological finding of nervous, respiratory, gastrointestinal and hemopoietic system

Case	Nervous system[a]						Respiratory system[b]		Gastrointestinal system[b]			Hemopoietic system[b]		
	Gliosis	Neuronophagia	Meningitis	MPC	Demyelination	Inclusions	INP	BINP	Catarrhal	Necrosis	LMP	Depletion	Necrosis	Histiocytic
BKK05/09	+	+	-	-	-	N, G		*	*			*	*	*
BKK03/10	+++	++	+++	+	+++	N, G	*				*	*		*
BKK04/10	+	+	-	+	+	N, G	*			MD			MD	
BKK05/10	-	-	-	-	-	-	*		*		*	*		*
BKK06/10	-	-	-	-	-	-		*		MD		*		*
BKK01/11	+++	++	++	++	+++	N, G		*			*	*		*
BKK04/11	-	-	-	-	-	-		*	*			*		
BKK07/11	+	++	+	-	+	G	*			MD		*	*	
BKK12/11	+	+	+	-		G		*			*	*	*	
BKKZ01/11	-	-	-	-	-	-	*		*	*		*		
BKKZ02/11	-	-	-	-	-	-	*		*			*	*	
BKKZ03/11	-	-	-	-	-	-	*			*		*		
BKKZ04/11	+	+	-	-	-	G	*			*	*	*		*

Note: [a] The severity of microscopic lesions on nervous system were scored as follows; +++: severe lesion, ++: moderate lesion, +: mild lesion.

[b] The presence of microscopic lesions on non-nervous system were noted as *.

MPC: mononuclear perivascular cuffing, N: inclusion bodies in neurons, G: inclusion bodies in glia cells, INP: interstitial pneumonia,

BINP: bronchointerstitial pneumonia, LMP: lymphoplasmacytic infiltration, MD: missing data.

Table 4 Pathological diagnosis of affected organs

Case No.	Organs	Gross diagnosis	Histopathology
BKK05/09	Brain	Congestion	Mild non-suppurative polioencephalitis
	Lung	Pneumonia	Acute suppurative bronchopneumonia
	Lymph node	Mild lymphadenopathy	Histiocytic lymphadenitis
	Spleen	Mild splenomegally	Multifocal follicular necrotic splenitis
	Intestine	NRL	Mild catarrhal enteritis
BKK03/10	Brain	Congestion	Severe focally extensive leukoencephalomalacia and non-supurative meningitis
	Lung	Pneumonia	Acute suppurative interstitial pneumonia
	Tonsil	Mild tonsilar enlargement	Histiocytic tonsilitis
	Spleen	Mild splenomegaly	Splenic congestion
	Stomach	NRL	Mild lymphocytic gastritis
	Intestine	Mild catarrhal enteritis	Mild lymphocytic catarrhal enteritis
	Liver	Hepatic congestion	Congestion, reactive Kupffer cells with INIB in bile duct epithelium
BKK04/10	Brain	Congestion	Moderate non-suppurative polioencephalitis and focal demyelination
BKK05/10	Brain	NRL	NRL
	Lung	Pulmonary congestion and edema	Moderate subacute suppurative-hemorrhagic interstitial pneumonia and congestion
	Lymph node	NRL	Mild histiocytic lymphadenitis
	Intestine	Catarrhal enteritis	Mild lymphocytic catarrhal enteritis
BKK06/10	Brain	NRL	NRL
	Lung	Pulmonary edema	Mild acute suppurative bronchointerstitial pneumonia and edema, proliferation of PAMs
	Lymph node	NRL	Histiocytic lymphadenitis

Case No.	Organs	Gross Lesions	Histopathology
BKK01/11	Brain	Brain congestion	Severe generalized leukoencephalomalacia and non-suppurative polioencephalitis
	Lung	Acute diffuse pneumonia	Suppurative bronchointerstitial pneumonia
	Spleen	Mild splenomegaly	Histiocytic splenitis and hemosiderosis
	Intestine	Mild catarrhal enteritis	Lymphoplasmacytic enteritis
	UB	NRL	Lymphocytic cystitis
BKK04/11	Brain	NRL	NRL
	Lung	Diffuse pneumonia and pulmonary edema	Acute hemorrhagic bronchointersitital pneumonia
	Spleen	NRL	Splenic follicular depletion
	Intestine	Catarrhal enteritis	Mild catarrhal enteritis
BKK07/11	Brain	NRL	Moderate non-suppurative polioencephalitis and focal demyelination
	Lung	Severe pneumonia and pulmonary edema	Severe acute diffuse suppurative bronchopneumonia
	Spleen	NRL	Severe multifocal necrotic suppurative splenitis
	Lymph node	NRL	Severe diffuse suppurative lymphadenitis
BKK12/11	Brain	NRL	Mild non-suppurative polionecephaliits and non-suppurative meningitis
	Lung	Multifocal extensive pneumonia and edema	Moderate subacute suppurative bronchointerstitial pneumonia
	Spleen	Mild splenomegaly	Multifocal necrotic splenitis
	Intestine	Severe diffuse catarrhal enteritis	Moderate lymphoplasmacytic enteritis
BKKZ01/11	Brain	Marked cerebral congestion	NRL
	Lung	Severe pneumonia and pulmonary edema	Multifocal interstitial pneumonia
	Spleen	NRL	Hemorrhagic splenitis
	Intestine	Mild thickening of intestinal mucosa	Moderate catarrhal necrotic enteritis

Case No.	Organs	Gross Lesions	Histopathology
BKKZ02/11	Brain	Cerebral congestion	NRL
	Lung	Pneumonia, pulmonary edema and congestion	Necrotizing interstitial pneumonia
	Lymph node	NRL	Necrotic lymphadenitis
	Gastrointestine	Catarrhal gastroenteritis	Moderate catarrhal gastroenteritis
	Foot pads/ nasal planum	Severe hyperkeratosis	Severe necrotic hyperplastic dermatitis
BKKZ03/11	Brain	Cerebral congestion	NRL
	Lung	Pneumonia, pulmonary edema and congestion	Moderate multifocal interstitial pneumonia
	Spleen	NRL	Splenic congestion
	stomach	Gas containing stomach and intestine	Moderate necrotic gastritis
	Foot pads/ nasal planum	Severe hyperkeratosis	Severe necrotic hyperplastic dermatitis
BKKZ04/11	Brain	Marked cerebral congestion	Mild non-suppurative polioencephalitis
	Lung	Pneumonia, severe pulmonary congestion	Moderate multifocal interstitial pneumonia
	Lymph node	NRL	Histiocytic lymphadenitis
	Stomach	Diffuse gastric ulcer	Severe necrotic gastritis
	Intestine	Mucohemorrhagic enteritis	Severe chronic lymphocytic-necrohemorrhagic enteritis

Note: UB: urinary bladder, PAMs: pulmonary alveolar macrophages, INIB: intranuclear inclusion body, ICIB: intracytoplasmic inclusion body, NRL: no remarkable lesion

2) Immunohistochemical study

According to histopathological lesion classification of nervous system, immunohistochemistry against of CDV antigen was analyzed between three individual groups. The number of CDV antigen-positive cell type were scored as follows; +++: marked positive, ++: moderate positive, +: slight positive. Area where positive cells predominated was scored as follow; ***: frequently found, **: occasionally found, *: rarely found (Table 5).

Samples in group I (no pathological change) showed slightly to moderately positive results against CDV antigen in astrocytes. Mildly positive neurons, ependymal cells and meningeal cells were also found in some samples (BKK05/10, BKK04/11, BKKZ02/11 and BKKZ03/11). Positive cells population mainly located surrounding blood vessels in cerebral cortex and submeningeal area. Thalamus, brain stem and nearby central canal were mildly affected (BKK05/10, BKK06/10, BKK04/11 and BKKZ03/11).

All samples in group II (acute lesion) presented markedly positive neurons excepting BKK07/11 that showed strongly positive result in astrocytes (Figure 8a-b). Ependymal cells and meningeal cells were slightly found positive. CDV antigen significantly involved with cerebral cortex and brain stem. Other affected locations such as thalamus (BKK12/11 and BKKZ04/11), cerebellum (BKK04/10 and BKK12/11) and spinal cord (BKK05/09) were also observed.

In group III (chronic lesion), immunolabeled CDV antigen was found extensively positive in astrocytes, one sample displayed moderately positive ependymal cells (BKK03/10). Neuron and meningeal cell were mildly affected. Positive cells population was noticed throughout the brain such as cerebral cortex, thalamus, choroid plexus, cerebellum, brain stem and spinal cord, especially surrounding blood vessels and the fourth ventricle (Figure 8c-d).

Table 5 Immunohistochemical results of nervous system

HP Grading	Case No.	Positive Cells				Affected Area					
		Astrocytes	Neurons	Ependymal cells	Meningeal cells	Cerebral cortex	Thalamus	Cerebellum	Brain stem	Spinal cord	Choroid plexus
No Remarkable lesion	BKK05/10	+	+		+	*				*	
	BKK06/10	+				*			*		
	BKK04/11	++	+	+		*	**			*	
	BKKZ01/11	+				*					
	BKKZ02/11	+	+			*					
	BKKZ03/11	++	+		+	*	**		*		
Acute lesions	BKK05/09		+		+	*			*	*	
	BKK04/10		+++	+		***		*			
	BKK07/11	+++	+	+		***			*		
	BKK12/11	+	+++			*	**	*	*		
	BKKZ04/11	+	+++	+	+	**	*		*		
Chronic lesions	BKK03/10	+++	+			**	**	*	*	*	
	BKK01/11	+++	+	++	+	**		**	**	*	*

Note: The number of CDV antigen-positive cells were scored as follows; +++: marked positive, ++: moderate positive, +: slight positive.

Area where positive cells predominated was scored as follow; ***: frequently found, **: occasionally found, *: rarely found.

HP: histopathology.

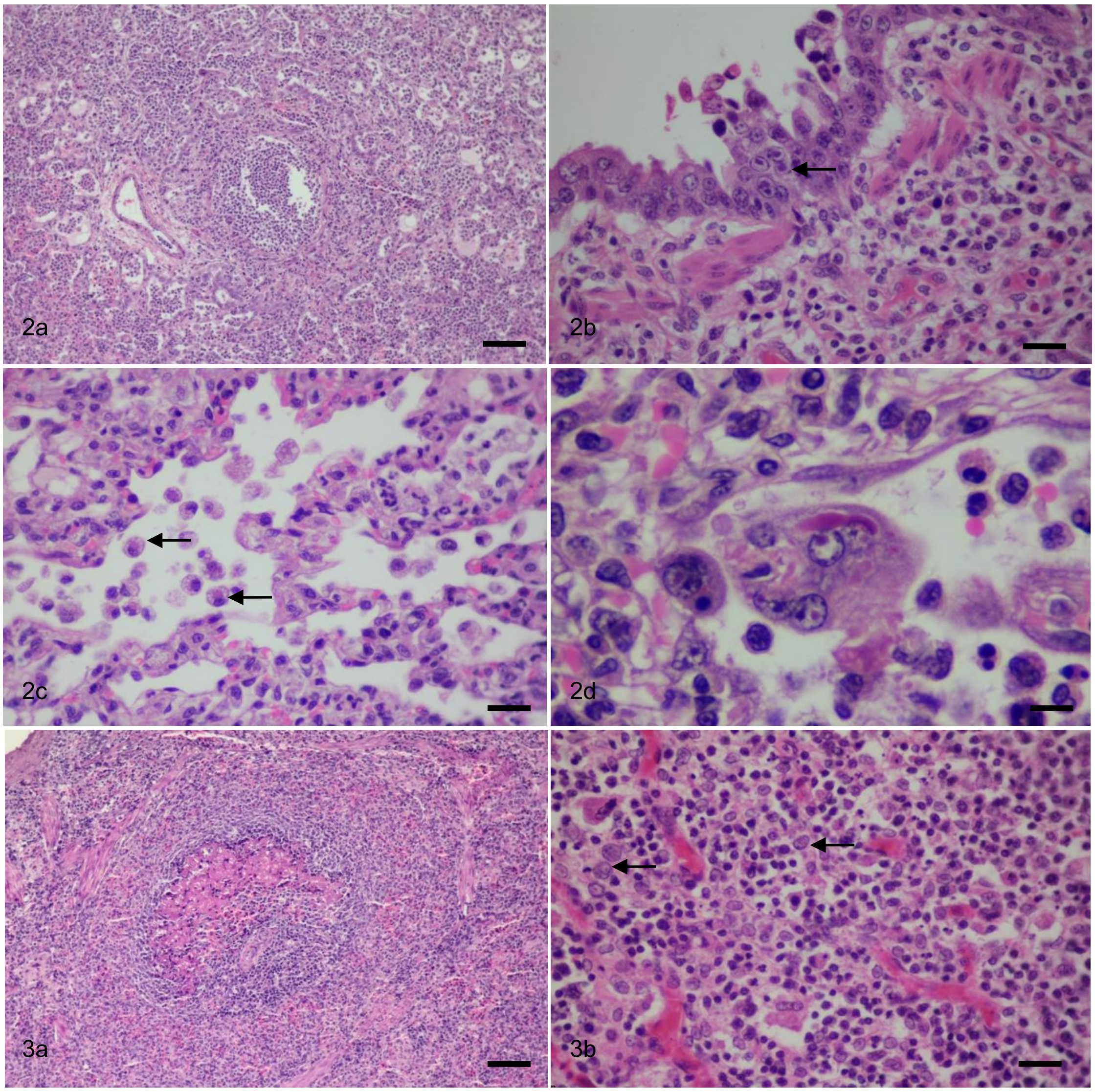


Figure 2a: Suppurative brochointerstitial pneumonia, lung; BKK05/09 (H&E staining, bar = 100 um).

Figure 2b: Syncytial bronchiolar epithelium with eosinophillic intranuclear inclusion bodies (arrow), lung; BKK04/11 (H&E staining, bar = 20 um).

Figure 2c: Proliferation of pulmonary alveolar macrophages (PAMs) with eosinophillic intracytoplasmic inclusion bodies, lung; BKK05/09 (H&E staining, bar = 20 um).

Figure 2d: Multinucleated syncytial cell with eosinophillic intracytoplasmic and intranuclear inclusion bodies, lung; BKK05/09 (H&E staining, bar = 10 um).

Figure 3a: Follicular necrosis, spleen; BKK03/10 (H&E staining, bar = 100 um).

Figure 3b: Eosinophilic intranuclear inclusion body (arrow) and lymphoid depletion, tonsil; BKK03/10 (H&E staining, bar = 20 um).

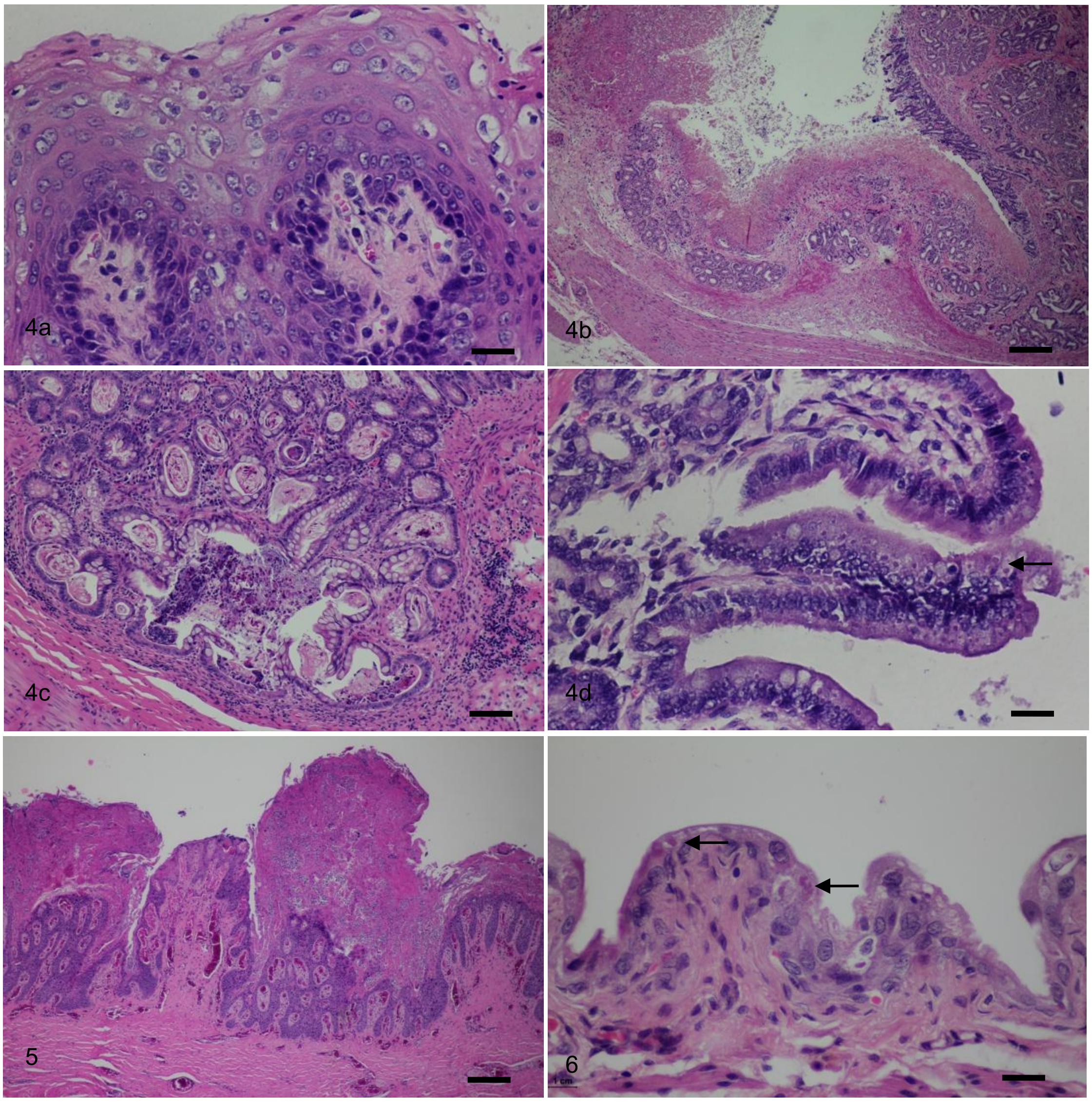


Figure 4a: Hydropic degeneration of squamous epithelium with eosinophillic intracytoplasmic inclusion bodies, tongue; BKKZ03/11 (H&E staining, bar = 20 um).

Figure 4b: Severe necrotic enteritis with shortened villi; BKKZ02/11 (H&E staining, bar = 200 um).

Figure 4c: Lymphoplasmacytic enteritis with crypt dilatation and necrosis, ileum; BKKZ04/11 (H&E staining, bar = 100 um).

Figure 4d: Shortening of villi and eosinophillic intracytoplasmic inclusion bodies (arrow), duodenum; BKKZ03/11(H&E staining, bar = 20 um).

Figure 5: Chronic dermatitis and hyperkeratosis, foot pad; BKKZ02/11 (H&E staining, bar = 200 um).

Figure 6: Eosinophilic intranuclear and intracytoplasmic inclusion bodies in transitional epithelium; urinary bladder (arrow); BKK05/09 (H&E staining, bar = 20 um).

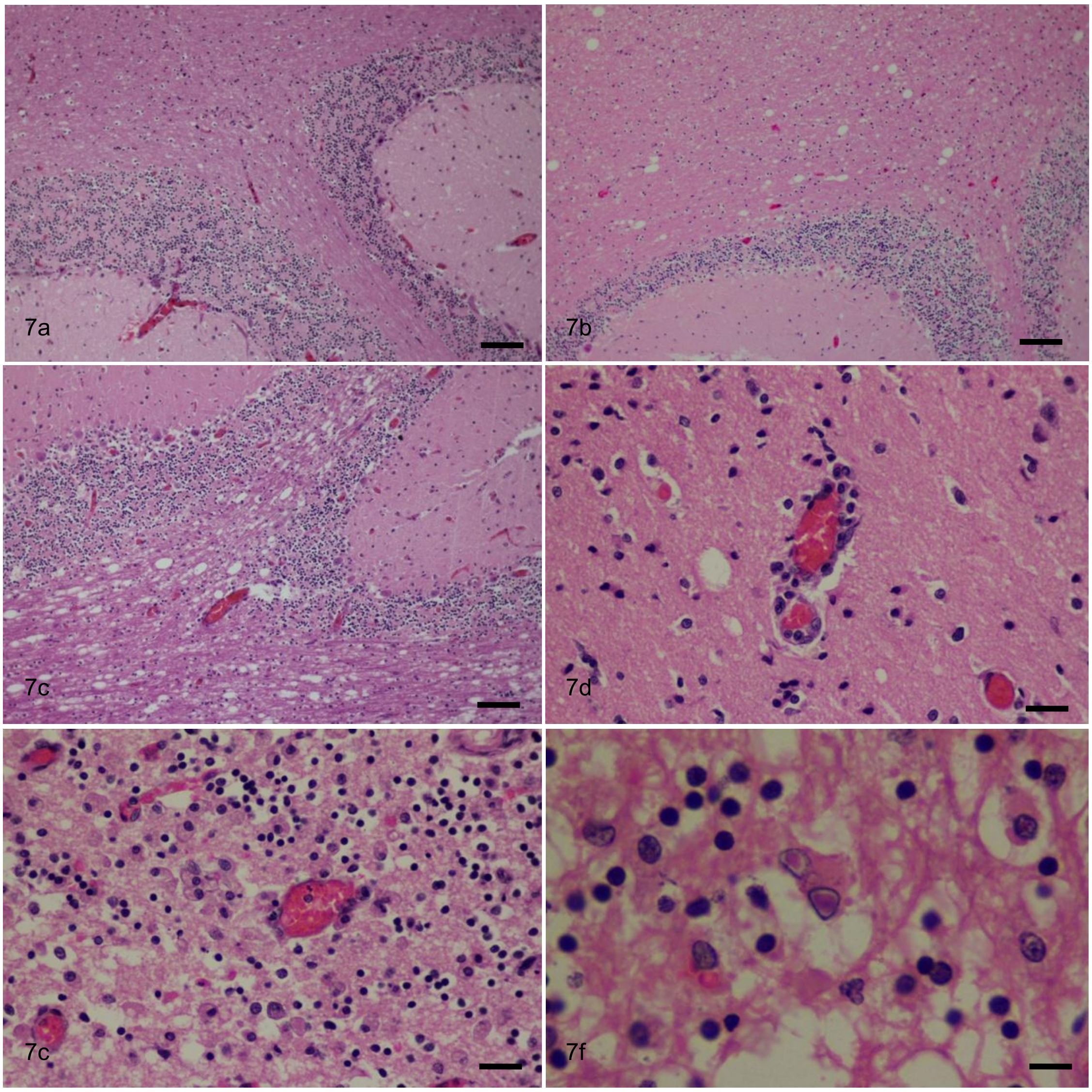


Figure 7a: No pathological lesion, cerebellum; BKK04/11 (H&E staining, bar = 100 um).

