Identification of the genetic attenuation-marker of canine parvovirus vaccine and methodological and epidemiological studies in canine serious infectious diseases

Summary of Doctoral Thesis

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Here, we describe basic studies for development and improvement of vaccines to prevent serious and fatal infectious diseases such as canine parvovirus (CPV) infection, canine distemper, as well as the highly incident canine infectious respiratory disease (CIRD) in owners of multiple dogs. Vaccines for these diseases have been developed and are widely used. However, many challenges remain in the development of more effective and safe vaccines. Regarding live attenuated vaccines for CPV infections, the genetic markers that define the attenuated phenotype have not yet been identified. Although reversion of virulence in a live attenuated CPV vaccine has never been demonstrated and the attenuation of virulence has been proven to be highly stable, concerns have arisen that enteric illnesses observed in animals following administration of a CPV vaccine may have resulted from vaccine reversion to a virulent form. It is important to clearly discriminate attenuated vaccine strains from wild-type strains to resolve this concern. Therefore, it is necessary for quality control of vaccines to determine the genetic markers that define the attenuated phenotype. Regarding canine distemper, evaluation of canine distemper virus (CDV) dynamics using quantitative reverse transcription and polymerase chain reaction (qRT-PCR) did not reveal any relationship to infection outcomes in experimentally infected dogs. Various methods have been applied to examine the CDV dynamics. CDV qRT-PCR is a rapid and handy method for the quantitative detection of CDV RNA in clinical specimens. However, there has been no report on changes in the CDV RNA load with time in infected dogs or the relationship between these changes and infectious outcomes. Further, the usefulness of this method under experimental infectious conditions has yet to be properly evaluated. Regarding CIRD, in Japan, studies on the surveillance of CIRD-causing pathogens have been limited. Although some research reports on individual CIRD pathogens were found in Japan, there are few reports on the recent field situation, particularly a comprehensive investigation of involvement of multiple pathogens,

including emerging pathogens. Additionally, vaccines are widely used for canine adenovirus type 2 (CAV-2), canine parainfluenza virus (CPIV), and CDV, but there are no reports comparing detection of each pathogen in vaccinated and unvaccinated dogs.

Based on the above background, in this study, we investigated genetic markers responsible for quality control of the CPV vaccine, evaluated the detection method of viral kinetics in experimentally infected dogs, which is important for development and improvement of canine distemper vaccine, and conducted an etiological investigation of CIRD in Japan. To this end, we identified genetic minimal determinants responsible for attenuation of CPV infection vaccine (Chapter 1), verified the usefulness of qRT-PCR in CDV experimental infection (Chapter 2), and conducted a pathogenetic investigation of CIRD in Japan (Chapter 3). In this paper, the results of these studies are summarized in three chapters.

Chapter 1

Identification of the minimal determinant for attenuation of the CPV vaccine strain 9985-46

CPV, which belongs to the genus *Protoparvovirus* in the family *Parvoviridae*, is an important canine pathogen that causes a severe, highly contagious gastroenteric disease in pups. CPV live attenuated vaccines have been widely used, and their usefulness has been confirmed. Identifying molecular determinants of virulence attenuation in the CPV vaccines is important to ensure their safety. To this end, we identified mutations in the attenuated CPV 9985-46 vaccine strain that arose during serial passage in Crandell-Rees feline kidney cells compared to their wild-type counterpart, as well as minimal determinants of virulence loss. Four amino acid substitutions (N93K, G300V, T389N, and V562L) in VP2 of strain 9985-46 significantly restricted infection in canine A72 cells. Using an infectious molecular clone system, we constructed isogenic CPVs of the

parental virulent 9985 strain carrying single or double mutations. We observed that only a single amino acid substitution in VP2, G300V or T389N, attenuated the virulent parental virus. Combinations of these mutations further attenuated CPV to a level comparable to that of 9985-46. Strains with G300V/T389N substitutions did not induce clinical symptoms in experimentally infected pups, and their ability to infect canine cells was highly restricted. We found that another G300V/V562L double mutation decreased affinity of the virus for canine cells, although its pathogenicity in dogs was maintained. These results indicate that mutation of residue 300, which plays a critical role in host tropism, is not sufficient for viral attenuation *in vivo*, and that attenuation of the 9985-46 strain is defined by at least two mutations in residues 300 and 389 of the VP2 capsid protein.

