CHAPTER V

DISCUSSION AND CONCLUSION

Discussion

Eventually, CDV can be divided in to 6 lineages but the clinical signs and pathologic lesion of respiratory, gastrointestinal and nervous system were not significantly different between each lineage. For the samples, the main clinical signs appeared in the respiratory and gastrointestinal system and a few dogs showed the nervous signs. The pathologic lesions were also presented in the respiratory and gastrointestinal systems. The routine histopathological diagnosis of the CDV lesions was difficult to observe on the brain section, while in some cases the immunohistochemistry demonstrated positive antigens in the astrocytes. This may be because of the phase of infection. After viremia, the virus arrived to the brain on day 17-20 after infection, then persistent infection occurred in the brain and nervous signs will show from day 21 forward (Greene and Appel, 2005). In other words, nervous signs or neurologic lesions should be found in subacute or chronic infection. For example, the wild-type virulent A75/17 strain typically produces a chronic demyelination associated with a persistent infection in the central nervous system. The antiviral immune response, which follows invasion of immune cells in the central nervous system, leads to viral clearance within the inflammatory lesion. However, this viral clearance is restricted to certain lesions and simultaneously; A75/17 strain has been shown to further spread in astrocytes in other areas of the brain without eliciting an inflammatory response. The persistent infection of A75/17 strain in central nervous system suggested to be in part determined by a defective immune response. In primary dog brain culture, wild-type CDV persistence was also associated with selective spread and very little virus release when compared to the Onderstepoort CDV vaccine strain, suggesting an important role also for viral factors in the establishment of persistent infection (Plattet et al., 2005).

Nucleotide and amino acid sequences of CDV have been studied for many years, in many countries throughout the world. This result from the re-emergence of the disease in the vaccinated populations after the vaccines had been invented. Those

influence the development of several techniques for advantage in studying CDV including cell lines and RT-PCR techniques, sequencing and phylogenetic analyses.

The cell lines had been developed for isolating viruses. The growing viruses in cell cultures allowed preparation of purified viruses for further proposes such as vaccine production. For CDV, the Vero cell, the kidney epithelial cell extracted from African green monkey, is the most used cell line for inoculation in the experiment. Later on, Vero cell expressed Human SLAM tag was invented and use for inoculation of the measles virus and adapted to use with CDV by expressed the Dog SLAM tag on the Vero cell (Vero-DST). This Vero-DST is the most efficient cell line to isolated CDV due to the early detected CPE and its stability. Its stability demonstrated that the nucleotide sequences of P, M and L genes did not change and a few change in amino acid of H gene after 20 passages. This made Vero-DST effective for inoculation, isolation, titration and further biological research due to its stability. (Lan et al., 2006[b]), and was developed to isolate wild-strain CDVs (Seki et al., 2003).

In this study, the viruses was isolated from fresh tissue samples in Vero-DST cell, but only 2 samples showed CPE. For CDV, the growth tropism and the fusogenic activities were regulated by H protein that interacts with F protein. The wild-type H gene induced very rare and small syncytia with limited virus production, compared to the attenuated CDV. These may lead the virus to cause persistent infection (Rivals et al., 2007).

The cytopatic effect of CDV depends on the degree of persistent infection of the virus. The receptor binding H protein of A75/17 which is represent the wild-type, neurovirulent and persistent CDV, induced very limited CPE, while the H protein of Onderstepoort strain which is represent the attenuated and non persistent CDV produced more pronounced cell to cell fusion. In addition, the persistence of cultured cells may be base on similar mechanism as persistence in animals. The wild-type CDV produces a persistent infection in the central nervous system, while it showed highly cytopathic in lymphoid organ which make the suggestive explanation that Vero-DST showed the CPE earlier. The reason to speculate that is because the presence or absence of SLAM may also determine cytolytic versus persistent infection and

unidentified low-binding affinity CDV receptor could be present in the CNS of dogs (Plattet et al., 2005).