Figure 7b: Acute lesion displayed focal demyelination and mild gliosis, cerebellum; BKK04/10 (H&E staining, bar = 100 um).

Figure 7c: Chronic lesion displayed severe spongiosis and gliosis, cerebellum; BKK01/11 (H&E staining, bar = 100 um).

Figure 7d: Lympho-plasmacytic perivascular cuffing with eosinophillic intranuclear inclusion bodies. cerebrum; BKK01/11 (H&E staining, bar = 20 um).

Figure 7e: Leukoencephalomalacia with massive infiltration of gitter cells, cerebellum; BKK01/11 (H&E staining, bar = 20 um).

Figure 7f: Syncytial cell formation with eosinophilic intranuclear and intracytoplasmic inclusion bodies, cerebellum; BKK01/11 (H&E staining, bar = 10 um).

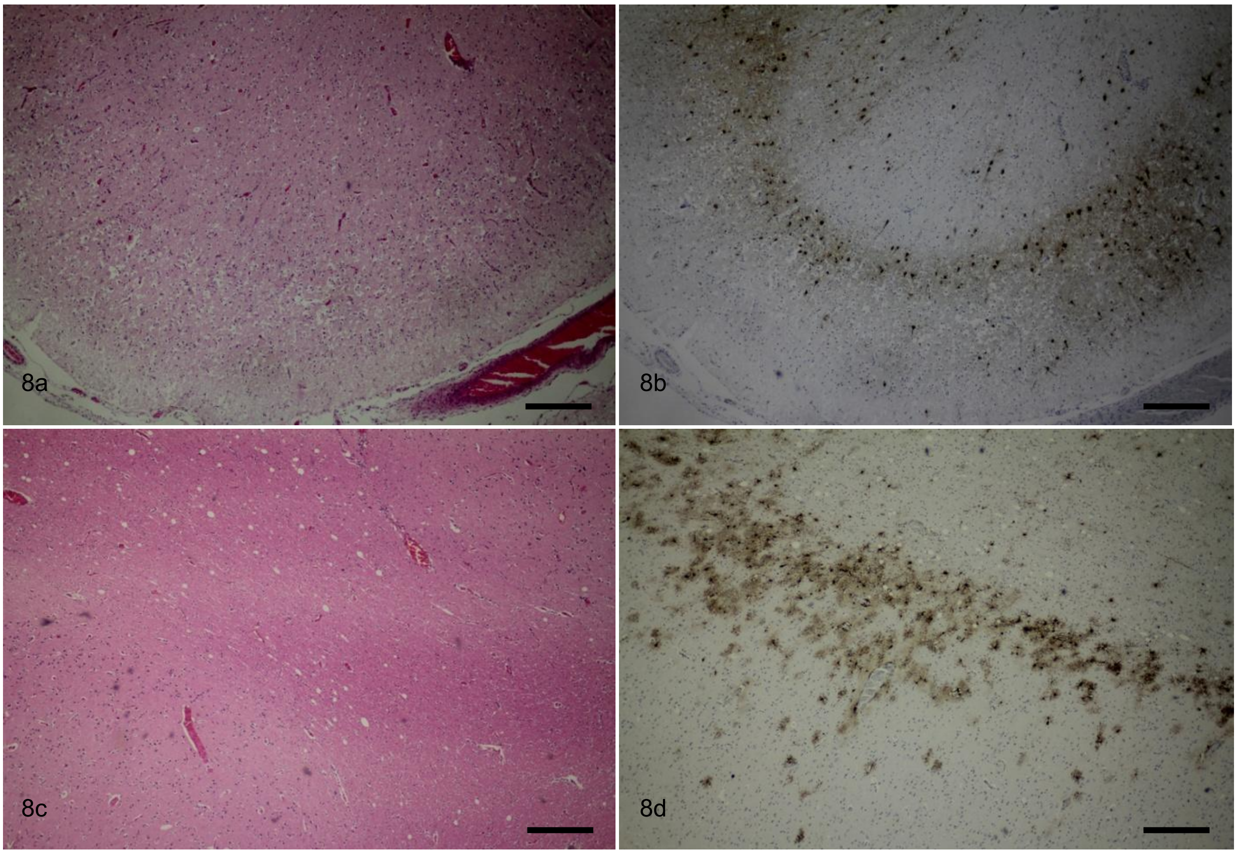


Figure 8a: Mild gliosis and cerebral congestion, cerebrum; BKK04/10 (H&E staining, bar = 200 um).

Figure 8b: Comparing with Fig. 8a, neuron-liked CDV-positive cells predominantly found at cortico-medullary junction, cerebrum; BKK04/10 (Envision system, bar = 200um).

Figure 8c: Focal demyelination in white matter, Cerebrum; BKK01/11 (H&E staining, bar = 200 um).

Figure 8d: Comparing with Fig. 8c, astrocyte-liked CDV-positive cells, predominantly found at cortico-medullary junction, cerebrum; BKK04/11(Envision system, bar = 200um).

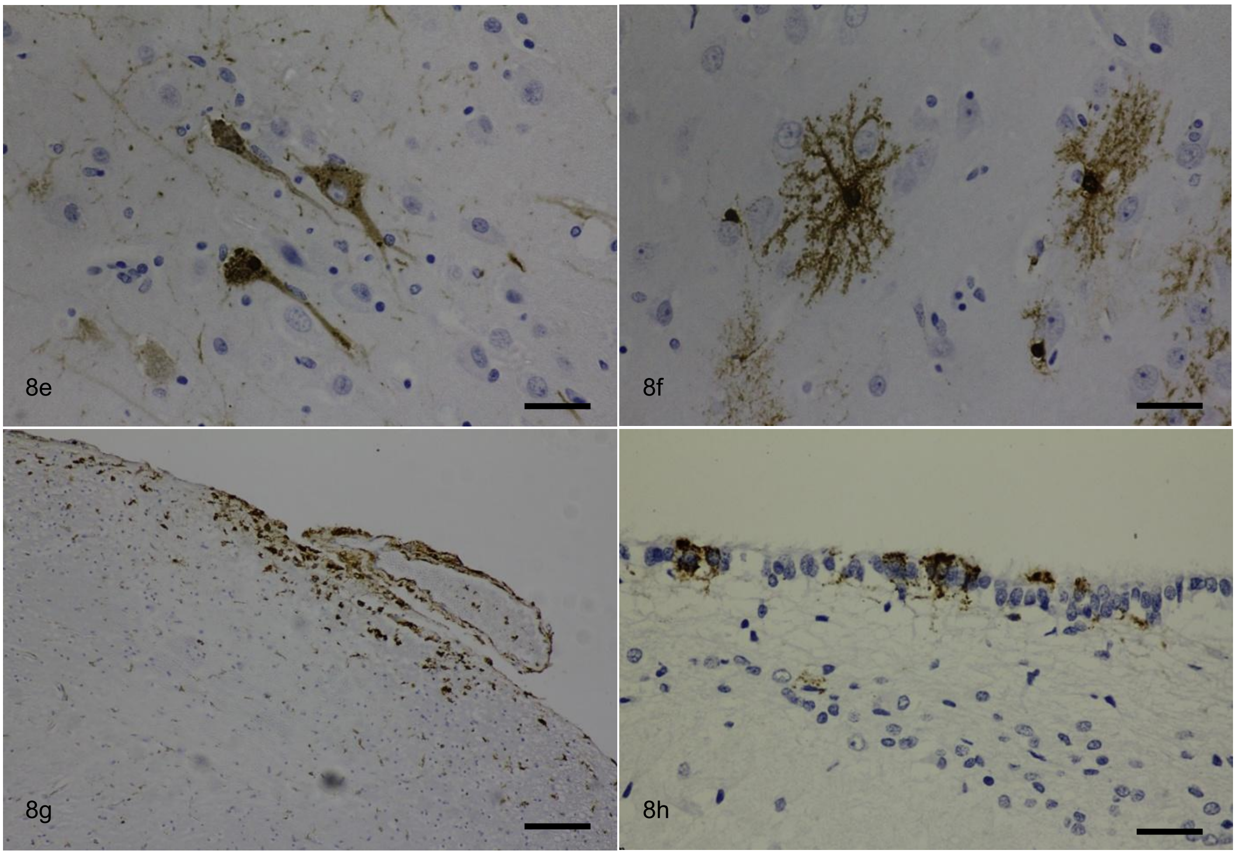


Figure 8e: Neuron-liked CDV-positive cells, cerebrum; BKK12/11(Envision system, bar = 20um).

Figure 8f: Astrocyte-liked CDV-positive cells, cerebrum; BKK03/10 (Envision system, bar = 20um).

Figure 8g: CDV-positive meningeal cells and blood vessels, cerebrum; BKK03/10 (Envision system, bar = 100um).

Figure 8h: CDV-positive ependymal cells, the forth ventricle; BKK04/11(Envision system, bar = 20um).

3. Viral Isolation

Supernatant of homogenized tissues, viral suspensions from conjunctival swabs and reconstituted vaccines from twenty-three samples were used for viral isolation in Vero-DST cell line. Twenty samples showed typical CPE identified by syncytial cell formation and cellular detachment (Figure 9b-c), one specimen (BKK/VG) presented different appearance of CPE with stellate cell formation (Figure 9d) and cellular detachment and the other two samples displayed no CPE (Table 6). Cell suspension from all samples showing CPE was collected for further studies.

4. Reverse - transcription polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP)

Viral RNA extract from different sources of samples (conjunctival swabs, tissue homogenates and CDV-infected Vero-DST cells) was used for amplification of partial P, H and F gene (Table 5). Agarose gel electrophoresis presented specific bands from of each target gene (Figure 10-12). All CDV suspected animals had positive results of 390 bp-fragment of P gene, 1,824 bp-fragment of H gene and 1,031 bp-fragments of F gene. Purified RT-PCR products from selected samples were submitted for nucleotide sequencing in P, H and F region. Oligonucleotide primers used for sequencing were same as for RT-PCR. Data was included in Table 1.

The 1,031 bp F gene fragments from RT-PCR reaction were allocated to perform RFLP analysis. After *Taq*$^{\alpha}$*I* digestion and visualizing on 2% agarose gel electrophoresis, the different cleaved fragments were divided into three groups (Figure 13). RT-PCR products from twenty-eight isolates showed two fragments with 393 and 638 bp length, eight isolates generated two fragments with 279 and 692 bp length (expected fragment of 60 bp length was indistinct), whereas four isolates was undigested and gave intact 1,031 bp products (Figure 17). Results of RFLP analysis on F gene fragments will be discussed with phylogenetic analysis.

Table 6 Positive sample sources and RFLP results

Case No.	RT- PCR positive samples			RFLP results	CPE in Vero-DST cell
	P gene	H gene	F gene		
BKK01/09	VI	VI	VI	New Asia	+
BKK02/09	VI	VI	VI	New Asia	+
BKK03/09	VI	VI	VI	Asia-1	+
BKK04/09	VI	VI	VI	New Asia	+
BKK05/09	VI	VI	VI	Asia-1	+
BKK01/10	VI	VI	VI	New Asia	+
BKK02/10	VI	VI	VI	Asia-1	+
BKK03/10	VI	VI	VI	New Asia	+
BKK04/10	HS	HS	HS	Asia-1	+
BKK05/10	HS	HS	HS	Asia-1	+
BKK06/10	HS	HS	HS	Asia-1	+
BKK01/11	HS	HS	HS	New Asia	-
BKK02/11	CS	VI	VI	Asia-1	+
BKK03/11	CS	CS	CS	Asia-1	+
BKK04/11	HS	HS	HS	Asia-1	+
BKK05/11	CS	CS	CS	Asia-1	ND
BKK06/11	CS	CS	CS	New Asia	+
BKK07/11	HS	HS	HS	New Asia	ND
BKK08/11	CS	CS	CS	Asia-1	-
BKK09/11	CS	CS	CS	Asia-1	ND
BKK10/11	CS	CS	CS	Asia-1	ND
BKK11/11	CS	CS	CS	Asia-1	ND
BKK12/11	HS	HS	VI	Asia-1	+
BKKZ01/11	HS	VI	VI	Asia-1	+
BKKZ02/11	HS	HS	VI	Asia-1	+
BKKZ03/11	HS	HS	VI	Asia-1	+
BKKZ04/11	HS	HS	VI	Asia-1	+

case No.	RT- PCR Samples			RFLP results	CPE in Vero-DST cell
	P gene	H gene	F gene		
BKKZ05/11	CS	CS	CS	Asia-1	ND
BKKZ06/11	CS	CS	CS	Asia-1	ND
BKKZ07/11	CS	CS	CS	Asia-1	ND
BKKZ08/11	CS	CS	CS	Asia-1	-
BKKZ09/11	CS	CS	CS	Asia-1	ND
BKKZ10/11	CS	CS	CS	Asia-1	ND
BKKZ11/11	CS	CS	CS	Asia-1	ND
BKKZ12/11	CS	CS	CS	Asia-1	ND
BKKZ13/11	CS	CS	CS	Asia-1	ND
BKK/CG	VC	VC	VC	Vaccine	ND
BKK/QT	VC	VC	VC	Vaccine	ND
BKK/TTD	VC	VC	VC	Vaccine	ND
BKK/VG	VC	VI	VI	Vaccine	+

Note: VI: viral isolation, HS: homogenized sample, CS: conjunctival swab, ND: Not done

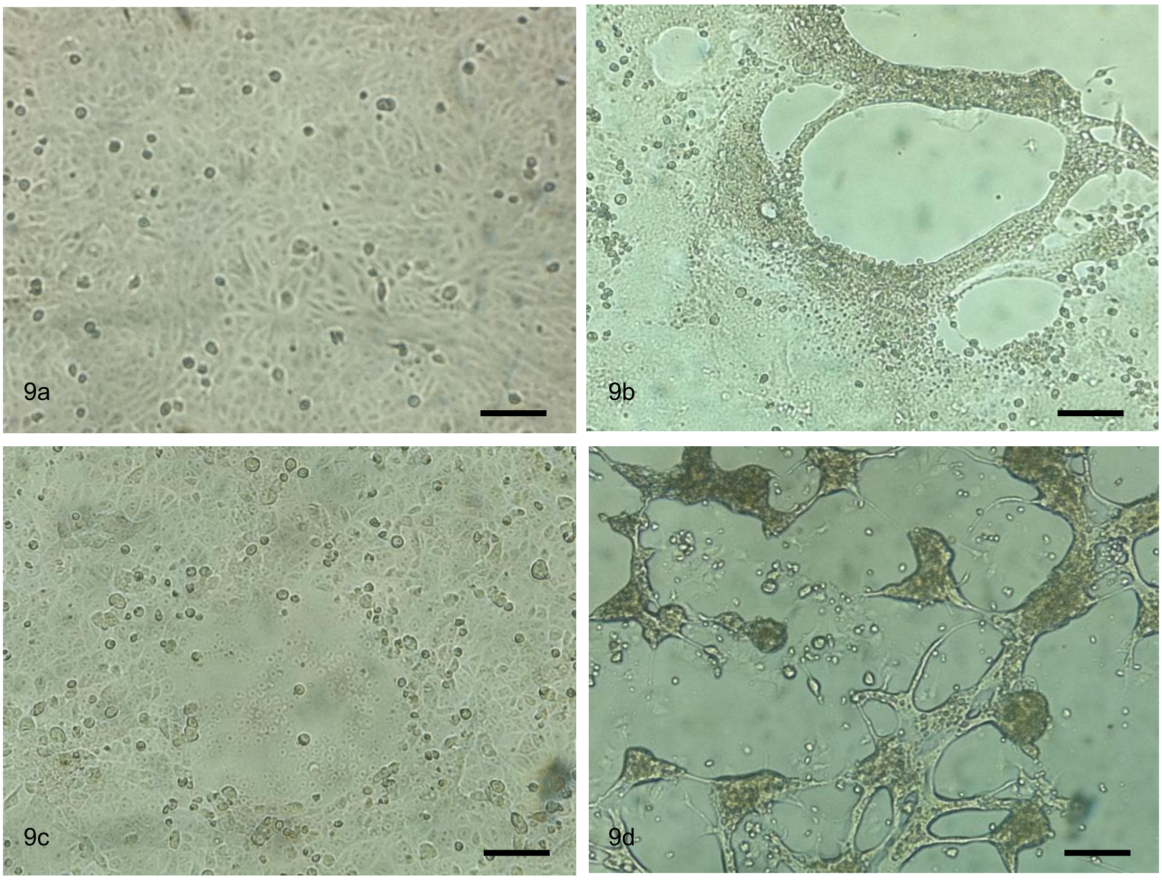


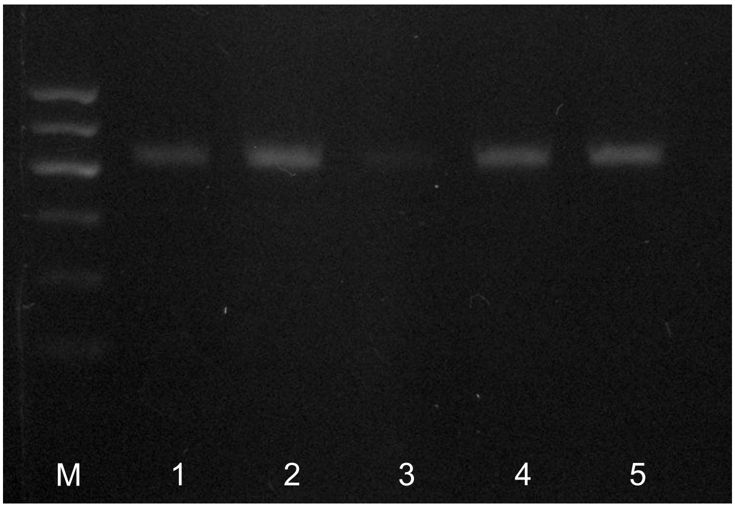
Figure 9a: Monolayer of normal Vero-DST cell line (bar = 40 um).

Figure 9b: Cellular detachment of CDV infected Vero-DST cell line (BKK02/11) (bar = 100 um).

Figure 9c: Syncytial cell formation of CDV infected Vero-DST cell line (BKK02/11) (bar = 40 um).

Figure 9d: Stellete cell formation of CDV infected Vero-DST cell line (BKK/VG) (bar = 40 um).

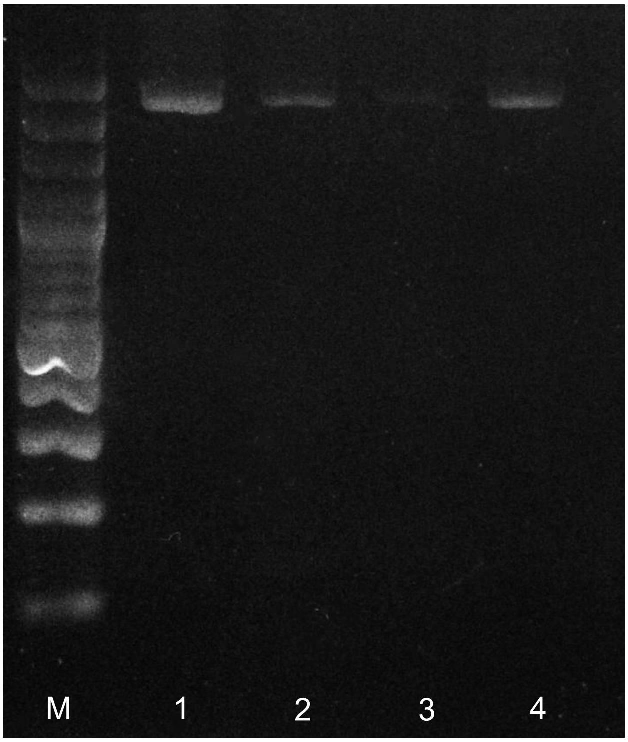
390 bp



P gene

10

1824 bp

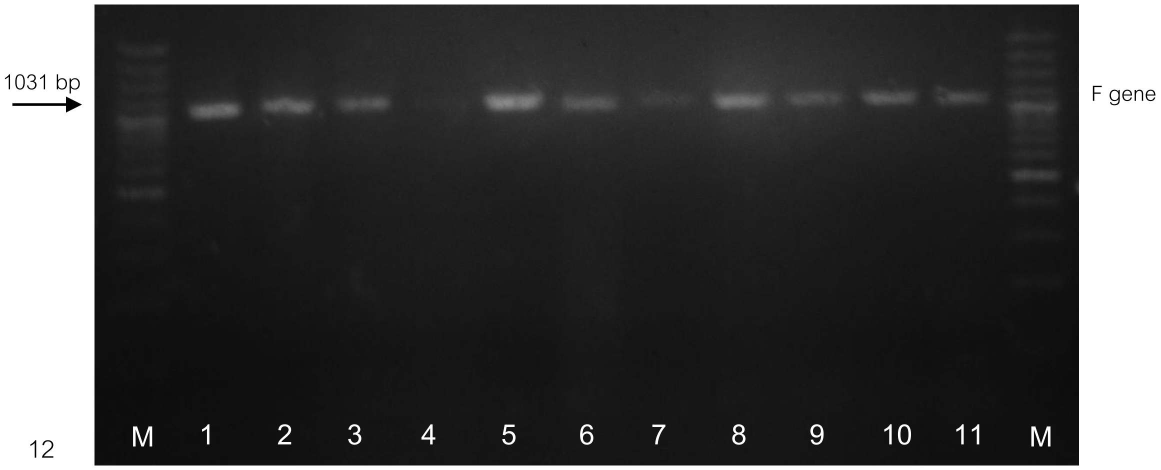


H gene

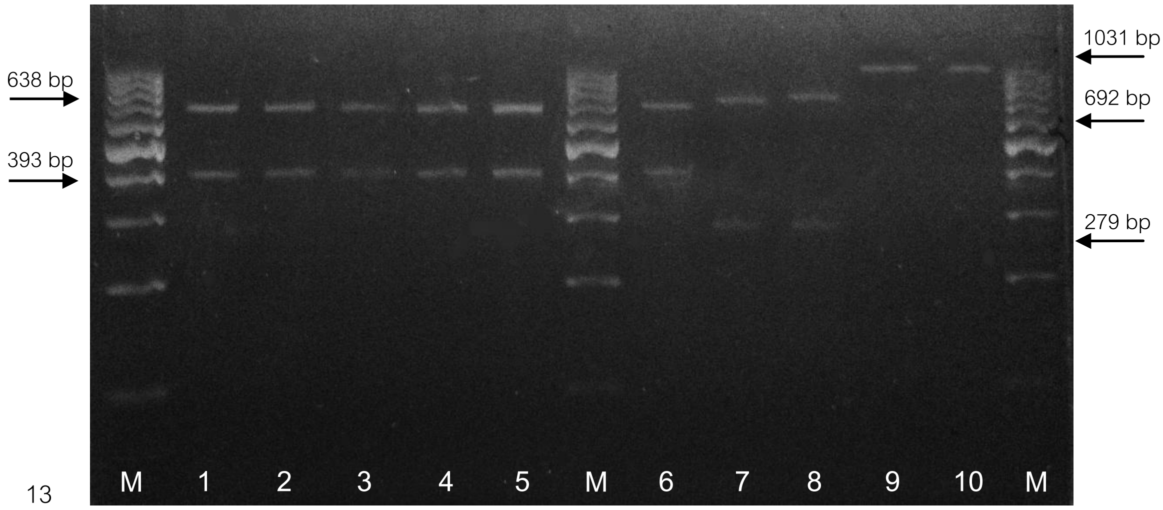
11

Figure 10: RT-PCR positive results of 390bp- P gene fragments; M: 100 bp DNA ladder, lane 1: BKK01/09, lane 2: BKK02/10, lane 3: BKK03/11, lane 4: BKKZ01/11, lane 5: BKK/CG.

