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**Study on the involvement of maternal immune response in the
natural occurrence of persistent infection with bovine viral
diarrhea virus**

(牛ウイルス性下痢ウイルス持続感染の自然発生における
母体の免疫応答に関する研究)

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ABBREVIATIONS

A: adenine

Bp: base pair

BVDV: bovine viral diarrhea virus

C: cytosine

cDNA: complementary DNA

Cp: cytopathic

Ct: threshold cycle

CXCL12: chemokine C-X-C-motif ligand 12

CXCR4: C-X-C chemokine receptor type 4

DNA: deoxyribonucleic acid

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme linked immunosorbent assay

F: forward

G: guanine

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

IFN: interferon

IFN- α : interferon alpha

IFN- γ : interferon gamma

IHC: immunohistochemical

IL: interleukin

MMLV-RT: moloney murine leukemia virus reverse transcriptase

Ncp: non cytopathic

NT: not tested

PCR: polymerase chain reaction

PI: persistently infected

Pmol: picomole

R: reverse

RNA: ribonucleic acid

RT: reverse transcription

T: thymine

TGF- β : transforming growth factor- β

Th2: T helper 2

UTR: untranslated region

UV: ultraviolet

β – ME: beta mercaptoethanol

GENERAL INTRODUCTION

Bovine viral diarrhea virus (BVDV) is an economically important viral pathogen in cattle that is widespread throughout the world. BVDV is a single-stranded positive-sense RNA virus belonging to the *Flaviviridae* family, genus *Pestivirus* (Heinz et al., 2000). Two genotypes, BVDV-1 and BVDV-2, are recognized as distinct species within the genus *Pestivirus*. Based on the genomic sequences of the 5'-untranslated region (5'UTR), N^{pro} and E2 region, several subgenotypes in each genotype are recognized (Pellerin et al., 1994; Ridpath et al., 1994; Tajima et al., 2001; Vilček et al., 2001). BVDV strains were also classified into two biotypes based on their lytic activity in cell culture: cytopathic (cp) and noncytopathic (ncp) (Meyers and Thiel, 1996).

Both cp and ncp BVDV strains are able to cause acute infection in immunocompetent animals resulting in varying clinical manifestations ranging from mild subclinical disease to severe systemic disease and reproductive failure, including embryonic death and abortion (Brownlie et al., 1989; Murray, 1990). While both cp and ncp BVDV viruses are able to cross the placenta and infect the fetus, only ncp BVDV strains have the ability to cause persistent infection in fetuses infected before about 150 days of gestation, due to the insufficient development of the fetal immune system. Infection with BVDV after about 120 days of gestation results in a transient infection that cleared by the dam and fetus, whose adaptive immune system is now sufficiently developed to mount an immune response to the infecting virus. The risk period for the production of persistently infected (PI) fetus is variable, long period in pregnancy has the potency to produce PI fetus (Baker, 1995; Grooms, 2004; Houe, 1999; McClurkin et al., 1984; Peterhans et al., 2010; Thurmond, 2005).

PI animals produce large quantities of virus in their secretions and excretions. These animals are considered to be responsible for most of the spread of BVDV because they are a permanent source of contamination within a herd (Houe, 1995). The infection is maintained on the herd by PI animals that are immunotolerant against BVDV. The PI animal is often born weak and undersized, but may also appear normal at birth. Due to an impaired immune system, it is particularly susceptible to other infections, which partly explains the high mortality during young age, compared to non-infected calves (Houe, 1999). Some PI animals, however, remain clinically unaffected and may breed satisfactorily (McClurkin et al., 1979). The main route of the spread of BVDV between herds is through trade or contact with infected animals, or through trade with dams carrying PI fetuses. In addition, infection can be introduced to a susceptible herd by indirect means using contaminated equipment or tools (Lang-Ree et al., 1994; Niskanen and Lindberg, 2003). Transmission of BVDV can also occur through the use of contaminated biological by-products such as live vaccines (Falcone et al., 1999) or contaminated embryos or semen (Givens and Waldrop, 2004). Biosecurity, early detection and elimination of PI animals are important tools in BVDV control programs (Houe, 1999; Lindberg and Alenius, 1999; Mainar-Jaime et al., 2001). There has been a predominance of studies showing that the prevalence of PI animals ranged from 0.5% to 2.0% under the uncontrolled conditions (Houe, 1999; Houe and Meyling, 1991; Reinhardt et al., 1990).