Chapter 2

Use of quantitative real-time RT-PCR to investigate CDV dynamics in experimentally infected dogs

CDV, which belongs to the genus *Morbillivirus* in the family *Paramyxoviridae*, is an essential canine pathogen, which causes a serious and often fatal disease. Dogs infected experimentally with field isolates of CDV commonly show diverse clinical symptoms and mild clinical features or subclinical infections. Thus, it is difficult to resolve and evaluate pathogenic differences among strains or to examine the protective efficacy of vaccines against field isolates. Although differences in the clinical responses of CDV-infected dogs have not been characterized, the degree of viral multiplication and spread throughout the body of infected dogs is considered a significant factor in CDV pathogenesis. Therefore, a sensitive and quantitative investigation of viremia and viral shedding in infected dogs is vital to understand CDV pathogenesis. We used qRT-PCR and virus titration to examine CDV kinetics in the peripheral blood and rectal

and nasal secretions of 12 experimentally infected dogs. qRT-PCR proved extremely sensitive, and the correlation between the two methods for rectal and nasal samples on the peak day of viral RNA was good. Although dogs showed diverse symptoms, the viral RNA kinetics were similar; specifically, the peak of viral RNA in the symptomatic dogs was consistent with the onset of symptoms. These results indicate that qRT-PCR is sufficiently sensitive to monitor CDV replication in experimentally infected dogs regardless of the degree of clinical manifestation. Further, it suggests that the peak of viral RNA reflects active CDV replication.

Chapter 3

Etiological investigation on CIRD in Japan

The oral, nasal, and ocular swabs collected from 119 dogs suffering from CIRD were tested for CAV-2, CPIV, CDV, canine herpes virus (CHV), canine respiratory coronavirus (CRCoV), and *Bordetella bronchiseptica* (Bb) genes by a polymerase chain reaction method. The most prominent pathogen detected in 47 dogs positive for a single pathogen was Bb followed by CRCoV, CPIV, CDV, CAV-2, and CHV, and they were found in 15, 13, 9, 6, 2, and 2 dogs, respectively. Similarly, Bb, CPIV, and CRCoV were more prominent in 16 dogs positive for plural pathogens, and they were detected in 13, 9, and 6 dogs, respectively. These appear to suggest that Bb, CPIV, and CRCoV are major pathogens to cause CIRD, and concomitant infection of Bb with other pathogens may exacerbate the disease. In addition, CPIV, CAV-2, and CDV were apt to be detected less frequently in dogs administered previously with the multivalent live vaccine, including those viruses than those unvaccinated, suggesting that the vaccine works effectively to prevent infection in dogs.

Overall,

1. The first study led to the identification of the minimal determinant

of attenuation as the genetic marker of the CPV vaccine strain 9985-46. This finding is relevant for quality control of the vaccine and provides insight into the rational design of second-generation live attenuated vaccine candidates.

2. The second study underlined the usefulness of qRT-PCR for investigating CDV dynamics in experimental infections. This method will be useful for comparing the multiplication and dissemination among different CDV strains and for determining the protective efficacy of vaccines.
3. The third study provided the occurrence of CIRD, which suggested that Bb, CPIV, and CRCoV are involved single or multiple infections. Further, the detection rates of CAV-2, CPIV, and CDV included in the current vaccine tended to be lower in vaccinated dogs as compared with unvaccinated dogs, indicating vaccine efficacy.

These findings obtained in this study provide useful and insightful information regarding the development of improved vaccines against important infectious canine diseases.