Figure 11: RT-PCR positive results of 1824bp- H gene fragments; M: 100 bp DNA ladder, lane 1: BKK01/09, lane 2: BKK02/10, lane 3: BKK03/11, lane 4: BKK/CG.



12



13

Figure 12: RT-PCR positive results of 1031bp- F gene fragments; M: 100 bp DNA ladder, lane 1: BKK04/10, lane 2: BKK05/10, lane 3: BKK02/11, lane 4: BKK03/11, lane 5: BKKZ01/11, lane 6: BKKZ02/11, lane 7: BKK01/11, lane 8: BKK06/11, lane 9: BKK07/11, lane 10: BKK/CG, lane 11: VG.

Figure 13: RFLP analysis on F gene fragments for identification of CDV lineages; M: 100 bp DNA ladder, Asia-1 lineage showed 393 and 638 bp fragments (lane 1-6: BKK04/10, BKK05/10, BKK02/11, BKK03/11, BKKZ01/11, BKKZ02/11), new Asia lineage showed 279 and 692 bp fragments (lane 7,8: BKK01/11, BKK06/11), Vaccine lineage showed intact-1031 bp fragments (lane 9,10: BKK/VG, BKK/VG).

5. Sequence and phylogenetic analyses

The phylogenetic tree of P gene region among CDV isolates presented that samples isolated in this study were mainly divided into three clusters based on reference strains which the accession numbers were mentioned in material and method chapter (Figure 14). Thirteen samples from civets; BKKZ01/11, BKKZ02/11, BKKZ03/11, BKKZ04/11, BKKZ05/11, BKKZ06/11, BKKZ07/11, BKKZ08/11, BKKZ9/11, BKKZ10/11, BKKZ11/11, BKKZ12/11, BKKZ13/11 and thirteen samples from dogs; BKK03/09, BKK05/09, BKK02/10, BKK04/10, BKK05/10, BKK06/10, BKK02/11, BKK03/11, BKK04/11, BKK05/11, BKK08/11, BKK09/11, BKK10/11 showing 99.7-100% nucleotide identity were included in cluster A. Eight samples from dogs; BKK01/09, BKK02/09, BKK04/09, BKK01/10, BKK03/10, BKK01/11, BKK06/11 and BKK07/11 displaying 96.9-100% nucleotide identity were included in cluster B. Three samples from commercial vaccine products; BKK/CG, BKK/TTD and BKK/QT presented 98.9-99.1% nucleotide identity and were in cluster C while BKK/VG was separated from the others in vaccine group because of low identity (96.9-98.0%) (Appendix C). Comparing to other published strains in GenBank, cluster A revealed significant similarity (98.0-99.1%) to reference strains in Asia-1 lineage. Cluster B presented high similarity (97.5-98.3%) to Th270Br and Th290Br while hardly related to vaccine or other lineages in database. Cluster C showed high similarity (98.6-99.7%) to Onderstepoort strain which represented vaccine lineage while BKK/VG showed more closely related (97.5-98.9%) to wild-type strains from the America-2 lineage.

The phylogenetic distances of H gene also indicates that the results tend to be categorized into three clusters based on reference strains which the accession numbers were mentioned in material and method chapter (Figure 15). Four sample from civets; BKKZ01/11, BKKZ02/11, BKKZ06/11 and BKKZ13/11 and seven samples from dogs; BKK03/09, BKK05/09, BKK02/10, BKK05/10, BKK06/10, BKK10/11 and BKK12/11 presenting 97.2-100% nucleotide identity were in cluster A. Six samples from dogs; BKK01/09, BKK02/09, BKK04/09, BKK01/10, BKK03/10 and BKK07/11 showing 99.0-99.8% nucleotide identity were included in cluster B. BKK/QT and BKKVG from commercial vaccine products having 92.6% identity were not in the same cluster. Whereas BKK/QT had 98.2% similarity to Onderstepoort strain in vaccine lineage, BKK/VG showed more closely related (98.0% homology) to the America-2 lineage (Appendix C). Likewise phylogenetic tree of P gene, cluster A revealed significant similarity (96.6-99.1%) to reference strains in Asia-1 lineage. Cluster B which clearly separated from cluster A (93.2-94.1% similarity) and presented high similarity (98.6-99.3%) to Th270Br and Th290Br while hardly related to other lineages in database.

The phylogeny of F gene emphasized the results from phylogenetic analyses of P and H gene (Figure 16). Nine samples from civets; BKKZ03/11, BKKZ05/11, BKKZ06/11, BKKZ07/11, BKKZ9/11, BKKZ10/11, BKKZ11/11, BKKZ12/11 and BKKZ13/11 and nine samples from dogs BKK03/09, BKK05/09, BKK02/10, BKK04/10, BKK05/10, BKK06/10, BKK02/11, BKK03/11, BKK04/11, BKK05/11, BKK08/11, BKK09/11 and BKK11/11 joining in cluster A showed 98.4-100% nucleotide identity. Eight samples from dogs; BKK01/09, BKK02/09, BKK04/09, BKK01/10, BKK03/10, BKK01/11, BKK06/11 and BKK07/11 participating in cluster B revealed 99.0-100% nucleotide identity. Three samples from commercial vaccine products; BKK/CG, BKK/TTD and BKK/QT presented 98.8-99.2% nucleotide identity and were included in cluster C while BKK/VG was separated from the others in vaccine group because of low identity (95.1-95.6%) (Appendix C). Agreeably with previous results, cluster A revealed significant similarity (98.0-99.3%) to reference strains in Asia-1 lineage. Cluster B which clearly distinguished from cluster A (95.4-96.4% similarity) related to neither vaccine nor other lineages in Genbank. Cluster C showed high similarity (99.1-99.3%) to Onderstepoort strain which represented vaccine lineage while BKK/VG revealed disparity from vaccine group.

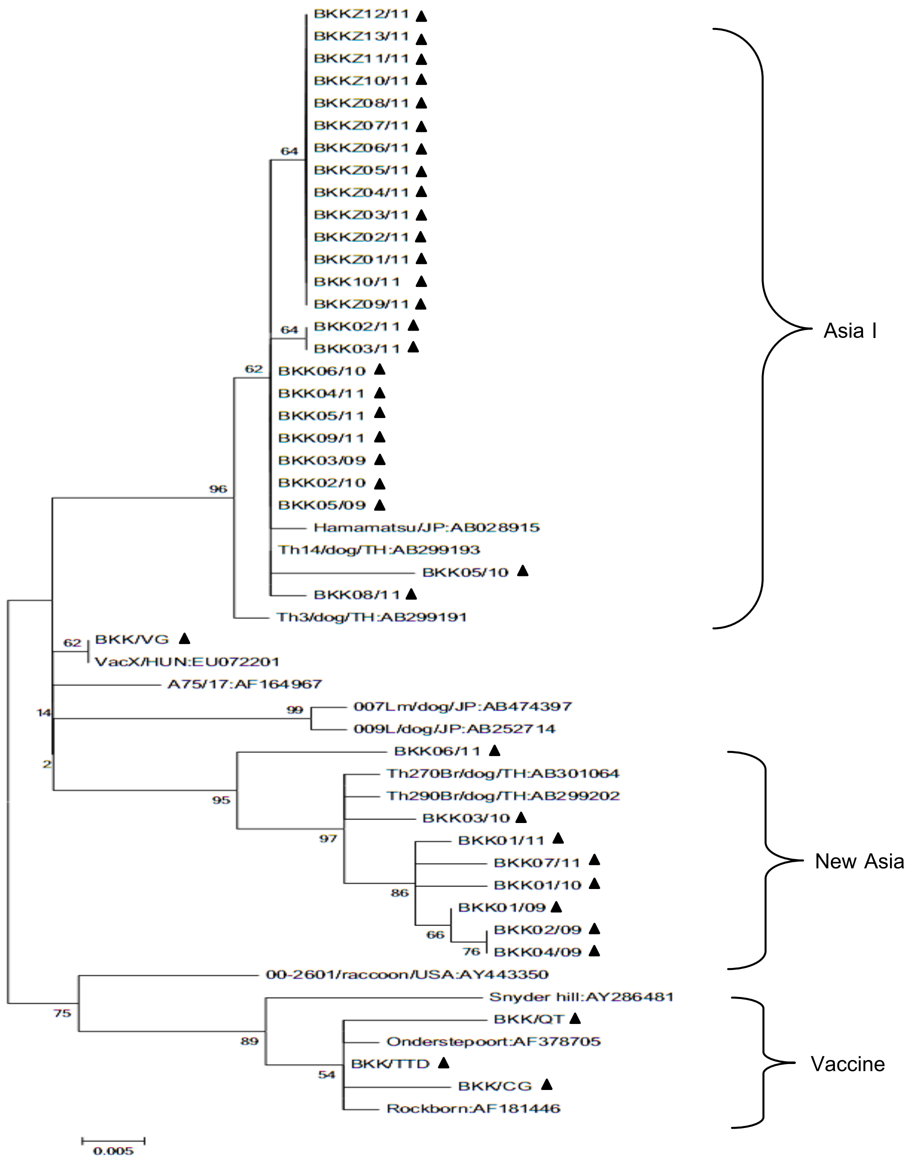


Figure 14: Phylogenetic tree of CDV isolates based on the nucleotide sequences of partial P gene fragments. Distance values were calculated by the ClustalW program within the MEGA 5.0 software package. Triangle (▲) indicates 37 CDV isolates analyzed in this study. The original country of each CDV isolates was indicated: CHN: China, JP: Japan, USA: United States of America, TH: Thailand, HUG: Hungary.

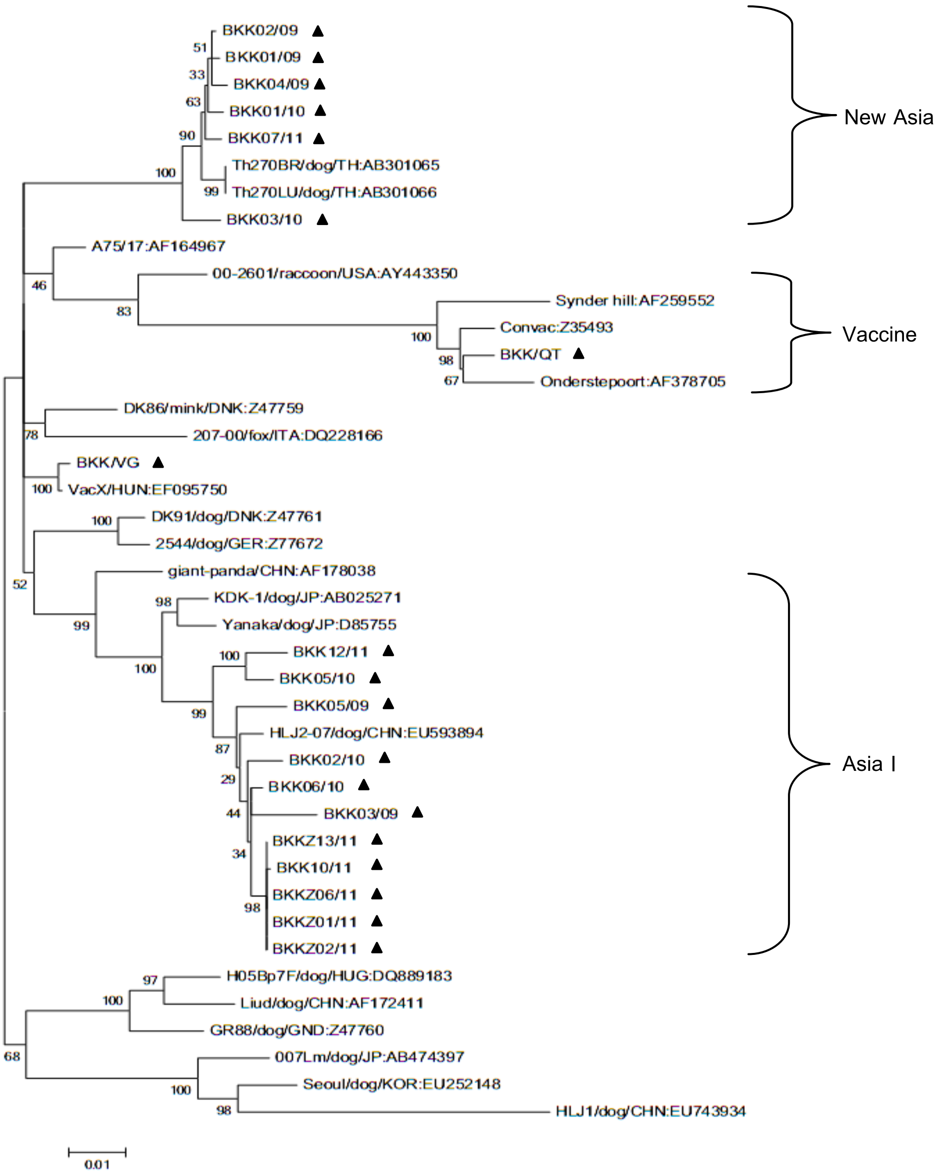


Figure 15: Phylogenetic tree of CDV isolates based on the nucleotide sequences of partial H gene fragments. Distance values were calculated by the ClustalW program within the MEGA 5.0 software package. Triangle (▲) indicates 19 CDV isolates analyzed in this study. The original country of each CDV isolates was indicated: CHN: China, JP: Japan, USA: United States of America, HUG: Hungary, GER: Germany, DNK: Denmark, TRK: Turkey, GND: Greenland, KOR: Korea, ITA: Italia, TH: Thailand.

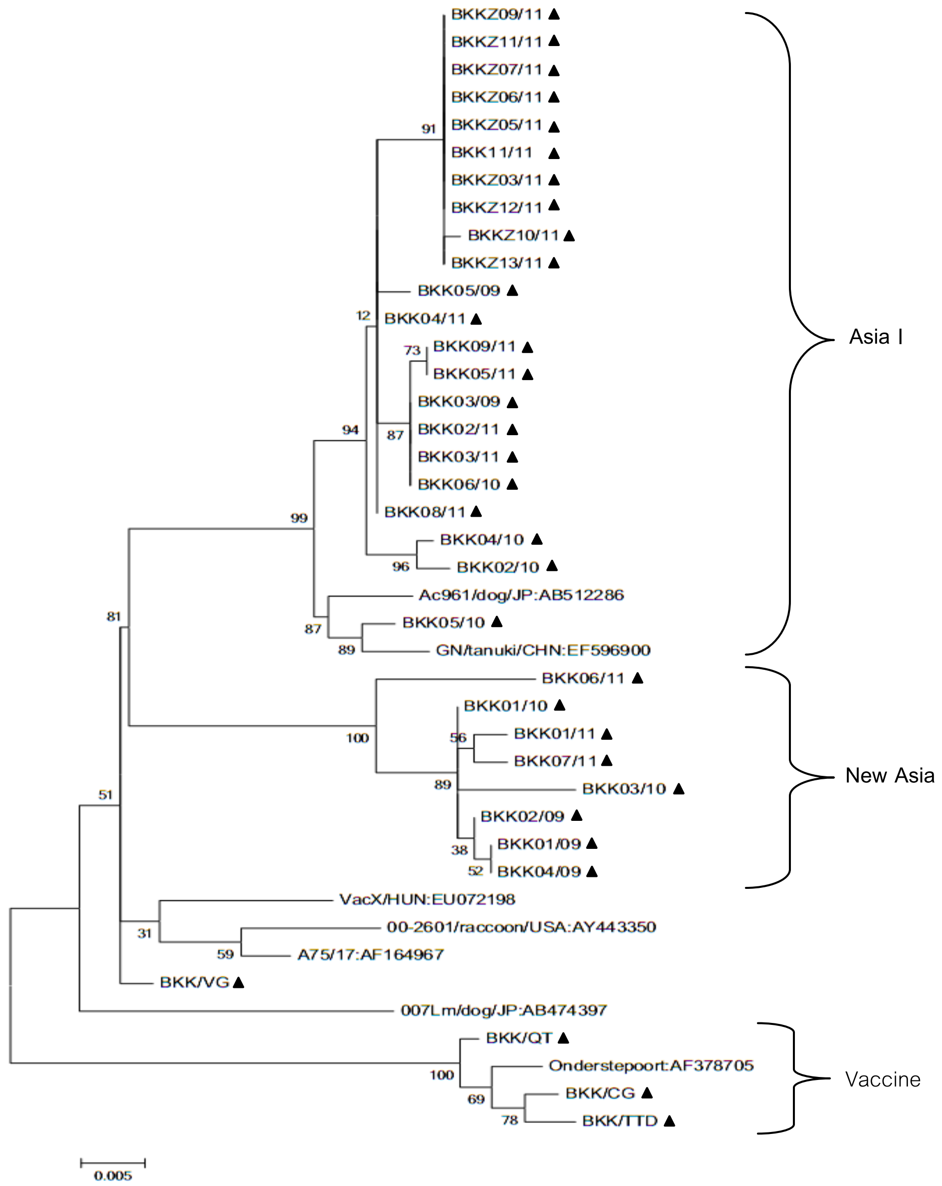
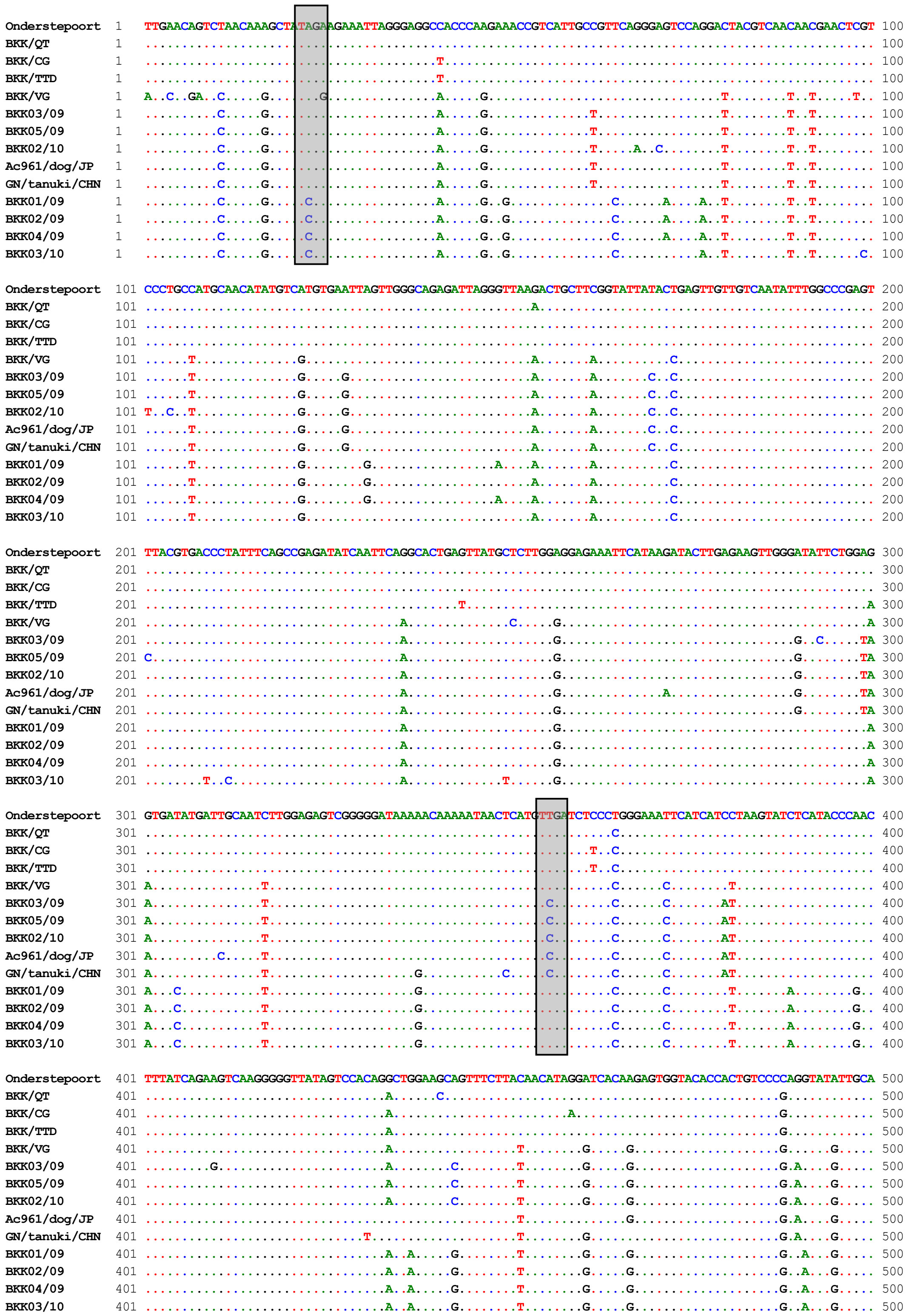


Figure 16: Phylogenetic tree of CDV isolates based on the nucleotide sequences of partial F gene fragments. Distance values were calculated by the ClustalW program within the MEGA 5.0 software package. Triangle (▲) indicates the 34 CDV isolates analyzed in this study. The original country of each CDV strain was indicated: CHN: China, JP: Japan, US: United States of America, HUG: Hungary.