In order for the BVDV to successfully infect the fetus, it has to replicate in maternal blood and tissues, reach and cross the placenta before maternal immune response clears the virus in the dam. Establishment of fetal persistent infection involves complex interactions between the maternal, fetal, and placental immune responses

(Smirnova et al., 2012). Currently, there is no examination method to detect cows carrying PI fetuses under the field conditions prior to birth of the PI calf. In the investigation of experimental fetal infections, some chemokines and cytokines in the dams of PI animals were involved in the production of PI animals (Baigent et al., 2002; Rhodes et al., 1999; Smirnova et al., 2009; Wiener et al., 2012). Maternal C-X-C chemokine receptor type 4 (CXCR4) expression was previously reported as concerned with BVDV infection (Smirnova et al., 2009; Wiener et al., 2012). CXCR4 is a G-protein-coupled 7-transmembrane receptor that is expressed on immune cells, platelets and the cells of the central nervous system (Nagasawa et al., 1998). It has a unique specific endogenous ligand, chemokine C-X-C-motif ligand 12 (CXCL12). Disruption of CXCL12/CXCR4 interaction has been shown to affect multiple biological processes, such as hematopoiesis, cardiogenesis, vasculogenesis, neuronal development, immune cell trafficking, and to cause embryonic lethality (Nagasawa et al., 1996; Nagasawa et al., 1998; Zou et al., 1998). Therefore, this disruption might cause immunosuppression and fetal defects similar to those observed in PI animals (Smirnova et al., 2009). In the natural occurrence of PI animals, it has not been clarified what influence of maternal immune response to the prevalence of PI calves.

The identification and removal of PI animals is an important component for the control of BVDV infection. In addition, the prevention of PI animal production should be considered. In order to prevent PI animal production, the mechanism of persistent infection should be thoroughly investigated. As described above, under the experimental conditions, the maternal immune response has been suspected as closely concerned to the production of PI calves. It has not been clarified, however, whether the experimental conditions corresponded to the field conditions or not. For the control of BVDV

infection, more detailed analyses of the maternal immune response under the natural occurrence of PI calves should be estimated. Under field conditions, the prevalence of PI animals is variable in cattle herds. Prevalence of PI calves might be related to the immunological status of BVDV-infected cattle. In this thesis, the involvement of maternal immune response in case of natural infection with BVDV was investigated and the mechanism of PI calf production was discussed. In chapter 1, a herd with high prevalence of PI calves within a short period having a single origin of infection was selected from natural cases of BVDV infection and was investigated epidemiologically. The herd condition was estimated for validity to the analysis of maternal immunological status. In chapter 2, using the high prevalence herd analyzed in chapter 1, the maternal immune response was investigated and the possible mechanism of PI calf production in the natural occurrence was discussed.

CHAPTER 1

ANALYSIS OF A DAIRY HERD WITH HIGH PREVALENCE OF CALVES PERSISTENTLY INFECTED WITH BOVINE VIRAL DIARRHEA VIRUS IN NATURAL OCCURRENCE

INTRODUCTION

PI animals are capable of shedding large quantities of virus throughout their lives and are considered as the primary reservoirs for BVDV (Houe, 1995). The prevalence of PI animals ranged from 0.5% to 2.0% in most of previous reports (Houe, 1999; Houe and Meyling, 1991; Reinhardt et al., 1990). Control of BVDV includes biosecurity, identification and elimination of PI animals, and vaccination (Brock, 2004).

Laboratory diagnosis of BVDV infection depends on the virus detection in PI animals or antibody detection in transiently infected animals. The virus detection is performed using virus isolation, antigen-capture enzyme linked immunosorbent assay (ELISA), immunohistochemical (IHC) analysis for the skin biopsy or reverse transcription polymerase chain reaction (RT-PCR). The antibody detection techniques most commonly used are the virus neutralization test (VNT) and ELISA (Houe et al., 2006). To date, most common methods for detecting PI animals are RT-PCR assays and antigen-capture ELISAs (Edmondson et al., 2007; Fulton et al., 2009; Hilbe et al., 2007).