The other samples that did not show the CPE, can be divided into 2 types, the first is only the homogenized samples that were positive for P gene RT-PCR, and the second is both the homogenized and the supernatant without CPE were positive for P gene RT-PCR. However, both evidences can be explained that the virus was already dead before starting the experiment and left some part of the nucleic acid in the supernatant, or the amount of virus in the supernatants were too little to produce the CPE.

All the samples were positive with P gene RT-PCR but only the two samples that showed CPE were positive with H gene RT-PCR. The positive P gene is because of 390 basepair P gene is part of conserve region. May be the virus left part of the nucleic acid and some of virus particle in the supernatant, although the virus was too little to grow in the cell culture or it was already dead. Therefore, this conserve region is benefit to screen the samples.

The H gene region is more complicated, because it has to bind with the host cell receptor as the same time as escape from the host defense mechanism. The high degree of H gene variation most probably reflects the role of H in binding to host cell receptor and this means that it is also the main target of host's humoral immune responses and neutralizing antibodies are mainly directed against the H protein. The amino acid changes in H that are potentially linked to the host's adaptive evolution, thus Morbilliviruses are located in B-cell epitopes and at sites linked to its interactions with the cell receptor (Barrett et al., 2006).

The negative of RT-PCR in H gene region may be because of the inappropriate H gene primers. As H gene is highly variation region, so it was difficult to find the sensitive and specific primer. To fill this gap, it had been suggested to design primer from the C-terminal of F gene as a forward primer and the N-terminal of L gene as a reverse primer. Because of F and L gene regions are the very conserve regions in CDV.

In the view of phylogenetic analyses, most researches focus on H gene, as H gene is the most variable region, the important attachment protein that bind with host cellular receptor and a main target of host's humoral immune response (Griffin and

Bellini, 1996). The fusogenicity begin with the H protein binds to the CDV receptor of the host cell membrane, and the F protein mediates the membrane fusion event, which allows the entry of the viral genome into the cytoplasm. The Onderstepoort cytolytic strain was demonstrated to spread through cell cultures both by producing infectious extracellular particles and by lateral cell to cell fusion (Prado et al., 2005).

Furthermore, the H gene of wild-type strain CDV has more potential glycosylation sites than the attenuated strains and has a higher apparent molecular weight which is consistent with increased oligosaccharide addition. It is possible that oligosaccharides on the CDV H protein may influence the strength of the interactions with cellular receptors. On the other hand, without significant altering receptor binding, the oligosaccharides could influence the extent of viral propagation by altering the fusion efficiency of the F-H protein complex expressed on infected cells (Von Messling et al., 2001). The number of potential N-glycosylation sites on H gene varying between and within each Morbilliviruses. These side chains are required to move the protein along the exocytic pathway to the cell membrane as well as influencing the antigenicity of the molecule. The degree of glycosylation may also affect virulence as has been shown for the haemagglutinin-neuraminidase (HN) protein of Newcastle disease virus (NDV) (Barrett et al., 2006).

The second most used CDV protein for phylogenetic analysis is the most conserve region, P protein, then the F and N gene also the conserve regions that have been used for analyzed. However, the nucleotide sequences of these 3 genes are not present in the Genbank as much as the H protein, because of it less interesting. On the other hand, this made these genes less frequently used for comparison because the reference strains in the Genbank were not enough for all lineages, and of course, it was impossible to compare the different genes.

The CDV phyogenetic analyses have been done in many parts of the world. In North America, Prado et al (2005) was analyzed from fresh tissues samples that were collected from necropsy cases, then analyzed by use the CDV H gene, P gene and F gene regions. The result showed that the new isolates CDV were distinct from the vaccine lineage, and two of the samples were in the same lineage with the CDV that isolated from a dog in Greenland, the Arctic lineage. One of the samples was closely

related to the CDV that was isolated from a Panda in China, a member of the Asia1 lineage. The last sample was closely related to the strain from a mink in Denmark, a member of Europe lineage. The result was in the same direction in H, P and F gene. In summary, the CDV strains detected in this study were genetically distinct from viruses previously detected in the continental United States and closely matched with strains from Asia or Europe. The author suggested that the CDV strains isolated from the infecting dogs in this study may have originated from non-canine species or may have been transmitted from dogs to the other species (Prado et al., 2005).