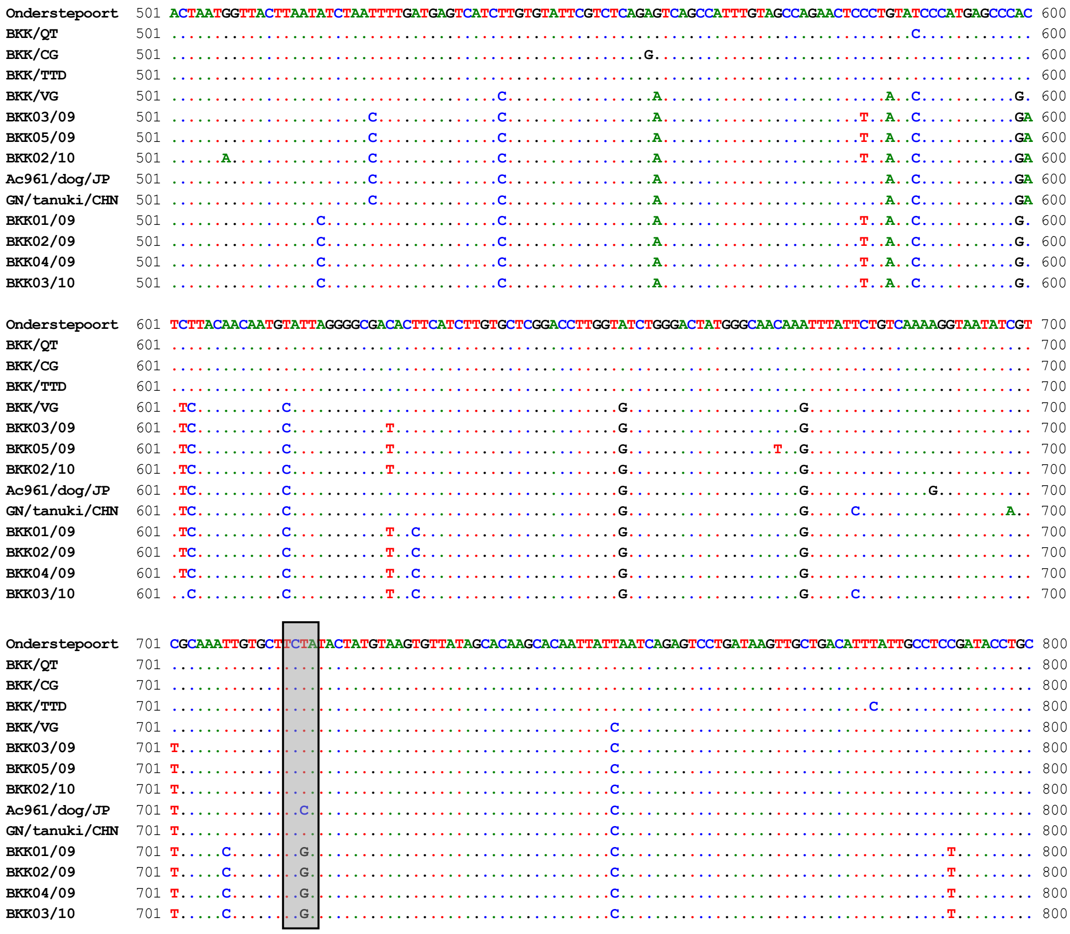


Figure 17: Alignment of nucleotide sequences of partial F gene of the CDV isolates and reference strains (Onderstepoort, Ac961/dog/JP and GN/tanuki/CHN). Onderstepoort, BKK/QT, BKK/CG, BKK/TTD and BKK/VG revealed no restriction site of *Tag*$^{\alpha}$*I*. BKK03/09, BKK05/09, BKK02/10, Ac961/dog/JP and GN/tanuki/CHN had C at 393 nucleotide position in 1,031-bp F gene fragments. BKK01/09, BKK02/09, BKK04/09 and BKK03/10 had C at 60 and G at 752 nucleotide position in 1,031-bp F gene fragments. The restriction sites of *Tag*$^{\alpha}$*I* (5'…T▾CGA…3') were shaded.

CHAPTER V

DISCUSSION AND CONCLUSION

CDV has been known causing multisystemic disease in all families of terrestrial carnivores and suggested to be nearly the most lethal infectious agent in either susceptible free-living or captive carnivores (Deem et al., 2000). However, there are limited studies of CDV infected wildlife in Thailand, while those have been frequently reported in several countries (Lednicky et al., 2004; Takayama et al., 2009; Wang et al., 2011). Recently, there was a CDV outbreak in the civet coffee farm in Kanchanaburi province and a number of clinical samples from affected civets (BKKZ01/11-BKKZ13/11) and a pet dog (BKK10/11) livings in the same farm were included in this study. Other samples from dogs infected with CDV were collected from private animal hospitals in Bangkok from December 2009 to December 2011.

Histopathological lesions indicating of CDV infection

History taking of both dogs and civets showed predominantly clinical signs of respiratory and nervous systems concordantly with gross lesions that mainly involved respiratory tract. However, other symptoms such as diarrhea and skin lesions were occasionally found. Due to non-specific clinical signs and macroscopic findings, in some cases, it may be imprecise in particular to give final diagnosis when systemic signs, preceding or lacking of neurological signs (Amude et al., 2007). Peracute CDV infection may display only respiratory symptom as well as chronic infection that may present only nervous disorder resulted in misinterpretation to other respiratory associated viruses, neurotoxicosis and epilepsy (Koutinas et al., 2002). Microscopic findings showed typically CDV-related lesions including eosinophilic intranuclear and intracytoplasmic inclusion bodies (INIB and ICIB) in both non-nervous and nervous tissues indicating viral distribution of various organs. However, histopathological findings of non-nervous tissues according to H&E staining were not distinctly different among CDV lineages and lesions found in civets were similar to those found in CDV infected dogs defined as interstitial pneumonia, lymphoid depletion, catarrhal enteritis and hyperkeratosis of skin (Ito et al., 2006; Takayama et al., 2009). Lymphoid depletion and necrosis were significantly noticed in all cases emphasizing lymphotrophic and immunosuppressive properties of virus. After initial infection occurred, three lymphocyte populations were attacked. Circulating B and T lymphocytes were infected leading to infection of thymocytes in primary lymphoid organs and lymphocytes residing in secondary lymphoid organs including splenic white pulps, tonsils, lymph nodes and organ-associated lymphoid tissues (von Messling et al., 2004). The histopathological destruction of lymphoid organs particularly tonsils and gut associated lymphoid tissues (GALTs) in Peyer's patch caused the compromise of mucosal defense mechanism and the high susceptibility to secondary bacterial infections as shown in histopathological lesion of respiratory and gastrointestinal tracts of both dogs and civets.

Closely related to measle virus (MV) in human, CDV has been wildly used as animal model of Paramyxovirus-induced demyelination. Canine distemper encephalitis (CDE) was categorized in different subtypes based on histopathological finding and distribution of lesions in central nervous system (CNS). Neuropathological manifestation of CDV is generally described in acute and chronic encephalomyelitis. However, other neuropathological classifications of CDV including acute encephalitis, polioencephalomalacia, inclusion body polioencephalitis, old dog encephalitis and post-vaccinal CDE have been recognized (Koutinas et al., 2002; Baumgärtner and Alldinger, 2005; Amude et al., 2010). The types of neuropathological lesion changed upon virus strains, age at time of infection and host immune responses. Due to limited number of CNS samples, we classified CNS samples of this study into 'no pathological', 'acute' and 'chronic' lesions.

The association of CDV lineages and neuronal cell tropism

Regarding histopathology of nervous tissue, CDV antigens were found in all histological staging groups. Samples in 'no pathological change group' (or per-acute lesion) displayed CDV-antigen positive cells predominantly in astrocytes and slightly in neurons that mostly localized surrounding blood vessels and submeningeal area of cerebral cortex and brain stem. This finding indicated that astrocytes were initialy infected before neuropathological alteration can be observed. Astrocytes were known to be the main target cells in early state of CDV infection. Nearly 64% of astrocytes in non-inflammatory lesions contained viral antigens and almost 95% of all positive cells proved to be astrocytes by GFAP labeling (Mutinelli et al., 1988).

Several studies stated that CDV approach to CNS mainly via hematogenous route which was compatable with other neurovirulent paramyxoviruses that prefer to infect circulating lymphocytes causing leukocyte-associated viremia to CNS. Antigen expression in CNS was primarily detected within capillary endothelial cells, perivascular areas, astrocytic foot processes and choroid plexus at 3-5 days after infection. Direct dissemination from meningeal cells of the pia matter to submeningeal area are has been noticed. Subsequently, CDV positive neurons and glial cells were found in white matter area. Two to three weeks after infection, virus was evident in neurons and glial cells throughout gray and white matter and also in ependymal and subependymal areas indicating that progeny of virus was released along cerebrospinal fluid (CSF) (Rudd et al., 2006; Beineke et al., 2009). Corresponding to this study, in samples of acute lesion group, neurons and ependymal cells expressing CDV antigen were considerably observed comparing to peracute lesion in no pathological change group and the affected area spread from cerebrum to thalamus and cerebellum during disease progression.

Histopathological change in chronic lesion group presented typical leukoencephalomalasia which referred to extensive demyelination in white matter of the cerebellum, periventricular and spinal cord (Vandevelde et al., 2005). The cause of demyelination was explained as biphasic events; acute and chronic demyelination. The initiation of demyelinating lesions took place at approximately 3 weeks post infection which developed during virus-induced immunosuppressive period. Therefore, early demyelinating changes characterized by ballooning degeneration of myelin sheets, focal vacuolization and active astrocytes with minimal inflammatory responses. CDV infection in early phase led to metabolic dysfunction of oligodendrocyte such as massive down-regulation of myelin gene transcription and decreased activity of cerebroside-sulfo transferase, an oligodendrocyte-specific enzyme revealing the direct effect of virus on demyelinating process. Although oligodendrocytes were exactly affected, several studies found that while viral RNA expression was observed, the number of oligodendrocytes expressing protein was extremely low. Therefore, the restriction infection in oligodendrocytes remained limited at the level of viral transcription but not translation has been proposed (Zurbriggen et al., 1998; Vandevelde and Zurbriggen, 2005).

Advanced demyelination was influenced by immunopathological complication. In contrast to rarely inflammatory influx in the early phase of infection, there was exceeding increase of inflammatory reaction displayed in perivascular cuffing of CD4+ lymphocytes following by migration of numerous plasma cells and overwhelming antibody synthesis. Excessive humoral immune production could result in antibody-dependent cell-mediated cytotoxicity (ADCC) that was suspected as the mechanism of myelin damage. Additionally, chronic demyelination might accelerate by overactive macrophages and microglia. Significantly increased phagocytic activity of microglia was observed and radical oxygen production from those cells could be a cause of myelin damage. Thus, chronic demyelination may so called a bystander phenomenon (Baumgärtner and Alldinger, 2005; Vandevelde and Zurbriggen, 2005; Beineke et al., 2009).

According to immunohistochemical studies, astrocytes were constantly found positive to CDV antigen in all disease stages, especially in ‘chronic lesion group’. Seehusen et al. (2007) revealed a modification of cell tropism in CDV infected astrocytes during disease progression. At the beginning, CDV antigen was detected prominently in GFAP positive mature astrocytes, while viral protein expressed distinctly in vimentin-positive cells immature astrocytes in advance demyelination. Moreover, vimentin-positive astrocytes were existent at either the center or the periphery of advanced lesions, whereas GFAP expressing astrocytes were existent only at the periphery of the lesion. It should be noted that astrocytes represented for a permanent source for harboring virus and may play an important role in future progression of the disease.

There was several investigations studied difference of CDV genotype associated with neurovirulence and cell tropism. Some strains correlated with a polioencephalitis (e.g. Snyder Hill strain) (Rudd et al., 2010) whereas others induced a demyelinating leukoencephalomyelitis (e.g. A75/17 and R252 strains) (Beineke et al., 2009). Interestingly, we found the characteristic cell tropism upon different CDV lineages isolated from Thailand. Samples belonging to a new discovered lineage (BKK03/10, BKK01/11 and BKK07/11) appeared to be positive against CDV antigen predominantly in astrocytes both in acute and chronic lesions. These findings were in agreement with previous investigations revealing the Th270 and Th290 strains which were belonged to the same new lineage and showed preferably CDV-positive astrocytes (Charoenvisal, 2008). However, the stage of infection associated cell tropism cannot be excluded, so the specific cell type labeling technique should be performed and more samples should be included to elucidate the pathogenesis of CDV-induced neuropatholgy.

Interspecies transmission was possible

In this study, partial H, P and F gene sequences were analyzed to investigate genetic relationships between CDV isolates and differentiate among wild-type and vaccine lineages in Thailand. Phylogenetic tree of those genes revealed compatible result that all samples in this study were classified into three lineages; Asia-1, novel Asia lineage and vaccine lineages.

Fifteen isolates from twenty-eight dogs and all thirteen civets belonged to Asia-1 that has been known circulating in Thailand consistently (Keawcharoen et al., 2005; Charoenvisal, 2008). Several domestic species from neighboring region were suffering from this CDV lineage including, Korea, Japan and China indicating geographic distribution rather than host species. (An et al., 2008; Sultan et al., 2009; Zhao et al., 2010). In 2010, Zhao et al. analyzed the complete H gene from both vaccinated and non-vaccinated minks, raccoon dogs and breeding foxes revealing that the three different CDV lineages, composing of Arctic, Asia-1 and Asia-3. In particularly Asia-1 lineage was predominantly circulating among wildlife in China. In addition, Masked Palm civets captured in Japan were naturally infected with CDV and then the H gene sequences from these samples were analyzed. Phylogenetic analysis was clarified that CDV isolates from those infected civets belonged to Asia-1 lineage and domestic dogs were suspected to be the carriers due to their intimate habitation (Hirama et al., 2004; Takayama et al., 2009).

Corresponding to our study, genetic characterization of three investigated genes from all civets isolates (BKKZ01/01-BKKZ13/11) was Asia-1 lineage and had a significantly high homology (99.9-100%) to BKK10/11 isolate which obtained from stray dog living in the same area. This finding indicated a strong evidence of viral spreading from dogs to civets representing as interspecies transmission. Nowadays, civet coffee farm business become progressive and civets were recruited from their natural dwelling into crowed farm which easily to access by other animals. Considering these facts, domestic dogs are supposed to play key roles in shedding CDV among endangered wildlife.

The existence of CDV new lineage

More than thirty percent (34.8%) of isolates obtained from CDV infected dogs were clustered in new discovered Asia lineage that was separated from either other virulent strains or vaccine lineage. Based on phylogeny of three target genes, we proposed this novel cluster as ‘new Asia lineage’. Similar to other regions, some novel discovered lineages were recognized in many countries including Argentina, Mexico and China (Calderon et al., 2007; Simon-Martínez et al., 2008; Zhao et al., 2010). Therefore, the causes of the anonymous lineage existence in several areas have been presupposed.

Interspecies transmission introducing unrecognized lineage has been widely suspected for CDV. In America, phylogenetic analysis of complete H, partial F and P gene sequences revealed that there were at least two genetically distant CDV lineages causing CDV outbreak in free-ranging raccoons living around the zoo. Viruses isolated in 1998 were closely related to old CDV lineage including Snyder Hill and Lederle, while viruses isolated in 2000 and 2001 appeared to originate from the America-2 lineage which has never been recognized in this area and some isolates produced large syncytial cell formation both *in vivo* and *in vitro* (Lednicky et al., 2004). The author suggested that this phenotypic feature which differed from the others might cause different pathogenesis. Moreover, the rotation of different CDV lineages within the same local might cause reintroductions of the virus to raccoons which speculated as reservoirs of genetically distinct CDV lineages (Lednicky et al., 2004). Pardo and colleagues (2005) also suggested this interspecies transmission concept upon the occurrence of novel lineages in North America. Complete sequences of H and F genes and partial sequence of P gene from dogs were analyzed and demonstrated that CDV strains circulating in this area were mostly associated to phocine distemper virus 2 (PDV-2) lineage and lesser panda in Asia-1 lineage rather than lineages previously reported in the United States. Thus, these were probable that virus strain infecting dogs might have originated from non canine species, alternatively transmitted from dogs to the other susceptible species.

Animal movement was possibly the cause of new lineages emergence. In Hungary, Demeter et al (2007) observed the genetic diversity of CDV on entirely H gene sequence by collecting large clinical samples from various background dogs during 2005-2006. This study demonstrated the significantly high diversity of CDV Hungarian isolates. Samples in the first cluster belonged to Arctic lineage that closely associated with CDV strains isolated from China, North America and Greenland. The second cluster joined Europe lineage and showed high a similarity to Italy, Denmark, German and Turkey strains while another cluster was categorized in new discovered lineage. The authors mentioned that the genotypes from geographically distant countries might result from travelling of dog and also uncontrolled animal movement. Lacking of geographic barriers as well as an import of exotic canine breeds conduced to the heterogeneity of CDV strains. Additionally, the existence of new variants due to point mutations was frequently observed in populations where CDV endemic took place.

Regarding to this study, 'new Asia lineage' presuming as an emergent lineage might have circulated in this area for long time ago. Yet there was a few investigation of genetic diversity and genotypic characterization of CDV lineage throughout Thailand accompanying with limited reports of CDV infection in neighboring countries including Laos, Vietnam and Myanmar. Therefore, phylogenetic studies of CDV currently circulation was necessary for controlling disease spread out and observing new genetic variants that might be associated with viral pathogenesis and also vaccination failure.

Controversial result of genetic analysis from commercial vaccine product

Phylogenetic tree of three interested genes revealed that the virus strains from three commercial vaccine products (BKK/CG, BKK/TTD and BKK/QT) belonged to vaccine lineage referred by Onderstepoort strain, unless virus strain from Vanguard vaccine (BKK/VG). BKK/VG was separated from the others in vaccine group and was more closely related to wild-type strains than other viruses from the group of vaccine lineage. This result was supported by previous studies showing that Vanguard vaccine strain demonstrated a higher level of identity with wild-type viruses from the America-2 lineage (Prado et al., 2005; Demeter et al., 2010). According to a previous study, various vaccine product including different batches of Vanguard vaccine obtained from different countries were used for RFLP analysis on partial sequence of H gene. A *PsiI* restriction site representing as a hallmark of vaccine strains was recognized in all isolates from vaccine products and resulted in two differentiable bands whereas the H gene fragments from wild-type viruses and all viruses in Vanguard products remained undigested. All batches of Vanguard vaccine were proved not have been containing the virus strain, 'Synder hill', informed by the manufacturer description since at least 1992 (Demeter et al., 2010).

The disease has been controlled by using lived attenuated vaccines for many decades, yet, in Thailand, a number of CDV cases have been reported in vaccinated dog populations. As demonstrated in this study, twenty-six percent of CDV infected dogs had a previous vaccination history. However, virus strains isolated from cases with vaccination history were genetically separated from vaccine lineage, as has been described in prior studies about vaccinated dogs suffering from CDV infection in Japan, Argentina, Mexico and Africa (Lan et al., 2006; Calderon et al., 2007; Simon-Martínez et al., 2008; Woma et al., 2010). These results suggested that the cause of clinically illed vaccinated dogs was due to the vaccination failure rather than the reversion of vaccine virus to virulence. Quality of the vaccine, inappropriate administration, failure of immunization due to CDV infected prior vaccination or poor immune response as well as vaccine strains that were not able to provide protection from the current circulating strains of CDV led to vaccination failure (Keawcharoen et al., 2005; Woma et al., 2010).

It is known that vaccine lineage have not been found circulating in domestic dogs over the last five decades, while vaccine-like CDV infection in wildlife were occasionally incident (Martella et al., 2007). Lan et al. (2009) isolated virus strains from clinically CDV suspected dogs in Vietnam and performed the molecular analysis. Phylogenetic relationship showed that virus strains obtained from CDV naturally infected dogs were clustered in vaccine lineage. Due to the fact that the dogs had never been vaccinated against CDV and vaccine was rarely used in Vietnam, the authors concluded that the virus in this study were not derived from vaccine virus but belonged to vaccine lineage that still circulated among Vietnam dogs. In Thailand, Kaewcharoen et al. (2005) reported there were at least two CDV lineages displaying a high homology to Asia-1 and vaccine lineages based on N gene analysis. Two virus strains that closely related to vaccine lineage were isolated from both vaccinated and non-vaccinated dogs suffering from CDV infection. In addition, Charoenvisal (2008) found the new lineage genetically different from the others reported in GenBank and subsequently joined our new Asia lineage.

RFLP as a potential tool for differentiation of CDV field strains from the vaccine

RFLP technique has been used widely as a tool for discrimination between vaccine and wild-type strains with a favorable nucleotide region on H protein gene due to their high variability (Uema et al., 2005; Calderon et al., 2007; Demeter et al., 2010; Zhao et al., 2010). This study mentioned on gene encoding F protein that was more conserved and easy to manipulate. Using *Tag*$^{\alpha}$*I* digestion, the target F gene fragments were cleaved and showed that all isolates were clustered into 3 groups by the different patterns of digested fragments.

According to phylogenetic characterization, all commercial vaccine exhibited no recognition site of *Tag*$^{\alpha}$*I* and gave an intact 1,031-bp F gene fragment by RFLP. The 393th base of a recognition site of a 1,031-bp F gene fragment (correlating to the 1,152nd base in the complete F gene) found in Asia-1 lineage was different from other strains. It was the transversion of the 393th base from A to C leading to an appearance of *Tag*$^{\alpha}$*I* digestion site. All samples belonged to Asia-1 lineage had agreeing RFLP results representing two fragments of 393 and 638 bp length. New lineage had nucleotide alternation at 60th and 752nd positions of F gene fragment (correlating to the 818th and 1,512nd base in the complete F gene respectively). The 60th base position changed from T to C (transition) and the 752nd base position changed from T to G (transversion) causing two *Taq*$^{\alpha}$*I* restriction site on 1,031-bp F gene fragment. From RFLP results, all samples in new lineage revealed two fragments with 279 and 692 bp length. Although expected fragment of 60 bp length was obscured, different patterns of *Taq*$^{\alpha}$*I* digested nucleotides were able to distinguish individual wild-type CDV lineages from vaccine lineage corresponding to phylogenetic characterization of P, H and F gene.

Digested pattern of BKK/VG from Vanguard vaccine which closely related to wild-type viruses appeared to act as vaccine lineage due to possess no *Taq*$^{\alpha}$*I* restriction sites on the amplified fragment. This finding resembled other wild-type lineages published in Genbank that were predicted not to contain *Taq*$^{\alpha}$*I* cleavage site on the target F gene sequence. Therefore it should be concerned the false negative of naturally infection from other wild-type lineages for instant America-2, Europe, Europe-wildlife, Arctic, Asia-2 and Asia-3. However, there were evidences of exactly three genotypes of CDV, Asia-1, new Asia and vaccine lineages, circulating in Thailand since 2005 (Kaewcharoen et al., 2005; Charoenvisal, 2008) and the confirmation of natural infection from field strains could be accomplished within five hours, so this RFLP technique might be use as screening test for identifying wild-type CDV infection.