For the detection of the BVDV gene by RT-PCR, 5'UTR and E2 primers were commonly used (Tajima et al., 2001). The 5'UTR, a highly conserved region of the genome, has shown to be a reliable and reproducible method for genetic characterization of BVDV isolates (Ridpath et al., 1994). The E2 glycoprotein has been described to be the main target of the neutralizing antibody response of the host (Donis, 1995). It is the least conserved and immunodominant protein (Bolin et al., 1988). The genetic diversity in the 5'UTR and E2 region was proven to be a useful epidemiological

tool for the detection of the origin of BVDV infection (Hamers et al., 1998, Luzzago et al., 2001; Tajima, 2004).

A dairy herd which produced many PI animals having a single origin of infection in a short period was detected in chapter 1. The herd condition was estimated for validity to the analysis of maternal immunological status.

MATERIALS AND METHODS

The Study Herd

A dairy herd in which 50 milking cows and 40 heifers and calves were housed during the study was investigated for BVDV infection. All animals in the herd had not been vaccinated against BVDV. There had been no occurrence of BVDV infection in the past few years. All cattle were fed in a tie stall served by three workers. There was no known contact with other herds. All cattle in the herd were not grazing about nine months before PI detection. A newborn PI calf (calf 1) was detected as a clinical case suffering from nervous manifestations. In order to eliminate BVDV from the herd, all cattle in this herd were examined and two PIs including a milking cow and a calf (calf 2) were detected. In order to estimate the prevalence of PI production, surveillance of all calves born in the herd was performed for 11 months after last PI detection until birth of all calves subjected to the risk of infection. The total period of surveillance was extended for over 15 months after the initial examination of all cattle in the herd.

Diagnosis of persistently infected animal

The PI animal was diagnosed in the case of both positive results of BVDV infection in two blood samples taken over two weeks interval. BVDV infection was confirmed by the detection of virus gene by RT-PCR using specific primers as described below.

Sampling

Blood samples collected from all cows and the newly born calves in the dairy herd were admitted to the laboratory of Veterinary Teaching Hospital, Hokkaido University from the veterinary practitioner. Serum or plasma was obtained by the centrifugation of the blood samples at 1,710 x g for 10 minutes.

Extraction of viral RNA from serum or plasma

Viral RNA was extracted from the serum or plasma using QIAamp Viral RNA Mini Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions. Briefly, 560 µl of prepared Buffer AVL containing 20 µl carrier RNA was pipetted into a 1.5-ml microcentrifuge tube. 140 µl of plasma or serum was added to the tube. The mixture was vortexed by pulse-vortexing for 30 seconds and incubated at room temperature (15–25°C) for 10 minutes then briefly centrifuged. 640 µl of 99.5% ethanol was added to the tube and mixed by pulse-vortexing for 30 seconds. 640 µl of the solution from previous step was carefully applied to the QIAamp spin column and centrifuged at 5,360 x g for 1 minute. Previous step was repeated once. 640 µl of Buffer AW1 was added to the QIAamp spin column and centrifuged at 5,360 x g for 1 minute. 640 µl of Buffer AW2 was carefully added to the QIAamp spin column and centrifuged at 16,400 x g for 2 minutes. The QIAamp spin column was placed into a clean 2-ml collection tube and centrifuged at 16,400 x g for 1 minute. The QIAamp spin column was placed into a clean 1.5-ml microcentrifuge tube. 60 µl of Buffer AVE equilibrated to room temperature was added to the spin column and kept at room temperature for 1 minute and then centrifuged at 5,360 x g for 1 minute to elute the RNA. The collected

solution was used as RNA. The RNA was heated at 96°C for 1 minute and quickly chilled on ice for denaturation. The RNA was immediately used or stored at –80°C until its use.

Reverse transcription (RT)

Synthesis of cDNA was carried out in the final reaction volume of 20 µl containing 8 µl of the RNA, 4 µl of 5X of buffer for reverse transcription (RT) (Invitrogen Inc., Carlsbad, CA, USA), 4 µl of dNTP mixture (Promega, Fitchburg, WI, USA), 2 µl of 0.1 M DDT (Invitrogen) and 1 µl of random hexamer (Promega). The mixture was heated at 70°C for 5 minutes. Then 1 µl of moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Invitrogen) was added and the mixture was incubated at 37°C for 60 minutes. After the incubation, the reaction was heated to 96°C for 5 minutes in order to inactivate MMLV-RT and quickly chilled on ice. The cDNA was stored at –30°C until use.

