Distribution and Dynamics of Quasispecies Related with Bovine Viral Diarrhea Virus Infection

Summary of Doctoral Thesis

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Bovine viral diarrhea virus (BVDV) is an enveloped positive-stranded RNA virus classified into the genus *Pestivirus* within the family *Flaviviridae*. Disease induced by BVDV leads to significant economic losses for cattle producers worldwide. BVDV can be classified into cytopathogenic (CP) and noncytopathogenic (NCP) biotypes; most field-isolated BVDV strains are NCP. Only NCP virus can establish a persistent infection. Persistently infected (PI) calves continuously excrete large amounts of virus particles throughout their lives. Because they show few symptoms, PI calves are a continual source of infection in a herd. Furthermore, PI calves are at risk of developing fatal mucosal disease (MD). A pair of closely related NCP and CP viruses can be isolated from animals that have developed MD.

NCP BVDV can be further divided into two additional biotypes, one of which shows the exaltation of Newcastle disease virus phenomenon (END^+) and the other of which does not (END^-). END^- virus does however interfere with vesicular stomatitis virus. END^+ virus suppresses the induction of type I interferon (IFN) production in cell cultures, whereas END^- virus does not. Previous studies have shown that these viruses coexist in the same strain as quasi-species. However, the distribution of these quasi-species in the field and in infected animals remains unclear, and whether a causal link exists between quasi-species and the clinical condition has not been addressed. The aims of the present study, therefore, were to collect basic data for understanding of the relation between BVDV quasispecies and the various disease state of BVDV infection by examining their distribution and fluctuation among field isolates and within PI cattle.

Chapter 1　Distribution of quasispecies in BVDV field isolates

END^+ and END^- viruses are known to coexist within several laboratory strains of BVDV. In a previous study, I found that END^+ and END^- viruses may be present in varying proportions in BVDV field isolates, but the geographical region examined

in that study was limited to Hokkaido Prefecture, Japan. In the present study, 39 field strains of BVDV isolated in another prefecture were analyzed using a peroxidase-linked assay (PLA) with anti-NS3 monoclonal antibody, observation of the cytopathic effect (CPE), the END method, and the reverse plaque formation (RPF) method for quantification of BVDV, CP virus, END^+ virus, and END^- virus, respectively.

The isolates were grouped as follows based on the determined virus composition: 32 isolates (82.0%) in which END^- viruses were the major component; 2 isolates (5.1%) containing similar titers of END^+ and END^- viruses; 1 isolate (2.6%) in which END^+ virus was the major component; and 4 isolates (10.3%) containing CP viruses. These results show that END^- viruses are distributed widely in the field and are the major component of many field isolates. Interestingly, these results differed from previous results obtained from Hokkaido Prefecture field isolates, 52% of which contained END^+ viruses as the major component. The data generated in the present study suggest that the distribution of quasi-species in the field varies geographically.

Chapter 2 Changes in quasi-species composition during virus passage in cultured cells

Although BVDV field isolates contain varying proportions of END^+ and END^- viruses, they are usually passaged several times in cultured cells, which calls into question whether virus composition estimates based on titers determined from cultured cells accurately reflect the composition in BVDV-infected animals. In this experiment, changes in virus composition during multiple passages in cultured cells were analyzed. Four field isolates, passaged once in a livestock hygiene center, were passaged 19 additional times at intervals of 4 days in cultured bovine testicular cells. The culture supernatant was sampled at each passage and analyzed using the PLA, END, and RPF methods. The results of these analyses indicated that the virus composition is relatively stable until the 10th passage,

suggesting that the composition of field isolates is similar to that within the host. However, after the 10th passage, END$^+$ viruses began to predominate over END$^-$ viruses. Therefore, the passage history must be taken into consideration in quantitative analyses of virus quasi-species.

Chapter 3 Direct detection of quasi-species from cattle persistently infected with BVDV

In this experiment, END$^+$ and END$^-$ viruses in PI cattle were examined by direct detection. A total of 50 blood samples were collected from subclinical PI cattle, and active viruses were isolated using the PLA method. Samples from which BVDV was isolated were then tested using the PLA, END, and RPF methods. Active BVDV was isolated from 26 of 50 samples, and END$^-$ virus was detected in 16 of the 26 samples. END$^+$ virus was detected in 22 of the 26 samples. The 26 samples with active BVDV were divided into 5 groups based on virus composition: 5 samples in which END$^+$ virus was the predominant component, 1 sample in which END$^-$ viruses were predominant, 1 sample in which the END$^+$ and END$^-$ virus titers were equal, 16 samples in which the titers determined by PLA were higher than the titers determined using the other methods, and 3 samples in which BVDV was detected only by PLA. These results show that END$^+$ and END$^-$ viruses, which differentially affect the innate immune response, coexist in PI animals. However, the relationship between these viruses and various aspects of the disease, such as symptoms and duration of persistent infection, has not been elucidated. Further studies are needed to determine whether any association exists between the quasi-species and BVDV infections. Interestingly, three samples did not show neither the END phenomenon nor reverse plaques, suggesting that either these samples contain a new BVDV biotype that cannot be detected by biological methods such as END and RPF or that interfering biologically active factors were present.

Chapter 4 Changes in quasi-species composition in PI cattle

It was hypothesized that the coexistence of quasispecies affects the clinical condition and mode of transmission of BVDV infection because both END^+ and END^- viruses were detected in the blood of PI cattle. In this experiment, changes in virus composition over time were examined in PI cattle. Blood samples were obtained regularly from three breeding PI animals, and the titer of BVDV, END^+ virus, and END^- virus in the serum were determined using the PLA, END, and RPF methods, respectively. Both END^+ and END^- viruses were detected in the serum of one of the animals at all samplings. In another animal, no END^+ virus was detected, although END^- virus was detected at all samplings. This result suggests that a variety of quasispecies compositions may be present even among cows that are born during the same epidemic. In the third PI cow, which survived for an extended period, END^+ virus was detected in all serum samples collected before the age of 23 months but in none of the samples collected after the age of 24 months. END^- virus was detected in all samples until the age of 68 months. Total RNA was extracted from five of the serum samples from the long-surviving PI cow. The BVDV N^{pro} gene region was amplified by reverse transcription polymerase chain reaction and sequenced using next-generation sequencing techniques. The abundance of an interferon-inducible nucleotide sequence within this region was higher in 2 samples that were collected after the age of 61 months, suggesting that the virus composition fluctuates in PI cattle and that END^- viruses eventually predominate in long-surviving PI cows.

The results of this study demonstrate that END^- and END^+ viruses are distributed widely in the field and that they coexist in varying proportions as quasispecies in viral strains. The results of this study also show that END^+ and END^- viruses coexist in PI animals and that their composition fluctuates with aging. In addition, the duration of persistent infection appears to be related to the proportion of END^- virus, which tends to increase over time. The results of this

study also suggest that the coexistence of END$^+$ and END$^-$ viruses and fluctuations in their proportions affect the clinical diversity of BVDV infections and the onset of MD. Further research into the distribution of these quasi-species during persistent BVDV infection may lead to elucidation of the mechanisms of both BVDV infection onset and the development of MD.