Conclusion

Phylogenetic analysis elucidated that there were at least three CDV lineages composing of vaccine, Asia-1 and new Asia circulating among the susceptible animals and such RFLP pattern was able to differentiate individual wild-type CDV strains from the vaccine viruses in Thailand. Since the accuracy of this method depend on the mutation within nucleotide sequences, using DNA polymerase with proofreading activity or cloning the virus into vectors should be performed for increasing the test reliability. However, this developed RFLP method has the potential for considerable savings time and effort within the laboratory and would be useful for several clinical applications such as confirmation of nature CDV infection, evaluation of vaccination status and epidemiological monitoring of the circulating viral genotype.

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APPENDICES

APPENDICES

Appendix A: Primers for sequence analyses of CDV P, H and F gene

Gene	Primer	Sequence (5'-3')
P	UPP1	ATGTTTATGATCACAGCGCGGT
P	UPP2	ATTGGGTTGCACCACTTGTC
H	CDV-HS1	AACTTAGGGCTCAGGTAGTCC
H	CDV-HS2	ATGCTGGAGATGGTTTAATTCAATCG
H	CDV-HforD	GACACTGGCTTCCTTGTGTGTAG
H	CDV-Hr2	GTTCTTCTTGTTTCTCAGAGG
F	CDVF-Fo1031	CCTCAATGCTCAAGCAATCC
F	CDVF-Re1031	CAAGGATCTGGTTAGAGGAG

Appendix B: Nucleotide sequences

The nucleotide sequences of P gene

BKK01/09, 366 nucleotides
TGACAGTCTCGTGGTACCTGCAGGCGCTGTCAATAGTCGAGGACTCGAGGAAGGAGAAGGAAGCATTGATGACAGCATTGAG
GATTCTAGCGAAGATTATTCCGAGGGAAATGCTTCATCTAACTGGGGATATTCTTTCGGCCTTAAACCAGACAGAGCAGCTGATG
TGAGCATGCTGATGGAAGAGGAATTGAGTGCTCTGCTCAGGACAAGCAGAAATGTAAGGATTCAGAAAAGGGATGGGAAGACT
CTGCAGTTCCCACACAATCCCGAAGGTAAGACAGGGGAGCCGGAGTGTGGATCCATTAAAAAGGGCACAGGAGAGAGGTCAG
CCTCACATGGAATGGGGATAGTTGCTGGATCGAC

BKK02/09, 366 nucleotides
TGACAGTCTCGTGGTACCTGCAGGCGCTGTCAATAGTCGAGGACTCGAGGAAGGAGAAGGAAGCATTGATGACAGCATTGAG
GATTCTAGCGAAGATTATTCCGAGGGAAATGCTTCATCTAACTGGGGATATTCTTTCGGCCTTAAACCAGACAGAGCAGCTGATG
TGAGCATGCTGATGGAAGAGGAATTGAGTGCCCTGCTCAGGACAAGCAGAAATGTAAGGATTCAGAAAAGGGATGGGAAGACT
CTGCAGTTCCCACACAATCCCGAAGGTAAGACAGGGGAGCCGGAGTGTGGATCCATTAAAAAGGGCACAGGAGAGAGGTCAG
CCTCACATGGAATGGGGATAGTTGCTGGATCGAC

BKK03/09, 366 nucleotides
TGACAGTCTCGTGGTACCTGCAGGCGCTGTCAGTAATCGAGGATTCGAGAGAGGAGAAGGAAGCCTTGATGATAGCACTGAGG
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GAGCATGCTGATGGAAGAGGAATTGAGTGCTCTGCTCAAGACAAGCAGAAATGTGGGGATTAAGAAAAGGGATGGGATGACTC
TGCAGTTCCCACACAATCCCGAAGGTAAGACAGAGGATCCGGAGTGTGGATCCATTAAAAAGGGCACAGGAGAGAGGTCAGC
CTCACATGGAATGGGGATAGTTGCTGGATCGAC

BKK04/09, 366 nucleotides
TGACAGTCTCGTGGTACCTGCAGGCGCTGTCAATAGTCGAGGACTCGAGGAAGGAGAAGGAAGCATTGATGACAGCATTGAG
GATTCTAGCGAAGATTATTCCGAGGGAAATGCTTCATCTAACTGGGGATATTCTTTCGGCCTTAAACCAGACAGAGCAGCTGATG
TGAGCATGCTGATGGAAGAGGAATTGAGTGCCCTGCTCAGGACAAGCAGAAATGTAAGGATTCAGAAAAGGGATGGGAAGACT
CTGCAGTTCCCACACAATCCCGAAGGTAAGACAGGGGAGCCGGAGTGTGGATCCATTAAAAAGGGCACAGGAGAGAGGTCAG
CCTCACATGGAATGGGGATAGTTGCTGGATCGAC

BKK05/09, 366 nucleotides
TGACAGTCTCGTGGTACCTGCAGGCGCTGTCAGTAATCGAGGATTCGAGAGAGGAGAAGGAAGCCTTGATGATAGCACTGAGG
ATTCTGGCGAAGATTATTCCGAGGGAAATGCTTCATCTAACTGGGGATATTCTTTCGGCCTTAAACCAGACAGAGCGGCTGATGT
GAGCATGCTGATGGAAGAGGAATTGAGTGCTCTGCTCAAGACAAGCAGAAATGTGGGGATTAAGAAAAGGGATGGGATGACTC
TGCAGTTCCCACACAATCCCGAAGGTAAGACAGAGGATCCGGAGTGTGGATCCATTAAAAAGGGCACAGGAGAGAGGTCAGC
CTCACATGGAATGGGGATAGTTGCTGGATCGAC

BKK01/10, 366 nucleotides
TGACAGTCTCGTGGTACCTGCAGGCGCTGTCAATAGTCGAGGACTCGAGGAAGGAGAAGGAAGCCTTGATGACAGCATTGAG
GATTCTAGCGAAGATTATTCCGAGGGAAATGCTTCATCTAACTGGGGATATTCTTTCGGCCTTAAACCAGACAGAGCAGCTGATG
TGAGCATGCTGATGGAAGAGGAATTGAGTGCTCTGCTCAGGACAAGCAGAAATGGAAAGATTCAGAAAAGGGATGGGAAGACT
CTGCAGTTCCCACACAATCCCGAAGGTAAGACAGGGGAGCCGGAGTGTGGATCCATTAAAAAGGGCACAGGAGAGAGGTCAG
CCTCACATGGAATGGGGATAGTTGCTGGATCGAC

BKK02/10, 366 nucleotides
TGACAGTCTCGTGGTACCTGCAGGCGCTGTCAGTAATCGAGGATTCGAGAGAGGAGAAGGAAGCCTTGATGATAGCACTGAGG
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TGCAGTTCCCACACAATCCCGAAGGTAAGACAGAGGATCCGGAGTGTGGATCCATTAAAAAGGGCACAGGAGAGAGGTCAGC
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BKK03/10, 366 nucleotides
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BKK05/10, 366 nucleotides
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BKK06/10, 366 nucleotides
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BKK01/11, 366 nucleotides
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BKK02/11, 366 nucleotides
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BKK03/11, 366 nucleotides
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BKK04/11, 366 nucleotides
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BKK06/11, 366 nucleotides
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BKK07/11, 366 nucleotides
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BKK09/11, 366 nucleotides
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BKK10/11, 366 nucleotides
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BKKZ01/11, 366 nucleotides
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BKKZ04/11, 366 nucleotides
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BKKZ05/11, 366 nucleotides
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BKKZ06/11, 366 nucleotides
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BKKZ07/11, 366 nucleotides
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BKKZ08/11, 366 nucleotides
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BKKZ09/11, 366 nucleotides
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BKKZ10/11, 366 nucleotides
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BKKZ11/11, 366 nucleotides
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BKKZ13/11, 366 nucleotides
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The nucleotide sequences of H gene

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CTACAACCAG

BKK02/09, 1547 nucleotides
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CTACAACCAG

BKK03/09, 1547 nucleotides
AGGATATGGAGAAATCAGAGGCCGTACATCACCAAGTCATAGATGTCTTGACACCGCTCTTCAAAATTATTGGAGATGAGATTGG
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CTCTACAACCAG

BKK04/09, 1547 nucleotides
AGGATATGGAGAAATCAGAGGCCGTACATCACCAGGTCATAGATGTCTTGACACCGCTATTCAAAATTATTGGAGATGAGATTGG
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CTACAACCAG

BKK05/09, 1547 nucleotides
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CTACAACCAG

BKK01/10, 1547 nucleotides
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CTACAACCAG

BKK02/10, 1547 nucleotides

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TCTACAACCAG

BKK03/10, 1547 nucleotides

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CTCTACAACCAG

BKK05/10, 1547 nucleotides

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CTCTACAACCAG

BKK06/10, 1547 nucleotides
AGGATATGAAGAAATCAGAGGCCGTACATCACCAAGTCATAGATGTCTTGACACCGCTCTTCAAAATTATTGGAGATGAGATTGG
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CTCTACAACCAG

BKK07/11, 1547 nucleotides
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CTACAACCAG

BKKZ01/11, 1547 nucleotides
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CTCTACAACCAG

BKKZ02/11, 1547 nucleotides
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GGCGATCATGCAATTGTTTATTATGTTTATGACCCAATCCGGACGATTTCTTATACACACCCATTTAGACTAACTACCAAGGGTAG
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CTCTACAACCAG

BKKZ06/11, 1547 nucleotides
AGGATATGGAGAAATCAGAGGCCGTACATCACCAAGTCATAGATGTCTTGACACCGCTCTTCAAAATTATTGGAGATGAGATTGG
GTTACGGTTGCCACAAAAACTAAACGAGATCAAACAATTTATCCTCCAAAAGACAAACTTCTTCAATCCGAACAGGGAATTCGAC
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TGGAGCTACTACTTCAGTAGGCAGAGTATTCCCCCTATCCGTATCATTATCCATGTCTTTGATATCAAGAACATCAGAGATAATCA
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AAAGATTCGAGTCTTTGAGATAGGGTTTATCAAACGGTGGCTGAATGACATGCCTTTACTCCAGACAACCAACTATATGGTCCTC
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TTGTTATATCATGACAGCAACGGTTCACAAGATGGTATTCTAGTAGTGACATTGGGAATATTTGGGGCAACACCTATGGATCAAGT
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ACCTGGATGATGCCTGTATTGGTCTCTGAGAAACAAGAGGAGCAAAAAAACTGTCTGGAGTCTGCTTGTCACAGAAAATCCTAC
CCTATGTGCAACCAAACGTCATGGGAACCCTTTGGAGGAGGACAGTTGCCTTCTTATGGGCGGTTGACATTACCTCTAGATCCA
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CACTGTGACCCCCCATGTGTTGACATTTGCGCCCAGGGAATCAAGTGGAAATTGTTATTTACCTATTCAAACATCCCAGATTATG
GATAAAGATGTCCTTACTGAGTCCAATTTAGTGGTGTTACCTACACAGAATTTTAGATATGTCATAGCAACATATGATATATCCCGG
GGCGATCATGCAATTGTTTATTATGTTTATGACCCAATCCGGACGATTTCTTATACACACCCATTTAGACTAACTACCAAGGGTAG
ACCTGATTTCCTAAGGATTGAATGTTTTGTGTGGGATGACGATTTGTGGTGTCATCAATTTTACCGATTCGAGGCTAACATCACTAA
CTCTACAACCAG

BKKZ10/11, 1547 nucleotides
AGGATATGGAGAAATCAGAGGCCGTACATCACCAAGTCATAGATGTCTTGACACCGCTCTTCAAAATTATTGGAGATGAGATTGG
GTTACGGTTGCCACAAAAACTAAACGAGATCAAACAATTTATCCTCCAAAAGACAAACTTCTTCAATCCGAACAGGGAATTCGAC
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TGGAGCTACTACTTCAGTAGGCAGAGTATTCCCCCTATCCGTATCATTATCCATGTCTTTGATATCAAGAACATCAGAGATAATCA
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AAAGATTCGAGTCTTTGAGATAGGGTTTATCAAACGGTGGCTGAATGACATGCCTTTACTCCAGACAACCAACTATATGGTCCTC
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TTGTTATATCATGACAGCAACGGTTCACAAGATGGTATTCTAGTAGTGACATTGGGAATATTTGGGGCAACACCTATGGATCAAGT
TGAAGAGGTGATACCTATCGCTCACCCATCAGTGGAGAGAATACATATAACAAATCACCGTGGGTTCATAAAAGACTCAATAGTA
ACCTGGATGATGCCTGTATTGGTCTCTGAGAAACAAGAGGAGCAAAAAAACTGTCTGGAGTCTGCTTGTCACAGAAAATCCTAC
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AGCATTGACCTTCAACTTAACATATCATTTACATATGGTCCGGTTATACTGAACGGAGACGGTATGGATTATTATGAAAGCCCACT
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GGCGATCATGCAATTGTTTATTATGTTTATGACCCAATCCGGACGATTTCTTATACACACCCATTTAGACTAACTACCAAGGGTAG
ACCTGATTTCCTAAGGATTGAATGTTTTGTGTGGGATGACGATTTGTGGTGTCATCAATTTTACCGATTCGAGGCTAACATCACTAA
CTCTACACCCAG

BKKZ13/11, 1547 nucleotides
AGGATATGGAGAAATCAGAGGCCGTACATCACCAAGTCATAGATGTCTTGACACCGCTCTTCAAAATTATTGGAGATGAGATTGG
GTTACGGTTGCCACAAAAACTAAACGAGATCAAACAATTTATCCTCCAAAAGACAAACTTCTTCAATCCGAACAGGGAATTCGAC
TTCCGCGATCTCCACTGGTGTATTAACCCACCTAGCAAGATCAAGGTGAATTTTACTAATTACTGTGATACAGTTGGGGTCAAAAA
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AAAGATTCGAGTCTTTGAGATAGGGTTTATCAAACGGTGGCTGAATGACATGCCTTTACTCCAGACAACCAACTATATGGTCCTC
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CCTATGTGCAACCAAACGTCATGGGAACCCTTTGGAGGAGGACAGTTGCCTTCTTATGGGCGGTTGACATTACCTCTAGATCCA
AGCATTGACCTTCAACTTAACATATCATTTACATATGGTCCGGTTATACTGAACGGAGACGGTATGGATTATTATGAAAGCCCACT
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CACTGTGACCCCCCATGTGTTGACATTTGCGCCCAGGGAATCAAGTGGAAATTGTTATTTACCTATTCAAACATCCCAGATTATG
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GGCGATCATGCAATTGTTTATTATGTTTATGACCCAATCCGGACGATTTCTTATACACACCCATTTAGACTAACTACCAAGGGTAG
ACCTGATTTCCTAAGGATTGAATGTTTTGTGTGGGATGACGATTTGTGGTGTCATCAATTTTACCGATTCGAGGCTAACATCACTAA
CTCTACAACCAG

The nucleotide sequences of F gene

BKK01/09, 906 nucleotides
ATTAGGGAGGCAACCCAGGAGACCGTCATTGCCGTCCAGGGAATCCAAGATTACGTCAATAATGAACTCGTCCCTGCTATGCAA
CATATGTCGTGTGAATTGGTTGGGCAGAGATTAGGATTAAAACTGCTTAGGTATTATACCGAGTTGTTGTCAATATTTGGCCCGAG
TTTACGTGACCCTATTTCAGCCGAGATATCAATTCAAGCACTGAGTTATGCTCTTGGGGGAGAAATTCATAAGATACTTGAGAAGT
TGGGATATTCTGGAAATGACATGATTGCAATTTTGGAGAGTCGGGGGATAAAGACAAAAATAACTCATGTTGATCTCCCCGGGAA
ACTCATCATCTTAAGTATATCATACCCGACTTTATCAGAAGTCAAGGGGGTTATAGTCCACAGACTAGAAGCGGTTTCTTATAACA
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TCGATACTATGTAAGTGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAGTTGCTGACATTTATTGCCTCTGATACCTGC
CCACTGGTTGAAATAGATGGTGTAACTATCCAGGTTGGAGGGAGGCAATACCCTGATATGGTATACGAAAGCAAAGTTGCCTTA
GGCCCTGCTATATCACTTGAGAGGTTAGATGTAGGTACAAATTTAGGGAAC

BKK02/09, 906 nucleotides
ATTAGGGAGGCAACCCAGGAGACCGTCATTGCCGTCCAGGGAATCCAAGATTACGTCAATAATGAACTCGTCCCTGCTATGCAA
CATATGTCGTGTGAATTGGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTATACCGAGTTGTTGTCAATATTTGGCCCGAG
TTTACGTGACCCTATTTCAGCCGAGATATCAATTCAAGCACTGAGTTATGCTCTTGGGGGAGAAATTCATAAGATACTTGAGAAGT
TGGGATATTCTGGAAATGACATGATTGCAATTTTGGAGAGTCGGGGGATAAAGACAAAAATAACTCATGTTGATCTCCCCGGGAA
ACTCATCATCTTAAGTATATCATACCCGACTTTATCAGAAGTCAAGGGGGTTATAGTCCACAGACTAGAAGCGGTTTCTTATAACA
TAGGGTCACAGGAGTGGTACACCACTGTCCCGAGATATGTTGCAACTAATGGTTACTTAATCTCTAATTTTGATGAGTCATCCTGT
GTATTCGTCTCAGAATCAGCCATTTGTAGCCAGAACTCTCTATACCCCATGAGCCCGCTTCTACAACAATGCATTAGGGGCGATA
CCTCATCTTGTGCTCGGACCTTGGTGTCTGGGACTATGGGCAACAAGTTTATTCTGTCAAAAGGTAATATCGTTGCAAACTGTGCT
TCGATACTATGTAAGTGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAGTTGCTGACATTTATTGCCTCTGATACCTGC
CCACTGGTTGAAATAGATGGTGTAACTATCCAGGTTGGAGGGAGGCAATACCCTGATATGGTATACGAAAGCAAAGTTGCCTTA
GGCCCTGCTATATCACTTGAGAGGTTAGATGTAGGTACAAATTTAGGGAAC

BKK03/09, 906 nucleotides
ATTAGGGAGGCAACCCAGGAAACCGTCATTGCTGTTCAGGGAGTCCAGGATTACGTCAATAATGAACTCGTCCCTGCTATGCAA
CATATGTCGTGTGAGTTAGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTACACCGAGTTGTTGTCAATATTTGGCCCGA
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GCCCACTGGTTGAAATAGATGGTGTAACTATCCAAGTTGGAGGGAGGCAATACCCTGATATGGTATACGAAAGCAAGGTTGCCT
TAGGACCTGCTATATCACTTGAGAGGTTGGATGTAGGTACAAATTTAGGGAAC

BKK04/09, 906 nucleotides
ATTAGGGAGGCAACCCAGGAGACCGTCATTGCCGTCCAGGGAATCCAAGATTACGTCAATAATGAACTCGTCCCTGCTATGCAA
CATATGTCGTGTGAATTGGTTGGGCAGAGATTAGGATTAAAACTGCTTAGGTATTATACCGAGTTGTTGTCAATATTTGGCCCGAG
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BKK05/09, 906 nucleotides
ATTAGGGAGGCAACCCAGGAAACCGTCATTGCTGTTCAGGGAGTCCAGGATTACGTCAATAATGAACTCGTCCCTGCTATGCAA
CATATGTCGTGTGAGTTAGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTACACCGAGTTGTTGTCAATATTTGGCCCGA
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AAACTCATCATATTAAGTATCTCATACCCAACTTTATCAGAAGTCAAGGGGGTTATAGTCCACAGACTGGAAGCCGTTTCTTATAA
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GCCCACTGGTTGAAATAGATGGTGTAACTATCCAAGTTGGAGGGAGGCAATACCCTGATATGGTATACGAAAGCAAAGTTGCCT
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BKK01/10, 906 nucleotides
ATTAGGGAGGCAACCCAGGAGACCGTCATTGCCGTCCAGGGAGTCCAAGATTACGTCAATAATGAACTCGTCCCTGCTATGCA
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CATAGGGTCACAGGAGTGGTACACCACTGTCCCGAGATATGTTGCAACTAATGGTTACTTAATCTCTAATTTTGATGAGTCATCCT
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BKK02/10, 906 nucleotides
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BKK03/10, 906 nucleotides
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ACATATGTCGTGTGAATTAGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTATACCGAGTTGTTGTCAATATTTGGCCCGA
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CTTCGATACTATGTAAGTGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAGTTGCTGACATTTATTGCCTCTGATACCT
GCCCACTGGTTGAAATAGATGGTGTAACTATCCAGGTTGGAGGGAGGCAATACCCTGATATGGTATACGAAAGCAAAGTTGCCT
TAGGCCCTGCTATATCACTTGAGAGGTTAGATGTAGGTACAAATTTAGGGAAC

BKK04/10, 906 nucleotides
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BKK05/10, 906 nucleotides
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CATATGTCGTGTGAGTTAGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTACACCGAGTTGTTGTCAATATTTGGCCCGA
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ACACTTCATCTTGTGCTCGGACCTTGGTGTCTGGGACTATGGGCAACAAGTTTATTCTGTCAAAAGGTAATATCGTTGCAAATTGT
GCTTCTATACTATGTAAGTGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAGTTGCTGACATTTATTGCCTCCGATACC
TGCCCACTGGTTGAAATAGATGGTGTAACTATCCAAGTTGGAGGGAGGCAATACCCTGATATGGTATACGAAAGCAAAGTTGCC
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BKK06/10, 906 nucleotides
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CATATGTCGTGTGAGTTAGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTACACCGAGTTGTTGTCAATATTTGGCCCGA
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BKK01/11, 906 nucleotides
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ACATATGTCGTGTGAATTGGTTGGGCAGAGATTAGGATTAAAACTGCTTAGGTATTATACCGAGTTGTTGTCAATATTTGGCCCGA
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TAGGCCCTGCTATATCACTTGAGAGGTTAGATGTAGGTACAAATTTAGGGAAC

BKK02/11, 906 nucleotides
ATTAGGGAGGCAACCCAGGAAACCGTCATTGCTGTTCAGGGAGTCCAGGATTACGTCAATAATGAACTCGTCCCTGCTATGCAA
CATATGTCGTGTGAGTTAGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTACACCGAGTTGTTGTCAATATTTGGCCCGA
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GCCCACTGGTTGAAATAGATGGTGTAACTATCCAAGTTGGAGGGAGGCAATACCCTGATATGGTATACGAAAGCAAGGTTGCCT
TAGGACCTGCTATATCACTTGAGAGGTTGGATGTAGGTACAAATTTAGGGAAC