Polymerase chain reaction (PCR)

In order to detect the virus gene, RT-PCR was used. PCR was done in the final reaction volume of 50 µl containing 5 µl of each cDNA template added to the PCR mix containing 5 µl of 10X Gene *Taq* universal buffer (Nippon gene, Tokyo, Japan), 4 µl of 25 mM dNTP mixture (Promega), 1 µl of 10 pmol sense and antisense primers, 33.75 µl of nuclease-free water and 0.25 µl Gene *Taq* polymerase (Nippon gene). For the detection of the BVDV gene, 5'UTR primers were used (Tajima et al., 2001). The nucleotide sequences of the primers used were described in Table 1. PCR was done after 94°C for 1 minute, 35 cycles of denaturation at 94°C for 30 seconds, annealing at

55°C for 30 seconds and extension at 72°C for 30 seconds and the final extension step at 72°C for 2.5 minutes. The amplification was confirmed by 1.5% agarose gel electrophoresis after RT-PCR and visualized under UV light by ethidium bromide staining.

Identification of viral genes from PI animals in the herd

To identify the origin of the PI virus, the homology of the virus genes was analyzed in PI animals. After the detection of the PI animal, highly conserved region of the 5'UTR region and a variable region in the E2 coding region were analyzed.

Purification of RT- PCR products

After the confirmation of persistent infection, a part of genes was amplified by RT-PCR using 5'UTR and E2 primers described in Table 1. The amplified RT-PCR products of the viral genes were purified using Wizard SV Gel and PCR Clean-Up System (Promega). 45 µl of membrane binding solution was added to 45 µl the PCR product. SV minicolumn was inserted into collection tube. Prepared PCR product was transferred to the minicolumn. Incubation was done at room temperature for 1 minute. After centrifugation at 16,400 x g for 1 minute, the flow-through was discarded and minicolumn was reinserted into collection tube. 700 µl of membrane wash solution was added to the tube. After centrifugation at 16,400 x g for 1 minute, the flow-through was discarded and minicolumn was reinserted into collection tube. Previous step was repeated with 500 µl membrane wash solution. After centrifugation at 16,400 x g for 5 minutes, the collection tube was emptied and the minicolumn was recentrifuged for 1 minute. The minicolumn was carefully transferred to a clean 1.5-ml microcentrifuge

tube. 50 µl of nuclease-free water was added to the minicolumn and kept at room temperature for 1 minute then centrifuged at 16,400 x g. The minicolumn was discarded and the purified PCR product was stored at 4°C or –30°C.

Sequencing of viral genes from PI animals

Sequencing of viral genes from PI animals was performed using ABI PRISM Big Dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Waltham, MA, USA) according to the manufacturer's protocol. Sequencing was performed using sequencing primers including 5'UTR, E2inf, and E2inr primers described in Table 1. Final sequencing reaction volume of 15 µl contained 6 µl of sequence reaction master mix and 2.4 µl sequencing primer of 1 pmol/µl concentration and 6.6 µl of purified PCR product. The reaction mixture was covered with a drop of mineral oil to prevent evaporation. The sequencing reaction was performed after 97°C for 60 seconds, 25 cycles of denaturation at 97°C for 60 seconds, annealing at 50°C for 15 seconds and extension at 60°C for 240 seconds. The elimination of the free dyes from the reaction product was done using the ethanol/EDTA precipitation method. The reaction mixture was moved to 1.5-ml microcentrifuge tube containing a mixture of 60 µl of 99.5% ethanol and 1 µl of 0.5 M EDTA. Then the tube was centrifuged at 16,400 x g for 15 minutes. After discarding the supernatant, 60 µl of 70% ethanol was added and the tube was centrifuged at 16,400 x g for 10 minutes. The supernatant was discarded and the tube was put in inverted position and kept 10 minutes for dryness. The tube was wrapped in foil to protect dyes from light and stored at 4 °C. The nucleotide sequences of the amplified products were determined with an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Life technologies Co., Grand

Island, NY, USA). Sequence analysis and comparison of the nucleotide sequences and their deduced amino acid sequences were performed using computer software GENETYX ver. 8.1.0 (Genetyx Co., Tokyo, Japan). These nucleotide sequences were confirmed by sequencing two directions of the genes. Corresponding sequences obtained from the GenBank data library were included as reference sequences in the analysis. Their reference strains were described in Table.2

Estimation of the origin of BVDV infection in the herd

In order to estimate the infectious origin of the epidemic of BVDV, the epidemiological profile of the herd was investigated, such as clinical record, purchase record of cows, vaccination, grazing place and history and so on. Nucleotide and amino acids sequences of the viral genes from PI animals in the herd were compared and the identity of each virus gene was estimated.