The research from Italy, Martella et al., 2006 showed that there were at least 3 different CDV lineages present in Italy, analyzed by H gene. Two of the lineages are Europe and Europe-wildlife which had been found circulating in Italy. Surprisingly, the third lineage is Arctic lineage, which is closely related to the virus isolated from dogs in Greenland and from seals in Lake Baikal. This may be an Arctic-related strain introduced by other dogs was imported to Italy from Eastern Europe or Northern Asia and spreaded throughout the canine population. This can be traced to the uncontrolled trading of low cost and high value breed pets that has been suggested in the last decades for Italy's case (Martella et al., 2006).

In Japan, there were two CDV lineages; Asia1 and Asia2 circulated in Japan, and both were not related with the vaccine lineage. One of the researches was carried out in the H and P gene regions. The samples were collected from vaccinated dogs, and isolated virus in Vero-DST cell line. The result of this research showed that the isolated viruses were closely related to the Asia1 lineage with both P and H gene region and many vaccinated dogs are still infected with CDV (Lan et al., 2006[a]). The possibility was considered that the vaccine efficacy was failed to have the intended result because of their poor quality or maternal immunity. Therefore, the existing of maternal immunity was eliminated because the dogs were checked for negative or low-level anti-CDV IgG and IgM before vaccination (Lan et al., 2006[a]).

In Thailand, Keawcharoen et al (2005) analyzed the N gene of CDV and suggested that Thai isolates could be divided in 2 groups; one was the group that closely related to vaccine strain (Onderstepoort) and the other one was homology to other new isolates strains in Genbank. The group that related to the vaccine may be

divided into 2 groups. The former group was isolated from the vaccinated dog, which could be related to the post-vaccination distemper encephalitis in puppies. The later one was from the unvaccinated group which can be explained in two reasons. Firstly, because of the dog had been exposed to the virus vaccine while kept among other vaccinated animals. Secondly is the dog might be infected with the wild-type CDV that closely related to the vaccine strain (Keawcharoen et al., 2005).

In 2007, Martella et al., have briefly divided CDV by 1824 bp H gene fragment in to 6 major genetic lineages; America-1 (vaccine strains), America-2, Asia-1, Asia-2, European and Arctic. Therefore, the CDV infected dogs in the vaccinated population have been explained in three explanations. First is the failure of vaccine, second is the reversion of attenuated CDV vaccine strains to virulence, and the third is the emergence of new strains that are sufficiently divergent to evade immune protection (Prado et al., 2005). Then, the emerging of new strains CDV is possibly by the uncontrolled trading dogs as discussed above and the interspecies transmission (Prado et al., 2005; Martella et al., 2006).

According to our study, the analyzed of H gene fragment of the isolates 270Brain and 270Lung were not categorized to any lineage, according above, and surely not join the vaccine lineage. In addition, the 390 bp P gene region of our isolates showed that they can be divided in to 2 groups, the first group was in the same lineage with Asia 1, the second group was distant from other virulent strains lineages and both groups are far from the vaccine lineage. The results of 270 BR and 270 LU, both H and P gene were shown in the same ways indicated that the isolates are not joining any found lineages including the vaccine.

However, the previous Thai CDV research used the N gene region and this study used the H and P genes to analyze with the other strains and it made the comparison not completely clear. Therefore, after we inquired the data, we found that number 3 of the isolate is the same sample as number 400 of the previous study which also closely related to the Asia-1 lineage. From the results, we suggested that there were 3 genotypes of CDV circulated in Thailand during year 2005 and 2007; the first was similar to the vaccine lineage, the second was join in the Asia-1 lineage and the third was independent from other lineage.

Therefore, the samples that joined Asia1 lineage were collected from the infected dogs in year 2001-2002, but the samples that were different from previously found lineages were collected in year 2006. In addition, our study used the RT-PCR products for sequencing, so the result of the third lineage should be confirmed by cloning the virus into vector and checking the sequences again. Furthermore, there are only 2 samples that are different from the other lineage, for this reason, more samples should be collected and further studied conducted.