BKK03/11, 906 nucleotides
ATTAGGGAGGCAACCCAGGAAACCGTCATTGCTGTTCAGGGAGTCCAGGATTACGTCAATAATGAACTCGTCCCTGCTATGCAA
CATATGTCGTGTGAGTTAGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTACACCGAGTTGTTGTCAATATTTGGCCCGA
GTTTACGTGACCCTATTTCAGCCGAGATATCAATTCAAGCACTGAGTTATGCTCTTGGGGGAGAAATTCATAAGATACTTGAGAA
GTTGGGGTACTCTGGTAATGATATGATTGCAATTTTGGAGAGTCGGGGGATAAAAACAAAAATAACTCATGTCGATCTCCCCGGG
AAACTCATCATATTAAGTATCTCATACCCAACTTTATCAGAGGTCAAGGGGGTTATAGTCCACAGACTGGAAGCCGTTTCTTATAA
CATAGGGTCACAGGAGTGGTACACCACTGTCCCGAAGTATGTTGCAACTAATGGTTACTTAATATCTAACTTTGATGAGTCATCCT
GTGTATTCGTCTCAGAATCAGCCATTTGTAGCCAGAACTCTCTATACCCCATGAGCCCGATTCTACAACAATGCATTAGGGGCGA
TACTTCATCTTGTGCTCGGACCTTGGTGTCTGGGACTATGGGCAACAAGTTTATTCTGTCAAAAGGTAATATCGTTGCAAATTGTG
CTTCTATACTATGTAAGTGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAGTTGCTGACATTTATTGCCTCCGATACCT
GCCCACTGGTTGAAATAGATGGTGTAACTATCCAAGTTGGAGGGAGGCAATACCCTGATATGGTATACGAAAGCAAGGTTGCCT
TAGGACCTGCTATATCACTTGAGAGGTTGGATGTAGGTACAAATTTAGGGAAC

BKK04/11, 906 nucleotides
ATTAGGGAGGCAACCCAGGAAACCGTCATTGCTGTTCAGGGAGTCCAGGATTACGTCAATAATGAACTCGTCCCTGCTATGCAA
CATATGTCGTGTGAGTTAGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTACACCGAGTTGTTGTCAATATTTGGCCCGA
GTTTACGTGACCCTATTTCAGCCGAGATATCAATTCAAGCACTGAGTTATGCTCTTGGGGGAGAAATTCATAAGATACTTGAGAA
GTTGGGGTATTCTGGTAATGATATGATTGCAATTTTGGAGAGTCGGGGGATAAAAACAAAAATAACTCATGTCGATCTCCCCGGG
AAACTCATCATATTAAGTATCTCATACCCAACTTTATCAGAAGTCAAGGGGGTTATAGTCCACAGACTGGAAGCCGTTTCTTATAA
CATAGGGTCACAGGAGTGGTACACCACTGTCCCGAAGTATGTTGCAACTAATGGTTACTTAATATCTAACTTTGATGAGTCATCCT
GTGTATTCGTCTCAGAATCAGCCATTTGTAGCCAGAACTCTCTATACCCCATGAGCCCGATTCTACAACAATGCATTAGGGGCGA
TACTTCATCTTGTGCTCGGACCTTGGTGTCTGGGACTATGGGCAACAAGTTTATTCTGTCAAAAGGTAATATCGTTGCAAATTGTG
CTTCTATACTATGTAAGTGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAGTTGCTGACATTTATTGCCTCCGATACCT
GCCCACTGGTTGAAATAGATGGTGTAACTATCCAAGTTGGAGGGAGGCAATACCCTGATATGGTATACGAAAGCAAAGTTGCCT
TAGGACCTGCTATATCACTTGAGAGGTTGGATGTAGGTACAAATTTAGGGAAC

BKK05/11, 780 nucleotides
ATTAGGGAGGCAACCCAGGAAACCGTCATTGCTGTTCAGGGAGTCCAGGATTACGTCAATAATGAACTCGTCCCTGCTATGCAA
CATATGTCGTGTGAGTTAGTTGGGCAGAGATTAGGGTTAAAGCTGCTTAGGTATTACACCGAGTTGTTGTCAATATTTGGCCCGA
GTTTACGTGACCCTATTTCAGCCGAGATATCAATTCAAGCACTGAGTTATGCTCTTGGGGGAGAAATTCATAAGATACTTGAGAA
GTTGGGGTACTCTGGTAATGATATGATTGCAATTTTGGAGAGTCGGGGGATAAAAACAAAAATAACTCATGTCGATCTCCCCGGG
AAACTCATCATATTAAGTATCTCATACCCAACTTTATCAGAGGTCAAGGGGGTTATAGTCCACAGACTGGAAGCCGTTTCTTATAA
CATAGGGTCACAGGAGTGGTACACCACTGTCCCGAAGTATGTTGCAACTAATGGTTACTTAATATCTAACTTTGATGAGTCATCCT
GTGTATTCGTCTCAGAATCAGCCATTTGTAGCCAGAACTCTCTATACCCCATGAGCCCGATTCTACAACAATGCATTAGGGGCGA

TACTTCATCTTGTGCTCGGACCTTGGTGTCTGGGACTATGGGCAACAAGTTTATTCTGTCAAAAGGTAATATCGTTGCAAATTGTG
CTTCTATACTATGTAAGTGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAGTTGCTGACATTTATTGCCTCCGATACCT
GCCCACTGGTT

BKK06/11, 906 nucleotides
ATTAGGGAGGCAACCCAGGAAACCGTCATTGCCGTCCAGGGAGTCCAGGATTACGTCAATAATGAACTCGTCCCTGCTATGCA
ACATATGTCGTGTGAATTGGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTATACCGAGTTGTTGTCAATATTTGGCCCGA
GTCTACGTGATCCTATTTCAGCCGAGATATCAATTCAAGCACTGAGTTATGCTCTTGGGGGAGAAATTCATAAGATACTTGAGAA
GTTGGGATATTCTGGAAATGATATGATTGCAATTTTGGAGAGTCGGGGGATAAAAACAAAAATAACTCATGTTGATCTCCCCGGG
AAACTCATTATCTTAAGTATATCATACCCGACCTTATCAGAAGTCAAGGGGGTTATAGTCCACAGACTAGAAGCGGTTTCTTATAA
CATAGGGTCACAAGAGTGGTACACCACTGTCCCGAGATATGTTGCAACTAATGGTTACTTAATCTCTAATTTTGATGAGTCATCCT
GTGTATTCGTCTCAGAATCAGCCATTTGTAGCCAGAATTCTCTATACCCCATGAGCCCGCTTCTACAACAATGCATTAGGGGCGA
TACCTCATCTTGTGCTCGGACCTTGGTGTCTGGGACTATGGGCAACAAGTTTATTCTGTCAAAAGGTAATATCGTTGCAAATTGTG
CTTCGATATTATGTAAATGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAGCTGCTGACATTTATTGCCTCTGATACCT
GCCCACTGGTTGAAATAGATGGTGTAACTATCCAGGTTGGAGGGAGACAATACCCTGATATGGTATACGAAAGCAAAGTTGCCT
TAGGCCCTGCCATCTCACTTGAGAGGTTAGATGTAGGTACAAATTTAGGGAAC

BKK07/11, 906 nucleotides
ATTAGGGAGGCAACCCAGGAGACCGTCATTGCCGTCCAGGGAGTCCAAGATTACGTCAATAATGAACTCGTCCCTGCTATGCA
ACATATGTCGTGTGAATTGGTTGGGCAGAGATTAGGATTAAAACTGCTTAGGTATTATACCGAGTTGTTGTCAATATTTGGTCCGA
GTTTACGTGACCCTATTTCAGCCGAGATATCAATTCAAGCACTGAGTTATGCTCTTGGAGGAGAAATTCATAAGATACTTGAGAAG
TTGGGATATTCTGGAAATGACATGATTGCAATTTTGGAGAGTCGGGGGATAAAGACAAAAATAACTCATGTTGATCTCCCCGGGA
AACTCATCATCTTAAGTATATCATACCCGACTTTATCAGAAGTCAAGGGGGTTATAGTCCACAGACTAGAAGCGGTTTCTTATAAC
ATAGGGTCACAGGAGTGGTACACCACTGTCCCGAGATATGTTGCAACTAATGGTTACTTAATCTCTAATTTTGATGAGTCATCCTG
TGTATTCGTCTCAGAATCAGCCATTTGTAGCCAGAACTCTCTATACCCCATGAGCCCGCTTCTACAACAATGCATTAGGGGCGAT
ACCTCATCTTGTGCTCGGACCTTGGTGTCTGGGACTATGGGCAACAAGTTTATTCTGTCAAAAGGTAATATCGTTGCAAACTGTG
CTTCGATACTATGTAAGTGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAGTTGCTGACATTTATTGCCTCTGATACCT
GCCCACTGGTTGAAATAGATGGTGTAACTATCCAGGTTGGAGGGAGGCAATACCCTGATATGGTATACGAAAGCAAAGTTGCCT
TAGGCCCTGCTATATCACTTGAGAGGTTAGATGTAGGTACAAATTTAGGGAAC

BKK08/11, 906 nucleotides
ATTAGGGAGGCAACCCAGGAAACCGTCATTGCTGTTCAGGGAGTCCAGGATTACGTCAATAATGAACTCGTCCCTGCTATGCAA
CATATGTCGTGTGAGTTAGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTACACCGAGTTGTTGTCAATATTTGGCCCGA
GTTTACGTGACCCTATTTCAGCCGAGATATCAATTCAAGCACTGAGTTATGCTCTTGGGGGAGAAATTCATAAGATACTTGAGAA
GTTGGGGTATTCTGGTAATGATATGATTGCAATTTTGGAGAGTCGGGGGATAAAAACAAAAATAACTCATGTCGATCTCCCCGGG
AAACTCATCATATTAAGTATCTCATACCCAACTTTATCAGAAGTCAAGGGGGTTATAGTCCACAGACTGGAAGCCGTTTCTTATAA
CATAGGGTCACAGGAGTGGTACACCACTGTCCCGAAGTATGTTGCAACTAATGGTTACTTAATATCTAACTTTGATGAGTCATCCT
GTGTATTCGTCTCAGAATCAGCCATTTGTAGCCAGAACTCTCTATACCCCATGAGCCCGATTCTACAACAATGCATTAGGGGCGA
TACTTCATCTTGTGCTCGGACCTTGGTGTCTGGGACTATGGGCAACAAGTTTATTCTGTCAAAAGGTAATATCGTTGCAAATTGTG
CTTCTATACTATGTAAGTGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAGTTGCTGACATTTATTGCCTCCGATACCT
GCCCACTGGTTGAAATAGATGGTGTAACTATCCAAGTTGGAGGGAGGCAATACCCTGATATGGTATACGAAAGCAAAGTTGCCT
TAGGACCTGCTATATCACTTGAGAGGTTGGATGTAGGTACAAATTTAGGGAAC

BKK09/11, 906 nucleotides
ATTAGGGAGGCAACCCAGGAAACCGTCATTGCTGTTCAGGGAGTCCAGGATTACGTCAATAATGAACTCGTCCCTGCTATGCAA
CATATGTCGTGTGAGTTAGTTGGGCAGAGATTAGGGTTAAAGCTGCTTAGGTATTACACCGAGTTGTTGTCAATATTTGGCCCGA
GTTTACGTGACCCTATTTCAGCCGAGATATCAATTCAAGCACTGAGTTATGCTCTTGGGGGAGAAATTCATAAGATACTTGAGAA
GTTGGGGTACTCTGGTAATGATATGATTGCAATTTTGGAGAGTCGGGGGATAAAAACAAAAATAACTCATGTCGATCTCCCCGGG
AAACTCATCATATTAAGTATCTCATACCCAACTTTATCAGAGGTCAAGGGGGTTATAGTCCACAGACTGGAAGCCGTTTCTTATAA
CATAGGGTCACAGGAGTGGTACACCACTGTCCCGAAGTATGTTGCAACTAATGGTTACTTAATATCTAACTTTGATGAGTCATCCT
GTGTATTCGTCTCAGAATCAGCCATTTGTAGCCAGAACTCTCTATACCCCATGAGCCCGATTCTACAACAATGCATTAGGGGCGA
TACTTCATCTTGTGCTCGGACCTTGGTGTCTGGGACTATGGGCAACAAGTTTATTCTGTCAAAAGGTAATATCGTTGCAAATTGTG
CTTCTATACTATGTAAGTGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAGTTGCTGACATTTATTGCCTCCGATACCT
GCCCACTGGTTGAAATAGATGGTGTAACTATCCAAGTTGGAGGGAGGCAATACCCTGATATGGTATACGAAAGCAAGGTTGCCT
TAGGACCTGCTATATCACTTGAGAGGTTGGATGTAGGTACAAATTTAGGGAAC

BKK10/11, 906 nucleotides
ATTAGGGAGGCAACCCAGGAAACCGTCATTGCTGTTCAGGGAGTCCAGGATTACGTCAATAATGAACTCGTCCCTGCTATGCAA
CATATGTCGTGTGAGTTAGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTACACCGAGTTGTTGTCAATATTTGGCCCGA
GTTTACGTGACCCTATTTCAGCCGAGATATCAATTCAAGCACTGAGTTATGCTCTTGGGGGAGAAATTCACAAGATACTTGAGAA

GTTGGGGTATTCTGGTAGTGATATGATTGCAATTTTGGAGAGTCGGGGGATAAAAACAAAAATAACTCATGTCGATCTCCCCGGG
AAACTCATCATATTAAGTATCTCATACCCAACTTTATCAGAAGTCAAGGGGGTGATAGTCCACAGACTGGAAGCCGTTTCTTATAA
CATAGGGTCACAGGAGTGGTACACCACTGTCCCGAAGTATGTTGCAACTAATGGTTACTTAATATCTAACTTTGATGAGTCATCCT
GTGTATTCGTCTCAGAATCAGCCATTTGTAGCCAGAACTCTCTATACCCCATGAGCCCGATTCTACAACAATGCATTAGGGGCGA
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CTTCTATACTATGTAAGTGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAATTGCTGACATTTATTGCCTCCGATACCT
GCCCACTGGTTGAAATAGATGGTGTAACTATCCAAGTTGGAGGGAGGCAATACCCTGATATGGTATACGAGAGCAAAGTTGCCT
TAGGACCTGCTATATCACTTGAGAGGTTGGATGTAGGTACAAATTTAGGGAAC

BKKZ03/11, 906 nucleotides
ATTAGGGAGGCAACCCAGGAAACCGTCATTGCTGTTCAGGGAGTCCAGGATTACGTCAATAATGAACTCGTCCCTGCTATGCAA
CATATGTCGTGTGAGTTAGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTACACCGAGTTGTTGTCAATATTTGGCCCGA
GTTTACGTGACCCTATTTCAGCCGAGATATCAATTCAAGCACTGAGTTATGCTCTTGGGGGAGAAATTCACAAGATACTTGAGAA
GTTGGGGTATTCTGGTAGTGATATGATTGCAATTTTGGAGAGTCGGGGGATAAAAACAAAAATAACTCATGTCGATCTCCCCGGG
AAACTCATCATATTAAGTATCTCATACCCAACTTTATCAGAAGTCAAGGGGGTGATAGTCCACAGACTGGAAGCCGTTTCTTATAA
CATAGGGTCACAGGAGTGGTACACCACTGTCCCGAAGTATGTTGCAACTAATGGTTACTTAATATCTAACTTTGATGAGTCATCCT
GTGTATTCGTCTCAGAATCAGCCATTTGTAGCCAGAACTCTCTATACCCCATGAGCCCGATTCTACAACAATGCATTAGGGGCGA
TACTTCATCTTGTGCTCGGACCTTGGTGTCTGGGACTATGGGCAACAAGTTTATTCTGTCAAAAGGTAATATCGTTGCAAATTGTG
CTTCTATACTATGTAAGTGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAATTGCTGACATTTATTGCCTCCGATACCT
GCCCACTGGTTGAAATAGATGGTGTAACTATCCAAGTTGGAGGGAGGCAATACCCTGATATGGTATACGAGAGCAAAGTTGCCT
TAGGACCTGCTATATCACTTGAGAGGTTGGATGTAGGTACAAATTTAGGGAAC

BKKZ05/11, 906 nucleotides
ATTAGGGAGGCAACCCAGGAAACCGTCATTGCTGTTCAGGGAGTCCAGGATTACGTCAATAATGAACTCGTCCCTGCTATGCAA
CATATGTCGTGTGAGTTAGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTACACCGAGTTGTTGTCAATATTTGGCCCGA
GTTTACGTGACCCTATTTCAGCCGAGATATCAATTCAAGCACTGAGTTATGCTCTTGGGGGAGAAATTCACAAGATACTTGAGAA
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AAACTCATCATATTAAGTATCTCATACCCAACTTTATCAGAAGTCAAGGGGGTGATAGTCCACAGACTGGAAGCCGTTTCTTATAA
CATAGGGTCACAGGAGTGGTACACCACTGTCCCGAAGTATGTTGCAACTAATGGTTACTTAATATCTAACTTTGATGAGTCATCCT
GTGTATTCGTCTCAGAATCAGCCATTTGTAGCCAGAACTCTCTATACCCCATGAGCCCGATTCTACAACAATGCATTAGGGGCGA
TACTTCATCTTGTGCTCGGACCTTGGTGTCTGGGACTATGGGCAACAAGTTTATTCTGTCAAAAGGTAATATCGTTGCAAATTGTG
CTTCTATACTATGTAAGTGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAATTGCTGACATTTATTGCCTCCGATACCT
GCCCACTGGTTGAAATAGATGGTGTAACTATCCAAGTTGGAGGGAGGCAATACCCTGATATGGTATACGAGAGCAAAGTTGCCT
TAGGACCTGCTATATCACTTGAGAGGTTGGATGTAGGTACAAATTTAGGGAAC

BKKZ06/11, 906 nucleotides
ATTAGGGAGGCAACCCAGGAAACCGTCATTGCTGTTCAGGGAGTCCAGGATTACGTCAATAATGAACTCGTCCCTGCTATGCAA
CATATGTCGTGTGAGTTAGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTACACCGAGTTGTTGTCAATATTTGGCCCGA
GTTTACGTGACCCTATTTCAGCCGAGATATCAATTCAAGCACTGAGTTATGCTCTTGGGGGAGAAATTCACAAGATACTTGAGAA
GTTGGGGTATTCTGGTAGTGATATGATTGCAATTTTGGAGAGTCGGGGGATAAAAACAAAAATAACTCATGTCGATCTCCCCGGG
AAACTCATCATATTAAGTATCTCATACCCAACTTTATCAGAAGTCAAGGGGGTGATAGTCCACAGACTGGAAGCCGTTTCTTATAA
CATAGGGTCACAGGAGTGGTACACCACTGTCCCGAAGTATGTTGCAACTAATGGTTACTTAATATCTAACTTTGATGAGTCATCCT
GTGTATTCGTCTCAGAATCAGCCATTTGTAGCCAGAACTCTCTATACCCCATGAGCCCGATTCTACAACAATGCATTAGGGGCGA
TACTTCATCTTGTGCTCGGACCTTGGTGTCTGGGACTATGGGCAACAAGTTTATTCTGTCAAAAGGTAATATCGTTGCAAATTGTG
CTTCTATACTATGTAAGTGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAATTGCTGACATTTATTGCCTCCGATACCT
GCCCACTGGTTGAAATAGATGGTGTAACTATCCAAGTTGGAGGGAGGCAATACCCTGATATGGTATACGAGAGCAAAGTTGCCT
TAGGACCTGCTATATCACTTGAGAGGTTGGATGTAGGTACAAATTTAGGGAAC

BKKZ07/11, 906 nucleotides
ATTAGGGAGGCAACCCAGGAAACCGTCATTGCTGTTCAGGGAGTCCAGGATTACGTCAATAATGAACTCGTCCCTGCTATGCAA
CATATGTCGTGTGAGTTAGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTACACCGAGTTGTTGTCAATATTTGGCCCGA
GTTTACGTGACCCTATTTCAGCCGAGATATCAATTCAAGCACTGAGTTATGCTCTTGGGGGAGAAATTCACAAGATACTTGAGAA
GTTGGGGTATTCTGGTAGTGATATGATTGCAATTTTGGAGAGTCGGGGGATAAAAACAAAAATAACTCATGTCGATCTCCCCGGG
AAACTCATCATATTAAGTATCTCATACCCAACTTTATCAGAAGTCAAGGGGGTGATAGTCCACAGACTGGAAGCCGTTTCTTATAA
CATAGGGTCACAGGAGTGGTACACCACTGTCCCGAAGTATGTTGCAACTAATGGTTACTTAATATCTAACTTTGATGAGTCATCCT
GTGTATTCGTCTCAGAATCAGCCATTTGTAGCCAGAACTCTCTATACCCCATGAGCCCGATTCTACAACAATGCATTAGGGGCGA
TACTTCATCTTGTGCTCGGACCTTGGTGTCTGGGACTATGGGCAACAAGTTTATTCTGTCAAAAGGTAATATCGTTGCAAATTGTG
CTTCTATACTATGTAAGTGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAATTGCTGACATTTATTGCCTCCGATACCT
GCCCACTGGTTGAAATAGATGGTGTAACTATCCAAGTTGGAGGGAGGCAATACCCTGATATGGTATACGAGAGCAAAGTTGCCT
TAGGACCTGCTATATCACTTGAGAGGTTGGATGTAGGTACAAATTTAGGGAAC

BKKZ09/11, 906 nucleotides
ATTAGGGAGGCAACCCAGGAAACCGTCATTGCTGTTCAGGGAGTCCAGGATTACGTCAATAATGAACTCGTCCCTGCTATGCAA
CATATGTCGTGTGAGTTAGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTACACCGAGTTGTTGTCAATATTTGGCCCGA
GTTTACGTGACCCTATTTCAGCCGAGATATCAATTCAAGCACTGAGTTATGCTCTTGGGGGAGAAATTCACAAGATACTTGAGAA
GTTGGGGTATTCTGGTAGTGATATGATTGCAATTTTGGAGAGTCGGGGGATAAAAACAAAAATAACTCATGTCGATCTCCCCGGG
AAACTCATCATATTAAGTATCTCATACCCAACTTTATCAGAAGTCAAGGGGGTGATAGTCCACAGACTGGAAGCCGTTTCTTATAA
CATAGGGTCACAGGAGTGGTACACCACTGTCCCGAAGTATGTTGCAACTAATGGTTACTTAATATCTAACTTTGATGAGTCATCCT
GTGTATTCGTCTCAGAATCAGCCATTTGTAGCCAGAACTCTCTATACCCCATGAGCCCGATTCTACAACAATGCATTAGGGGCGA
TACTTCATCTTGTGCTCGGACCTTGGTGTCTGGGACTATGGGCAACAAGTTTATTCTGTCAAAAGGTAATATCGTTGCAAATTGTG
CTTCTATACTATGTAAGTGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAATTGCTGACATTTATTGCCTCCGATACCT
GCCCACTGGTTGAAATAGATGGTGTAACTATCCAAGTTGGAGGGAGGCAATACCCTGATATGGTATACGAGAGCAAAGTTGCCT
TAGGACCTGCTATATCACTTGAGAGGTTGGATGTAGGTACAAATTTAGGGAAC