Table 1. Primers used for detection and sequencing of BVDV genes

Gene		Primer Sequence (5'–3')	Genome position	Reference
5'UTR	F:	GGCTAGCCATGCCCTTAG	(100'–117')	Radwan et al., 1995
	R:	GCCTCTGCAGCACCTAT	(328'–345')	
E2	E2F:	ACTTTGAATTTGGACTYTGCC	(2,754'–2,774')	Tajima et al., 2001
	E2R:	TCCAGGTCAAACCACTATTG	(3,453'–3,434')	
	E2inf:	AACGGACCGG CCTTCCAGATGG	(2,825'–2,846')	
	E2inr:	TCATATGGTCTGCAAGGCAT	(3,306'–3,287')	

Y and R denote C or T and A or G, respectively.

Table 2. Reference strains obtained from the GenBank data library used for genotyping of viral genes from PI animals

Genotype	Subgenotype	Reference strain	Accession number
BVDV1	1a	SD-1	M96751
		Nose	AB033752
		NADL	M31182
		Singer	L35852
		Singer-A	AF083348
	1b	Osloss	M96687
		NY1	AY027671
		CP7	U63479
	1c	Deer-NZ	AF144614
		519/93	AF144610
	1d	721/96	AF144609
	1e	Deer-GB1	AF144615
	1f	22146/81	AJ302967
	1g	1891/99	AJ303001
		KS86-1	AB033753
BVDV2	2	890	U18059
		MU3	AF104030
		MU2	AF144612
		104/98	AJ302961

RESULTS

Detection of persistently infected animals

The time course scheme of the PI detection in the herd is shown in Fig. 1. Details about dates of introduction of PI animals to the herd and dates of detection and elimination from the herd are shown in Table 3. During 15 months of surveillance in the period from June 2010 to August 2011, 36 newborn calves were produced in the herd. Six calves were identified as PI (from calf 3 to calf 8) and 8 calves were identified as not PI within the first 4 months of surveillance. All dams of PI calves were not PI. Three PI calves (calves 1, 6 and 7) could not be examined by a second RT-PCR due to death or elimination from the herd. However, the result of first RT-PCR strongly indicated that these calves were PI as recognized from the intensity of the amplified RT-PCR product reflecting high quantity of virus. Although the examination of calves was done using sera which might contain maternal antibodies to BVDV acquired from colostrum feeding, a strong amplified RT-PCR product was observed in the case of PI calves. In the following 11 months of surveillance, 22 calves were born and all of them were recognized as not PI. The PI prevalence represented 7.0% of 128 tested animals in the herd. All PIs were removed from the herd immediately after detection.

Identification of viral genes from PI animals in the herd

All viruses from PI animals in the herd were classified as BVDV1b based on the nucleotide sequences of the 5'UTR and E2 region. Comparison of 220 bp of the 5'UTR of viral genes from PI animals revealed 100% sequence homology (Fig. 2). Comparison of 420 bp of the E2 coding region of viral genes from PI animals revealed a high degree

of sequence homology. The homology was 99% to 100% among nucleotide sequences (Fig. 3) and 96.4-100% among amino acid sequences translated from nucleotide sequences (Fig. 4). Differences in sequences in the E2 region among the PI viruses ranged from 0 to 4 nucleotide substitutions (0-1%) and 0 to 5 amino acid substitutions (0-3.6%).

Epidemiological estimation of the origin of BVDV infection

From the epidemiological information of the herd, the PI milking cow was purchased from other herd located in different local area. The PI milking cow was fed in the herd for about 165 days until detected as PI (Fig.1). During this period all cows in the herd had the same opportunity to contact with the PI milking cow. There was no known contact with other herds because the cows in the herd had not utilized the public grazing field and not joined cattle exhibitions. There was no occurrence of previous BVDV infection in the herd. Based on the epidemiological information of the herd and the identity of viral genes from PI animals, the PI milking cow was strongly suspected as the origin of BVDV infection to the herd. All the PI calves were produced within the herd from single origin of infection.