BKKZ10/11, 906 nucleotides
ATTAGGGAGGCAACCCAGGAAACCGTCATTGCTGTTCAGGGAGTCCAGGATTACGTCAATAATGAACTCGTCCCTGCTATGCAA
CATATGTCGTGTGAGTTAGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTACACCGAGTTGTTGTCAATATTTGGCCCGA
GTTTACGTGACCCTATTTCAGCCGAGATATCAATTCAAGCACTGAGTTATGCTCTTGGGGGAGAAATTCACAAGATACTTGAGAA
GTTGGGGTATTCTGGTAGTGATATGATTGCAATTTTGGAGAGTCGGGGGATAAAAACAAAAATAACTCATGTCGATCTCCCCGGG
AAACTCATCATATTAAGTATCTCATACCCAACTTTATCAGAAGTCAAGGGGGTGATAGTCCACAGACTGGAAGCCGTTTCTTATAA
CATAGGGTCACAGGAGTGGTACACCACTGTCCCGAAGTATGTTGCAACTAATGGTTACTTAATATCTAACTTTGATGAGTCATCCT
GTGTATTCGTCTCAGAATCAGCCATTTGTAGCCAGAACTCCCTATACCCCATGAGCCCGATTCTACAACAATGCATTAGGGGCGA
TACTTCATCTTGTGCTCGGACCTTGGTGTCTGGGACTATGGGCAACAAGTTTATTCTGTCAAAAGGTAATATCGTTGCAAATTGTG
CTTCTATACTATGTAAGTGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAATTGCTGACATTTATTGCCTCCGATACCT
GCCCACTGGTTGAAATAGATGGTGTAACTATCCAAGTTGGAGGGAGGCAATACCCTGATATGGTATACGAGAGCAAAGTTGCCT
TAGGACCTGCTATATCACTTGAGAGGTTGGATGTAGGTACAAATTTAGGGAAC

BKKZ11/11, 906 nucleotides
ATTAGGGAGGCAACCCAGGAAACCGTCATTGCTGTTCAGGGAGTCCAGGATTACGTCAATAATGAACTCGTCCCTGCTATGCAA
CATATGTCGTGTGAGTTAGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTACACCGAGTTGTTGTCAATATTTGGCCCGA
GTTTACGTGACCCTATTTCAGCCGAGATATCAATTCAAGCACTGAGTTATGCTCTTGGGGGAGAAATTCACAAGATACTTGAGAA
GTTGGGGTATTCTGGTAGTGATATGATTGCAATTTTGGAGAGTCGGGGGATAAAAACAAAAATAACTCATGTCGATCTCCCCGGG
AAACTCATCATATTAAGTATCTCATACCCAACTTTATCAGAAGTCAAGGGGGTGATAGTCCACAGACTGGAAGCCGTTTCTTATAA
CATAGGGTCACAGGAGTGGTACACCACTGTCCCGAAGTATGTTGCAACTAATGGTTACTTAATATCTAACTTTGATGAGTCATCCT
GTGTATTCGTCTCAGAATCAGCCATTTGTAGCCAGAACTCTCTATACCCCATGAGCCCGATTCTACAACAATGCATTAGGGGCGA
TACTTCATCTTGTGCTCGGACCTTGGTGTCTGGGACTATGGGCAACAAGTTTATTCTGTCAAAAGGTAATATCGTTGCAAATTGTG
CTTCTATACTATGTAAGTGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAATTGCTGACATTTATTGCCTCCGATACCT
GCCCACTGGTTGAAATAGATGGTGTAACTATCCAAGTTGGAGGGAGGCAATACCCTGATATGGTATACGAGAGCAAAGTTGCCT
TAGGACCTGCTATATCACTTGAGAGGTTGGATGTAGGTACAAATTTAGGGAAC

BKKZ12/11, 906 nucleotides
ATTAGGGAGGCAACCCAGGAAACCGTCATTGCTGTTCAGGGAGTCCAGGATTACGTCAATAATGAACTCGTCCCTGCTATGCAA
CATATGTCGTGTGAGTTAGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTACACCGAGTTGTTGTCAATATTTGGCCCGA
GTTTACGTGACCCTATTTCAGCCGAGATATCAATTCAAGCACTGAGTTATGCTCTTGGGGGAGAAATTCACAAGATACTTGAGAA
GTTGGGGTATTCTGGTAGTGATATGATTGCAATTTTGGAGAGTCGGGGGATAAAAACAAAAATAACTCATGTCGATCTCCCCGGG
AAACTCATCATATTAAGTATCTCATACCCAACTTTATCAGAAGTCAAGGGGGTGATAGTCCACAGACTGGAAGCCGTTTCTTATAA
CATAGGGTCACAGGAGTGGTACACCACTGTCCCGAAGTATGTTGCAACTAATGGTTACTTAATATCTAACTTTGATGAGTCATCCT
GTGTATTCGTCTCAGAATCAGCCATTTGTAGCCAGAACTCTCTATACCCCATGAGCCCGATTCTACAACAATGCATTAGGGGCGA
TACTTCATCTTGTGCTCGGACCTTGGTGTCTGGGACTATGGGCAACAAGTTTATTCTGTCAAAAGGTAATATCGTTGCAAATTGTG
CTTCTATACTATGTAAGTGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAATTGCTGACATTTATTGCCTCCGATACCT
GCCCACTGGTTGAAATAGATGGTGTAACTATCCAAGTTGGAGGGAGGCAATACCCTGATATGGTATACGAGAGCAAAGTTGCCT
TAGGACCTGCTATATCACTTGAGAGGTTGGATGTAGGTACAAATTTAGGGAAC

BKKZ13/11, 906 nucleotides
ATTAGGGAGGCAACCCAGGAAACCGTCATTGCTGTTCAGGGAGTCCAGGATTACGTCAATAATGAACTCGTCCCTGCTATGCAA
CATATGTCGTGTGAGTTAGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTACACCGAGTTGTTGTCAATATTTGGCCCGA
GTTTACGTGACCCTATTTCAGCCGAGATATCAATTCAAGCACTGAGTTATGCTCTTGGGGGAGAAATTCACAAGATACTTGAGAA
GTTGGGGTATTCTGGTAGTGATATGATTGCAATTTTGGAGAGTCGGGGGATAAAAACAAAAATAACTCATGTCGATCTCCCCGGG
AAACTCATCATATTAAGTATCTCATACCCAACTTTATCAGAAGTCAAGGGGGTGATAGTCCACAGACTGGAAGCCGTTTCTTATAA
CATAGGGTCACAGGAGTGGTACACCACTGTCCCGAAGTATGTTGCAACTAATGGTTACTTAATATCTAACTTTGATGAGTCATCCT
GTGTATTCGTCTCAGAATCAGCCATTTGTAGCCAGAACTCTCTATACCCCATGAGCCCGATTCTACAACAATGCATTAGGGGCGA

TACTTCATCTTGTGCTCGGACCTTGGTGTCTGGGACTATGGGCAACAAGTTTATTCTGTCAAAAGGTAATATCGTTGCAAATTGTG
CTTCTATACTATGTAAGTGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAATTGCTGACATTTATTGCCTCCGATACCT
GCCCACTGGTTGAAATAGATGGTGTAACTATCCAAGTTGGAGGGAGGCAATACCCTGATATGGTATACGAGAGCAAAGTTGCCT
TAGGACCTGCTATATCACTTGAGAGGTTGGATGTAGGTACAAATTTAGGGAAC

BKK/CG, 906 nucleotides
ATTAGGGAGGCTACCCAAGAAACCGTCATTGCCGTTCAGGGAGTCCAGGACTACGTCAACAACGAACTCGTCCCTGCCATGCA
ACATATGTCATGTGAATTAGTTGGGCAGAGATTAGGGTTAAGACTGCTTCGGTATTATACTGAGTTGTTGTCAATATTTGGCCCGA
GTTTACGTGACCCTATTTCAGCCGAGATATCAATTCAGGCACTGAGTTATGCTCTTGGAGGAGAAATTCATAAGATACTTGAGAA
GTTGGGATATTCTGGAGGTGATATGATTGCAATCTTGGAGAGTCGGGGGATAAAAACAAAAATAACTCATGTTGATCTTCCCGGG
AAATTCATCATCCTAAGTATCTCATACCCAACTTTATCAGAAGTCAAGGGGGTTATAGTCCACAGACTGGAAGCAGTTTCTTACAA
CATAAGATCACAAGAGTGGTACACCACTGTCCCGAGGTATATTGCAACTAATGGTTACTTAATATCTAATTTTGATGAGTCATCTTG
TGTATTCGTCTCAGGGTCAGCCATTTGTAGCCAGAACTCCCTGTATCCCATGAGCCCACTCTTACAACAATGTATTAGGGGCGAC
ACTTCATCTTGTGCTCGGACCTTGGTATCTGGGACTATGGGCAACAAATTTATTCTGTCAAAAGGTAATATCGTCGCAAATTGTGC
TTCTATACTATGTAAGTGTTATAGCACAAGCACAATTATTAATCAGAGTCCTGATAAGTTGCTGACATTTATTGCCTCCGATACCTG
CCCACTGGTTGAAATAGATGGTGTTACTATCCAAGTTGGAGGCAGGCAATACCCTGATATGGTATACGAAGGCAAAGTTGCCTTA
GGCCCTGCTATATCACTTGAGAGGTTAGATGTAGGTACAAACTTAGGGAAC

BKK/QT, 906 nucleotides
ATTAGGGAGGCCACCCAAGAAACCGTCATTGCCGTTCAGGGAGTCCAGGACTACGTCAACAACGAACTCGTCCCTGCCATGCA
ACATATGTCATGTGAATTAGTTGGGCAGAGATTAGGGTTAAAACTGCTTCGGTATTATACTGAGTTGTTGTCAATATTTGGCCCGA
GTTTACGTGACCCTATTTCAGCCGAGATATCAATTCAGGCACTGAGTTATGCTCTTGGAGGAGAAATTCATAAGATACTTGAGAA
GTTGGGATATTCTGGAGGTGATATGATTGCAATCTTGGAGAGTCGGGGGATAAAAACAAAAATAACTCATGTTGATCTCCCCGGG
AAATTCATCATCCTAAGTATCTCATACCCAACTTTATCAGAAGTCAAGGGGGTTATAGTCCACAGACTGGAACCAGTTTCTTACAA
CATAGGATCACAAGAGTGGTACACCACTGTCCCGAGGTATATTGCAACTAATGGTTACTTAATATCTAATTTTGATGAGTCATCTT
GTGTATTCGTCTCAGAGTCAGCCATTTGTAGCCAGAACTCCCTGTACCCCATGAGCCCACTCTTACAACAATGTATTAGGGGCGA
CACTTCATCTTGTGCTCGGACCTTGGTATCTGGGACTATGGGCAACAAATTTATTCTGTCAAAAGGTAATATCGTCGCAAATTGTG
CTTCTATACTATGTAAGTGTTATAGCACAAGCACAATTATTAATCAGAGTCCTGATAAGTTGCTGACATTTATTGCCTCCGATACCT
GCCCACTGGTTGAAATAGATGGTGTTACTATCCAAGTTGGAGGCAGGCAATACCCTGATATGGTATACGAAGGCAAAGTTGCCT
TAGGCCCTGCTATATCACTTGAGAGGTTAGATGTAGGTACAAATTTAGGGAAC

BKK/TTD, 906 nucleotides
ATTAGGGAGGCTACCCAAGAAACCGTCATTGCCGTTCAGGGAGTCCAGGACTACGTCAACAACGAACTCGTCCCTGCCATGCA
ACATATGTCATGTGAATTAGTTGGGCAGAGATTAGGGTTAAGACTGCTTCGGTATTATACTGAGTTGTTGTCAATATTTGGCCCGA
GTTTACGTGACCCTATTTCAGCCGAGATATCAATTCAGGCACTGATTTATGCTCTTGGAGGAGAAATTCATAAGATACTTGAGAAG
TTGGGATATTCTGGAAGTGATATGATTGCAATCTTGGAGAGTCGGGGGATAAAAACAAAAATAACTCATGTTGATCTTCCCGGGA
AATTCATCATCCTAAGTATCTCATACCCAACTTTATCAGAAGTCAAGGGGGTTATAGTCCACAGACTGGAAGCAGTTTCTTACAAC
ATAGGATCACAAGAGTGGTACACCACTGTCCCGAGGTATATTGCAACTAATGGTTACTTAATATCTAATTTTGATGAGTCATCTTGT
GTATTCGTCTCAGAGTCAGCCATTTGTAGCCAGAACTCCCTGTATCCCATGAGCCCACTCTTACAACAATGTATTAGGGGCGACA
CTTCATCTTGTGCTCGGACCTTGGTATCTGGGACTATGGGCAACAAATTTATTCTGTCAAAAGGTAATATCGTCGCAAATTGTGCT
TCTATACTATGTAAGTGTTATAGCACAAGCACAATTATTAATCAGAGTCCTGATAAGTTGCTGACATTCATTGCCTCCGATACCTGC
CCACTGGTTGAAATAGATGGTGCTACTATCCAAGTTGGAGGCGGGCAATACCCTGATATGGTATACGAAGGCAAAGTTGCCTTA
GGCCCTGCTATATCACTTGAGAGGTTAGATGTAGGTACAAATTTAGGGAAC

BKK/VG, 906 nucleotides
ATTAGGGAGGCAACCCAGGAAACCGTCATTGCCGTTCAGGGAGTCCAGGATTACGTCAATAATGAACTTGTCCCTGCTATGCAA
CATATGTCGTGTGAATTAGTTGGGCAGAGATTAGGGTTAAAACTGCTTAGGTATTATACCGAGTTGTTGTCAATATTTGGCCCGAG
TTTACGTGACCCTATTTCAGCCGAGATATCAATTCAAGCACTGAGTTATGCCCTTGGGGGAGAAATTCATAAGATACTTGAGAAGT
TGGGATATTCTGGAAATGATATGATTGCAATTTTGGAGAGTCGGGGGATAAAAACAAAAATAACTCATGTTGATCTCCCCGGGAA
ACTCATCATCTTAAGTATCTCATACCCAACTTTATCAGAAGTCAAGGGGGTTATAGTCCACAGACTGGAAGCAGTTTCTTATAACA
TAGGGTCACAGGAGTGGTACACCACTGTCCCGAGGTATGTTGCAACTAATGGTTACTTAATATCTAATTTTGATGAGTCATCCTGT
GTATTCGTCTCAGAATCAGCCATTTGTAGCCAGAACTCCCTATACCCCATGAGCCCGCTTCTACAACAATGCATTAGGGGCGAC
ACTTCATCTTGTGCTCGGACCTTGGTGTCTGGGACTATGGGCAACAAGTTTATTCTGTCAAAAGGTAATATCGTCGCAAATTGTGC
TTCTATACTATGTAAGTGTTATAGCACAAGCACAATTATCAATCAGAGTCCTGATAAGTTGCTGACATTTATTGCCTCCGATACCTG
CCCACTGGTTGAAATAGATGGTGTAACTATCCAGGTTGGAGGGAGGCAATACCCTGATATGGTATACGAAAGCAAAGTTGCCTT
AGGCCCTGCTATATCACTTGAGAGGTTAGATGTAGGTACAAATTTAGGAAAC

Appendix C: Homology of nucleotide sequences

Homology of nucleotide sequences of P gene

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
		BKK02/11	BKK03/11	BKK05/10	BKK06/10	BKK04/11	BKK01/11	BKK05/11	BKK/QT	BKK/TTD	BKK/CG	BKK/VG	BKK09/11	BKK06/11	BKK07/11	BKK10/11	BKK08/11	BKKZ01/11	BKKZ02/11	BKKZ03/11	BKKZ04/11	BKKZ05/11	BKKZ06/11	BKKZ07/11
1	BKK02/11																							
2	BKK03/11	1																						
3	BKK05/10	0.986	0.986																					
4	BKK06/10	0.997	0.997	0.989																				
5	BKK04/11	0.997	0.997	0.989	1																			
6	BKK01/11	0.948	0.948	0.945	0.95	0.95																		
7	BKK05/11	0.997	0.997	0.989	1	1	0.95																	
8	BKK/QT	0.953	0.953	0.939	0.95	0.95	0.934	0.95																
9	BKK/TTD	0.953	0.953	0.945	0.958	0.958	0.939	0.958	0.989															
10	BKK/CG	0.945	0.945	0.937	0.948	0.948	0.937	0.948	0.98	0.991														
11	BKK/VG	0.978	0.978	0.989	0.98	0.98	0.984	0.98	0.984	0.989	0.961													
12	BKK09/11	0.997	0.997	0.989	1	1	0.95	1	0.95	0.956	0.948	0.98												
13	BKK06/11	0.961	0.961	0.959	0.964	0.964	0.989	0.964	0.942	0.948	0.939	0.972	0.984											
14	BKK07/11	0.948	0.948	0.945	0.95	0.95	0.989	0.95	0.934	0.939	0.937	0.984	0.95	0.969										
15	BKK10/11	0.972	0.972	0.964	0.975	0.975	0.931	0.975	0.931	0.937	0.928	0.958	0.975	0.939	0.928									
16	BKK08/11	0.994	0.994	0.986	0.997	0.997	0.948	0.997	0.948	0.953	0.945	0.978	0.997	0.961	0.948	0.972								
17	BKKZ01/11	0.994	0.994	0.986	0.997	0.997	0.948	0.997	0.953	0.959	0.95	0.978	0.997	0.961	0.948	0.978	0.994							
18	BKKZ02/11	0.994	0.994	0.986	0.997	0.997	0.948	0.997	0.953	0.959	0.95	0.978	0.997	0.961	0.948	0.978	0.994	1						
19	BKKZ03/11	0.994	0.994	0.986	0.997	0.997	0.948	0.997	0.953	0.959	0.95	0.978	0.997	0.961	0.948	0.978	0.994	1	1					
20	BKKZ04/11	0.994	0.994	0.986	0.997	0.997	0.948	0.997	0.953	0.959	0.95	0.978	0.997	0.961	0.948	0.978	0.994	1	1	1				
21	BKKZ05/11	0.994	0.994	0.986	0.997	0.997	0.948	0.997	0.953	0.959	0.95	0.978	0.997	0.961	0.948	0.978	0.994	1	1	1	1			
22	BKKZ06/11	0.994	0.994	0.986	0.997	0.997	0.948	0.997	0.953	0.959	0.95	0.978	0.997	0.961	0.948	0.978	0.994	1	1	1	1	1		
23	BKKZ07/11	0.994	0.994	0.986	0.997	0.997	0.948	0.997	0.953	0.959	0.95	0.978	0.997	0.961	0.948	0.978	0.994	1	1	1	1	1	1	
24	BKKZ08/11	0.959	0.959	0.95	0.961	0.961	0.917	0.961	0.918	0.923	0.915	0.942	0.961	0.928	0.912	0.977	0.959	0.984	0.984	0.984	0.984	0.984	0.984	0.984
25	BKKZ10/11	0.994	0.994	0.986	0.997	0.997	0.948	0.997	0.953	0.959	0.95	0.978	0.997	0.961	0.948	0.978	0.994	1	1	1	1	1	1	1
26	BKKZ11/11	0.994	0.994	0.986	0.997	0.997	0.948	0.997	0.953	0.959	0.95	0.978	0.997	0.961	0.948	0.978	0.994	1	1	1	1	1	1	1
27	BKKZ12/11	0.994	0.994	0.986	0.997	0.997	0.948	0.997	0.953	0.959	0.95	0.978	0.997	0.961	0.948	0.978	0.994	1	1	1	1	1	1	1
28	BKKZ13/11	0.994	0.994	0.986	0.997	0.997	0.948	0.997	0.953	0.959	0.95	0.978	0.997	0.961	0.948	0.978	0.994	1	1	1	1	1	1	1
29	BKKZ09/11	0.994	0.994	0.986	0.997	0.997	0.948	0.997	0.953	0.959	0.95	0.978	0.997	0.961	0.948	0.978	0.994	1	1	1	1	1	1	1
30	BKK02/09	0.948	0.948	0.945	0.95	0.95	0.989	0.95	0.934	0.939	0.937	0.984	0.95	0.969	0.989	0.928	0.948	0.948	0.948	0.948	0.948	0.948	0.948	0.948
31	BKK01/09	0.95	0.95	0.948	0.953	0.953	0.991	0.953	0.937	0.942	0.939	0.987	0.953	0.972	0.991	0.928	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95
32	BKK03/10	0.953	0.953	0.95	0.958	0.958	0.983	0.958	0.939	0.945	0.942	0.989	0.958	0.975	0.983	0.931	0.953	0.953	0.953	0.953	0.953	0.953	0.953	0.953
33	BKK03/09	0.997	0.997	0.989	1	1	0.95	1	0.95	0.956	0.948	0.98	1	0.964	0.95	0.975	0.997	0.997	0.997	0.997	0.997	0.997	0.997	0.997
34	BKK01/10	0.948	0.948	0.945	0.95	0.95	0.989	0.95	0.934	0.939	0.937	0.984	0.95	0.969	0.989	0.928	0.948	0.948	0.948	0.948	0.948	0.948	0.948	0.948
35	BKK02/10	0.997	0.997	0.989	1	1	0.95	1	0.95	0.956	0.948	0.98	1	0.964	0.95	0.975	0.997	0.997	0.997	0.997	0.997	0.997	0.997	0.997
36	BKK05/09	0.997	0.997	0.989	1	1	0.95	1	0.95	0.956	0.948	0.98	1	0.964	0.95	0.975	0.997	0.997	0.997	0.997	0.997	0.997	0.997	0.997
37	BKK04/09	0.948	0.948	0.945	0.95	0.95	0.989	0.95	0.934	0.939	0.937	0.984	0.95	0.969	0.989	0.928	0.948	0.948	0.948	0.948	0.948	0.948	0.948	0.948
38	Onderste	0.95	0.95	0.942	0.953	0.953	0.942	0.953	0.986	0.997	0.989	0.987	0.953	0.95	0.942	0.934	0.95	0.956	0.956	0.956	0.956	0.956	0.956	0.956
39	Snyder hill	0.95	0.95	0.937	0.948	0.948	0.931	0.948	0.989	0.975	0.967	0.958	0.948	0.945	0.931	0.928	0.945	0.95	0.95	0.95	0.95	0.95	0.95	0.95
41	00-2601	0.959	0.959	0.956	0.961	0.961	0.95	0.961	0.961	0.967	0.959	0.975	0.961	0.959	0.95	0.942	0.959	0.964	0.964	0.964	0.964	0.964	0.964	0.964
42	AT6/17	0.972	0.972	0.964	0.975	0.975	0.959	0.975	0.959	0.964	0.956	0.989	0.975	0.967	0.959	0.95	0.972	0.972	0.972	0.972	0.972	0.972	0.972	0.972
43	Hamamat	0.994	0.994	0.986	0.997	0.997	0.948	0.997	0.948	0.953	0.945	0.978	0.997	0.961	0.948	0.972	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994
44	Th3	0.989	0.989	0.98	0.991	0.991	0.948	0.991	0.948	0.953	0.945	0.978	0.991	0.961	0.948	0.967	0.989	0.989	0.989	0.989	0.989	0.989	0.989	0.989
45	Th14	0.994	0.994	0.986	0.997	0.997	0.948	0.997	0.948	0.953	0.945	0.978	0.997	0.961	0.948	0.972	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994
46	007Lm	0.959	0.959	0.95	0.961	0.961	0.95	0.961	0.95	0.956	0.948	0.975	0.961	0.953	0.95	0.937	0.959	0.959	0.959	0.959	0.959	0.959	0.959	0.959
47	Th270Br	0.953	0.953	0.95	0.958	0.958	0.983	0.958	0.939	0.945	0.942	0.989	0.958	0.975	0.983	0.931	0.953	0.953	0.953	0.953	0.953	0.953	0.953	0.953
48	Th290Br	0.953	0.953	0.95	0.958	0.958	0.983	0.958	0.945	0.95	0.948	0.989	0.958	0.975	0.983	0.931	0.953	0.953	0.953	0.953	0.953	0.953	0.953	0.953
49	VacX	0.978	0.978	0.989	0.98	0.98	0.984	0.98	0.984	0.989	0.961	1	0.98	0.972	0.984	0.956	0.978	0.978	0.978	0.978	0.978	0.978	0.978	0.978