Table 3. Dates of introduction, examination and elimination of PI animals

PI animal	Introduction to the herd ^a	Examination		Elimination or isolation
		First RT-PCR	Second RT-PCR	
Milking cow	2009-12-05	2010-06-16	2010-06-30	2010-06-23
Calf 1	2010-05-19	2010-05-20	NT ^b	2010-05-20
Calf 2	2010-05-31	2010-06-07	2010-06-21	2010-06-23
Calf 3	2010-06-25	2010-06-30	2010-07-12	2010-07-30
Calf 4	2010-07-13	2010-08-13	2010-08-24	2010-09-24
Calf 5	2010-07-24	2010-08-13	2010-08-24	2010-09-24
Calf 6	2010-08-09	2010-08-13	NT ^c	2010-08-21
Calf 7	2010-08-19	2010-08-24	NT ^d	2010-08-21
Calf 8	2010-09-14	2010-09-21	2010-10-05	2010-09-24

All dates in the table were written in year-month-day format.

^a The milking cow was purchased from other herd on December, 2009, calves 1-8 were born in the herd. Birth dates were indicated except for the milking cow.

^b Calf 1 died 2 days after birth.

^c Calf 6 was sold and not examined by second RT-PCR.

^d Calf 7 was culled from the herd and not examined by second RT-PCR.

PI: persistently infected, RT-PCR: reverse transcription polymerase chain reaction, NT: not tested

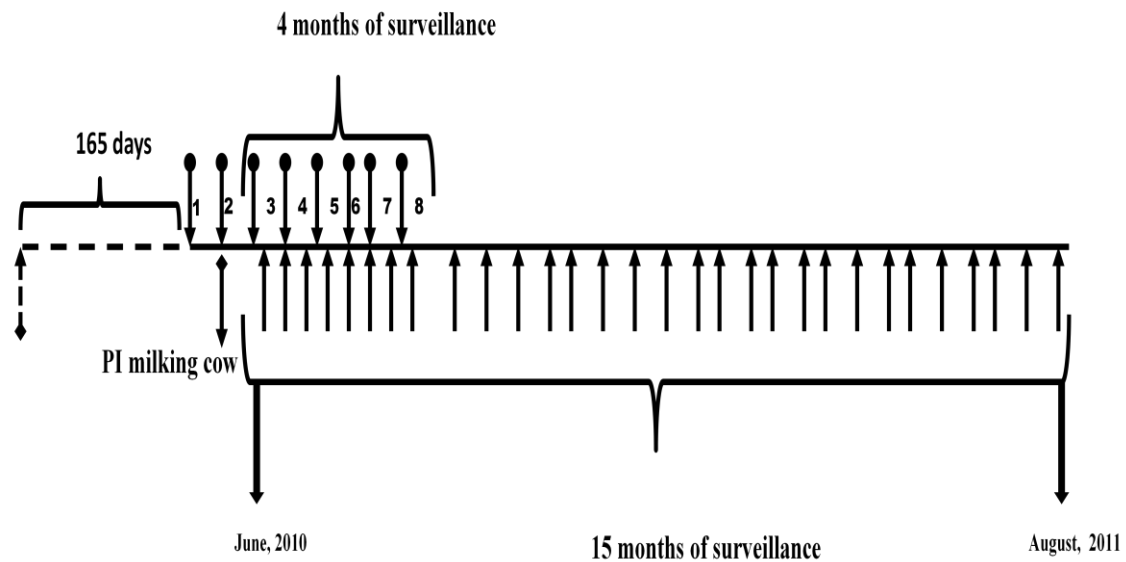


Fig. 1. The time course scheme of the PI detection.

Calf 1 was detected at first as an index clinical case. Two more PIs (a milking cow and calf 2) were newly detected. Fourteen calves were born within the first 4 months of surveillance. 6 of them were identified as PI and 8 calves were normal. In the following 11 months of surveillance 22 calves were born and all of them were recognized as not PI. $\updownarrow$: PI milking cow, $\downarrow$: PI calves, $\uparrow$: non-PI calves. $\updownarrow$: The PI milking cow had been introduced to the herd 165 days before detection (dashed line) of calf 1.