24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	41	42	43	44	45	46	47	48	49
BKKZ08/11	BKKZ10/11	BKKZ11/11	BKKZ12/11	BKKZ13/11	BKKZ09/11	BKK02/09	BKK01/09	BKK03/10	BKK03/09	BKK01/10	BKK02/10	BKK05/09	BKK04/09	Onderste	Snyder hill	00-2601	A75/17	Hamamat	Th3	Th14	007Lm	Th270Br	Th290Br	VacX
0.984																								
0.984	1																							
0.984	1	1																						
0.984	1	1	1																					
0.984	1	1	1	1																				
0.912	0.948	0.948	0.948	0.948	0.948																			
0.915	0.95	0.95	0.95	0.95	0.95	0.997																		
0.918	0.953	0.953	0.953	0.953	0.953	0.983	0.986																	
0.961	0.997	0.997	0.997	0.997	0.997	0.95	0.953	0.958																
0.912	0.948	0.948	0.948	0.948	0.948	0.989	0.991	0.983	0.95															
0.961	0.997	0.997	0.997	0.997	0.997	0.95	0.953	0.958	1	0.95														
0.961	0.997	0.997	0.997	0.997	0.997	0.95	0.953	0.958	1	0.95	1													
0.912	0.948	0.948	0.948	0.948	0.948	1	0.997	0.983	0.95	0.989	0.95	0.95												
0.92	0.958	0.958	0.958	0.958	0.958	0.942	0.945	0.948	0.953	0.942	0.953	0.953	0.942											
0.915	0.95	0.95	0.95	0.95	0.95	0.931	0.934	0.937	0.948	0.931	0.948	0.948	0.931	0.978										
0.926	0.984	0.984	0.984	0.984	0.984	0.95	0.953	0.958	0.961	0.95	0.961	0.961	0.95	0.984	0.959									
0.937	0.972	0.972	0.972	0.972	0.972	0.959	0.961	0.964	0.975	0.959	0.975	0.975	0.959	0.961	0.95	0.989								
0.959	0.994	0.994	0.994	0.994	0.994	0.948	0.95	0.953	0.997	0.948	0.997	0.997	0.948	0.95	0.945	0.959	0.972							
0.953	0.989	0.989	0.989	0.989	0.989	0.948	0.95	0.953	0.991	0.948	0.991	0.991	0.948	0.95	0.95	0.959	0.972	0.989						
0.959	0.994	0.994	0.994	0.994	0.994	0.948	0.95	0.953	0.997	0.948	0.997	0.997	0.948	0.95	0.95	0.959	0.972	0.994	0.994					
0.923	0.959	0.959	0.959	0.959	0.959	0.95	0.953	0.95	0.961	0.945	0.961	0.961	0.95	0.953	0.942	0.958	0.969	0.959	0.959	0.959				
0.918	0.953	0.953	0.953	0.953	0.953	0.983	0.986	0.989	0.958	0.983	0.958	0.958	0.983	0.948	0.942	0.958	0.964	0.953	0.958	0.958	0.95			
0.918	0.953	0.953	0.953	0.953	0.953	0.983	0.986	0.989	0.958	0.983	0.958	0.958	0.983	0.953	0.947	0.958	0.964	0.953	0.958	0.958	0.95	0.994		
0.942	0.978	0.978	0.978	0.978	0.978	0.984	0.987	0.989	0.98	0.984	0.98	0.98	0.984	0.987	0.958	0.975	0.989	0.978	0.978	0.978	0.975	0.989	0.989	

Homology of nucleotide sequences of H gene

	1 BKKZ01/11	2 BKKZ02/11	3 BKKZ06/11	4 BKKZ13/11	5 BKK10/11	6 BKK12/11	7 BKK05/10	8 BKK06/10	9 BKK07/11	10 BKK/QT	11 BKK/VG	12 BKK02/09	13 BKK01/09	14 BKK03/10	15 BKK03/09	16 BKK01/10	17 BKK02/10
1 BKKZ01/11																	
2 BKKZ02/11	1																
3 BKKZ06/11	1	1															
4 BKKZ13/11	0.992	0.992	0.992														
5 BKK10/11	0.999	0.999	0.999	0.992													
6 BKK12/11	0.973	0.973	0.973	0.974	0.972												
7 BKK05/10	0.981	0.981	0.981	0.974	0.98	0.983											
8 BKK06/10	0.994	0.994	0.994	0.988	0.994	0.974	0.981										
9 BKK07/11	0.935	0.935	0.935	0.928	0.935	0.925	0.932	0.934									
10 BKK/QT	0.911	0.911	0.911	0.904	0.91	0.902	0.906	0.908	0.908								
11 BKK/VG	0.954	0.954	0.954	0.946	0.953	0.944	0.953	0.954	0.959	0.926							
12 BKK02/09	0.936	0.936	0.936	0.929	0.935	0.926	0.933	0.935	0.995	0.908	0.96						
13 BKK01/09	0.935	0.935	0.935	0.928	0.935	0.925	0.932	0.934	0.994	0.908	0.959	0.998					
14 BKK03/10	0.935	0.935	0.935	0.928	0.935	0.925	0.932	0.934	0.987	0.906	0.959	0.99	0.989				
15 BKK03/09	0.986	0.986	0.986	0.979	0.985	0.968	0.975	0.986	0.94	0.915	0.961	0.941	0.94	0.94			
16 BKK01/10	0.935	0.935	0.935	0.928	0.935	0.925	0.932	0.934	0.994	0.909	0.959	0.996	0.995	0.987	0.94		
17 BKK02/10	0.99	0.99	0.99	0.983	0.99	0.97	0.978	0.99	0.933	0.906	0.952	0.935	0.933	0.934	0.982	0.933	
18 BKK05/09	0.987	0.987	0.987	0.98	0.987	0.97	0.978	0.987	0.932	0.906	0.95	0.932	0.932	0.932	0.979	0.932	0.983
19 BKK04/09	0.936	0.936	0.936	0.929	0.935	0.925	0.932	0.935	0.994	0.908	0.959	0.996	0.996	0.987	0.941	0.994	0.933
20 Onderste	0.906	0.906	0.906	0.899	0.905	0.898	0.903	0.904	0.906	0.982	0.923	0.906	0.906	0.904	0.912	0.907	0.902
21 Convac	0.912	0.912	0.912	0.904	0.911	0.901	0.906	0.909	0.908	0.988	0.926	0.908	0.908	0.906	0.916	0.909	0.906
22 Synder hill	0.909	0.909	0.909	0.902	0.908	0.897	0.904	0.908	0.905	0.972	0.925	0.906	0.906	0.905	0.913	0.906	0.905
23 00-2601	0.94	0.94	0.94	0.933	0.939	0.93	0.936	0.937	0.939	0.935	0.961	0.943	0.941	0.942	0.946	0.94	0.936
24 A75/17	0.953	0.953	0.953	0.946	0.952	0.946	0.954	0.953	0.959	0.926	0.98	0.962	0.96	0.961	0.965	0.959	0.953
25 GR88	0.938	0.938	0.938	0.931	0.937	0.931	0.94	0.937	0.935	0.923	0.957	0.935	0.935	0.935	0.941	0.936	0.937
26 H05Bp7F	0.933	0.933	0.933	0.926	0.932	0.923	0.932	0.933	0.932	0.919	0.954	0.934	0.932	0.932	0.936	0.934	0.934
27 Liud	0.933	0.933	0.933	0.926	0.932	0.921	0.932	0.932	0.929	0.919	0.952	0.93	0.929	0.929	0.935	0.93	0.932
28 Yanaka	0.976	0.976	0.976	0.968	0.975	0.966	0.972	0.976	0.94	0.911	0.961	0.941	0.94	0.94	0.972	0.94	0.973
29 007Lm	0.927	0.927	0.927	0.921	0.926	0.917	0.928	0.926	0.927	0.907	0.948	0.929	0.927	0.928	0.932	0.928	0.926
30 Seoul	0.921	0.921	0.921	0.915	0.921	0.915	0.923	0.921	0.921	0.906	0.941	0.92	0.919	0.919	0.927	0.92	0.921
31 HLJ1	0.892	0.892	0.892	0.886	0.891	0.886	0.894	0.891	0.89	0.875	0.908	0.89	0.889	0.889	0.896	0.89	0.891
32 Th270BR	0.937	0.937	0.937	0.93	0.936	0.925	0.934	0.935	0.992	0.907	0.959	0.993	0.992	0.986	0.939	0.992	0.934
33 Th270LU	0.937	0.937	0.937	0.93	0.936	0.925	0.934	0.935	0.992	0.907	0.959	0.993	0.992	0.986	0.939	0.992	0.934
34 DK91	0.948	0.948	0.948	0.941	0.947	0.938	0.95	0.946	0.95	0.923	0.97	0.949	0.948	0.948	0.951	0.948	0.945
35 VacX	0.955	0.955	0.955	0.948	0.954	0.945	0.954	0.955	0.961	0.927	0.996	0.961	0.961	0.961	0.962	0.96	0.954

18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
BKK05/09	BKK04/09	Onderste	Convac	Synder hill	00-2601	A75/17	GR88	H05Bp7F	Liud	Yanaka	007Lm	Seoul	HLJ1	Th270BR	Th270LU	DK91	VacX
0.932																	
0.903	0.906																
0.906	0.908	0.981															
0.905	0.906	0.965	0.972														
0.934	0.94	0.93	0.935	0.927													
0.949	0.959	0.924	0.926	0.922	0.971												
0.934	0.935	0.919	0.924	0.922	0.942	0.956											
0.93	0.932	0.915	0.919	0.919	0.94	0.954	0.974										
0.928	0.929	0.916	0.923	0.917	0.94	0.951	0.97	0.978									
0.97	0.94	0.907	0.912	0.908	0.941	0.96	0.941	0.938	0.935								
0.921	0.926	0.904	0.908	0.906	0.936	0.947	0.934	0.936	0.931	0.932							
0.919	0.919	0.902	0.906	0.904	0.927	0.941	0.932	0.932	0.929	0.926	0.97						
0.888	0.889	0.874	0.875	0.873	0.897	0.908	0.901	0.901	0.897	0.898	0.936	0.943					
0.934	0.992	0.905	0.907	0.906	0.939	0.958	0.935	0.934	0.93	0.94	0.926	0.921	0.89				
0.934	0.992	0.905	0.907	0.906	0.939	0.958	0.935	0.934	0.93	0.94	0.926	0.921	0.89	1			
0.943	0.948	0.918	0.923	0.921	0.955	0.97	0.952	0.946	0.946	0.954	0.939	0.933	0.899	0.948	0.948		
0.951	0.961	0.924	0.928	0.926	0.962	0.981	0.959	0.956	0.954	0.962	0.949	0.943	0.909	0.96	0.96	0.972	

Homology of nucleotide sequences of F gene

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
		BKK02/11	BKK/CG	BKK01/11	BKKZ03/11	BKK05/10	BKK03/11	BKK11/11	BKKZ05/11	BKKZ06/11	BKKZ07/11	BKKZ09/11	BKKZ10/11	BKKZ11/11	BKKZ12/11	BKKZ13/11	BKK07/11	BKK04/10	BKK06/10	BKK04/11	BKK/QT
1	BKK02/11																				
2	BKK/CG	0.935																			
3	BKK01/11	0.961	0.931																		
4	BKKZ03/11	0.991	0.935	0.959																	
5	BKK05/10	0.986	0.94	0.96	0.984																
6	BKK03/11	1	0.935	0.961	0.991	0.986															
7	BKK11/11	0.991	0.935	0.959	1	0.984	0.991														
8	BKKZ05/11	0.991	0.935	0.959	1	0.984	0.991	1													
9	BKKZ06/11	0.991	0.935	0.959	1	0.984	0.991	1	1												
10	BKKZ07/11	0.991	0.935	0.959	1	0.984	0.991	1	1	1											
11	BKKZ09/11	0.991	0.935	0.959	1	0.984	0.991	1	1	1	1										
12	BKKZ10/11	0.99	0.937	0.958	0.998	0.985	0.99	0.998	0.998	0.998	0.998	0.998									
13	BKKZ11/11	0.991	0.935	0.959	1	0.984	0.991	1	1	1	1	1	0.998								
14	BKKZ12/11	0.991	0.935	0.959	1	0.984	0.991	1	1	1	1	1	0.998	1							
15	BKKZ13/11	0.991	0.935	0.959	1	0.984	0.991	1	1	1	1	1	0.998	1	1						
16	BKK07/11	0.961	0.933	0.995	0.959	0.96	0.961	0.959	0.959	0.959	0.959	0.959	0.958	0.959	0.959	0.959					
17	BKK04/10	0.988	0.933	0.956	0.986	0.982	0.988	0.986	0.986	0.986	0.986	0.986	0.985	0.986	0.986	0.986	0.956				
18	BKK06/10	1	0.935	0.961	0.991	0.986	1	0.991	0.991	0.991	0.991	0.991	0.99	0.991	0.991	0.991	0.961	0.988			
19	BKK04/11	0.996	0.939	0.964	0.994	0.99	0.996	0.994	0.994	0.994	0.994	0.994	0.993	0.994	0.994	0.994	0.964	0.992	0.996		
20	BKK/QT	0.941	0.991	0.937	0.941	0.945	0.941	0.941	0.941	0.941	0.941	0.941	0.942	0.941	0.941	0.941	0.939	0.937	0.941	0.944	
21	BKK06/11	0.956	0.929	0.977	0.954	0.953	0.956	0.954	0.954	0.954	0.954	0.954	0.953	0.954	0.954	0.954	0.977	0.952	0.956	0.96	0.934
22	BKK08/11	0.996	0.939	0.964	0.994	0.99	0.996	0.994	0.994	0.994	0.994	0.994	0.993	0.994	0.994	0.994	0.964	0.992	0.996	1	0.944
23	BKK/TTD	0.836	0.887	0.828	0.836	0.838	0.836	0.836	0.836	0.836	0.836	0.836	0.837	0.836	0.836	0.836	0.831	0.833	0.836	0.838	0.884
24	BKK09/11	0.998	0.934	0.96	0.99	0.985	0.998	0.99	0.99	0.99	0.99	0.99	0.988	0.99	0.99	0.99	0.96	0.987	0.998	0.995	0.94
25	BKK/VG	0.974	0.952	0.972	0.972	0.976	0.974	0.972	0.972	0.972	0.972	0.972	0.973	0.972	0.972	0.972	0.972	0.972	0.974	0.977	0.958
26	BKK05/11	0.859	0.803	0.825	0.853	0.848	0.859	0.853	0.853	0.853	0.853	0.853	0.852	0.853	0.853	0.853	0.825	0.852	0.859	0.857	0.807
27	BKK02/09	0.963	0.933	0.995	0.961	0.962	0.963	0.961	0.961	0.961	0.961	0.961	0.96	0.961	0.961	0.961	0.995	0.959	0.963	0.966	0.939
28	BKK01/09	0.962	0.932	0.996	0.96	0.961	0.962	0.96	0.96	0.96	0.96	0.96	0.959	0.96	0.96	0.96	0.996	0.958	0.962	0.965	0.938
29	BKK03/10	0.959	0.931	0.988	0.956	0.958	0.959	0.956	0.956	0.956	0.956	0.956	0.955	0.956	0.956	0.956	0.988	0.954	0.959	0.962	0.937
30	BKK03/09	1	0.935	0.961	0.991	0.986	1	0.991	0.991	0.991	0.991	0.991	0.99	0.991	0.991	0.991	0.961	0.988	1	0.996	0.941
31	BKK01/10	0.964	0.934	0.996	0.962	0.963	0.964	0.962	0.962	0.962	0.962	0.962	0.961	0.962	0.962	0.962	0.996	0.96	0.964	0.967	0.94
32	BKK02/10	0.99	0.932	0.958	0.987	0.983	0.99	0.987	0.987	0.987	0.987	0.987	0.986	0.987	0.987	0.987	0.958	0.994	0.99	0.993	0.938
33	BKK05/09	0.994	0.937	0.964	0.992	0.987	0.994	0.992	0.992	0.992	0.992	0.992	0.991	0.992	0.992	0.992	0.962	0.99	0.994	0.997	0.942
34	BKK04/09	0.962	0.932	0.996	0.96	0.961	0.962	0.96	0.96	0.96	0.96	0.96	0.959	0.96	0.96	0.96	0.996	0.958	0.962	0.965	0.938
35	Onderste	0.937	0.991	0.932	0.937	0.943	0.937	0.937	0.937	0.937	0.937	0.937	0.938	0.937	0.937	0.937	0.934	0.932	0.937	0.94	0.993
36	00-2601	0.961	0.956	0.956	0.961	0.963	0.961	0.961	0.961	0.961	0.961	0.961	0.962	0.961	0.961	0.961	0.959	0.956	0.961	0.964	0.96
37	A75/17	0.965	0.943	0.963	0.966	0.97	0.965	0.965	0.965	0.965	0.965	0.965	0.966	0.965	0.965	0.965	0.963	0.961	0.965	0.969	0.949
38	Ac961	0.985	0.939	0.958	0.983	0.99	0.985	0.983	0.983	0.983	0.983	0.983	0.984	0.983	0.983	0.983	0.958	0.981	0.985	0.988	0.944
39	007Lm	0.945	0.937	0.945	0.943	0.95	0.945	0.943	0.943	0.943	0.943	0.943	0.944	0.943	0.943	0.943	0.945	0.941	0.945	0.949	0.94
40	GN	0.984	0.938	0.958	0.982	0.993	0.984	0.982	0.982	0.982	0.982	0.982	0.983	0.982	0.982	0.982	0.958	0.98	0.984	0.987	0.943
41	VacX	0.962	0.94	0.96	0.96	0.984	0.962	0.96	0.96	0.96	0.96	0.96	0.961	0.96	0.96	0.96	0.96	0.96	0.962	0.965	0.945

21 BKK06/11	22 BKK08/11	23 BKK/TTD	24 BKK09/11	25 BKK/VG	26 BKK05/11	27 BKK02/09	28 BKK01/09	29 BKK03/10	30 BKK03/09	31 BKK01/10	32 BKK02/10	33 BKK06/09	34 BKK04/09	35 Onderste	36 00-2601	37 A75/17	38 Ac981	39 007Lm	40 GN	41 VacX
0.96																				
0.83	0.838																			
0.956	0.995	0.835																		
0.967	0.977	0.85	0.973																	
0.824	0.857	0.9	0.86	0.839																
0.98	0.968	0.831	0.962	0.974	0.827															
0.979	0.965	0.83	0.961	0.973	0.826	0.998														
0.975	0.962	0.828	0.958	0.97	0.823	0.991	0.99													
0.956	0.996	0.836	0.998	0.974	0.859	0.963	0.962	0.959												
0.981	0.967	0.832	0.963	0.975	0.828	0.998	0.997	0.992	0.964											
0.953	0.993	0.833	0.988	0.973	0.852	0.96	0.959	0.955	0.99	0.961										
0.96	0.997	0.836	0.993	0.975	0.855	0.964	0.963	0.96	0.994	0.965	0.991									
0.979	0.965	0.83	0.961	0.973	0.826	0.998	1	0.99	0.962	0.997	0.959	0.963								
0.93	0.94	0.884	0.935	0.953	0.803	0.934	0.933	0.932	0.937	0.935	0.933	0.938	0.933							
0.956	0.964	0.854	0.96	0.977	0.825	0.959	0.958	0.959	0.961	0.96	0.958	0.962	0.958	0.96						
0.961	0.969	0.841	0.964	0.984	0.831	0.965	0.964	0.963	0.965	0.968	0.962	0.966	0.964	0.944	0.984					
0.953	0.988	0.837	0.984	0.975	0.847	0.96	0.959	0.955	0.985	0.961	0.982	0.986	0.959	0.942	0.962	0.966				
0.948	0.949	0.837	0.944	0.984	0.82	0.948	0.947	0.945	0.945	0.949	0.942	0.947	0.947	0.935	0.951	0.962	0.949			

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Miss Araya Radtanakatikanon was born on September 19, 1985 in Bangkok, Thailand. She graduated Bachelor Degree of Veterinary Science (DVM) with a first class honors in academic year 2009 from Faculty of Veterinary Science, Chulalongkorn University. She was granted by H.M. King Bhumibol Adulyadej's 72nd Birthday Anniversary Scholarship - Chulalongkorn University during studying for Master degree in Pathobiology program, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University. She is currently a staff of Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University.