Milking cow	1:AAATAAGGGGGGTAGCAACAGTGGTGAATTTCGTTGGATGGCTGAAGCCCTGAGTACAGGG	60
Calf 1	1:.....	60
Calf 2	1:.....	60
Calf 3	1:.....	60
Calf 4	1:.....	60
Calf 5	1:.....	60
Calf 6	1:.....	60
Calf 7	1:.....	60
Calf 8	1:.....	60
Milking cow	61:TAGTCGTCAGTGGTTCGACGCTTTGGAGGACAAGCCTCGAGATGCCACGTGGACGAGGGC	120
Calf 1	61:.....	120
Calf 2	61:.....	120
Calf 3	61:.....	120
Calf 4	61:.....	120
Calf 5	61:.....	120
Calf 6	61:.....	120
Calf 7	61:.....	120
Calf 8	61:.....	120
Milking cow	121:ACGCCCCACAGCACATCTTAACCTGAACGGGGGTCGTTCAAGGTGAAAACGGTTTAACCGAC	180
Calf 1	121:.....	180
Calf 2	121:.....	180
Calf 3	121:.....	180
Calf 4	121:.....	180
Calf 5	121:.....	180
Calf 6	121:.....	180
Calf 7	121:.....	180
Calf 8	121:.....	180
Milking cow	181:CGCTACGAATACAGCCTGATAGGGTGCTGCAGAGGCATTA	220
Calf 1	181:.....	220
Calf 2	181:.....	220
Calf 3	181:.....	220
Calf 4	181:.....	220
Calf 5	181:.....	220
Calf 6	181:.....	220
Calf 7	181:.....	220
Calf 8	181:.....	220

Fig. 2. Comparison of a part of the 5'UTR region of the BVDV genes from PI animals in the herd.

Comparison of 220 bp of nucleotide sequences in the 5'UTR region in the PI milking cow and PI calves revealed 100% homology. This sequence corresponds to positions 113-332 in BVDV reference strains. Dots indicate the identity of the nucleotides.

Milking cow	1: TGCAATTGCGCAATAGGATACGCTAGCCACAACCCTGCTGCGAACGTACAAGAGGACAGGCCCTTTCATATAGGCAGGACTGTGTCACCCAGAAAACATTGGGGAGACCTCTAC	120
Calf 1	1:	120
Calf 2	1: GA.....	120
Calf 3	1: T.....	120
Calf 4	1:	120
Calf 5	1:	120
Calf 6	1:	120
Calf 7	1:	120
Calf 8	1:	120
Milking cow	121: GACTGTGCTTAGGAGGGAAATTGGACCTGTGTACCGGGGGATGCACATAATATGTAGTGAGCCGTCGAGTCTTGTAGTGGTGTGTCACAGTTTATCAAAAGTGAGGGTCTGCCG	240
Calf 1	121: T.....	240
Calf 2	121: T.....	240
Calf 3	121:	240
Calf 4	121: T.....	240
Calf 5	121:	240
Calf 6	121:	240
Calf 7	121:	240
Calf 8	121:	240
Milking cow	241: CATTTTCCAAATTGGCAATGCAAGCTTAGAATGAGAGTGCTATAGACAAGTGGATGAGACTTCTTGCACAGAGACGGTGTGGCTATAGTGTCTATCTGGCCAGGTCAATGCAAGATA	360
Calf 1	241: T.....	360
Calf 2	241:	360
Calf 3	241: T.....	360
Calf 4	241: T.....	360
Calf 5	241:	360
Calf 6	241: G.....	360
Calf 7	241: G.....	360
Calf 8	241:	360
Milking cow	361: GGGGACACAGTAGTGCAGTCATTGCAATGGATGATTAATTAGGCTTATGCCCTGCAGA	420
Calf 1	361: 420 (99.5%)	
Calf 2	361: A..... 420 (99.0%)	
Calf 3	361: C.....A 420 (99.0%)	
Calf 4	361: C..... 420 (99.3%)	
Calf 5	361: 420 (100.0%)	
Calf 6	361: 420 (99.8%)	
Calf 7	361: 420 (99.8%)	
Calf 8	361: 420 (100.0%)	

Fig. 3. Comparison of a part of the E2 region of the BVDV genes from PI animals in the herd.

Comparison of 420 bp of nucleotide sequences in the E2 region in the PI milking cow and PI calves. This sequence corresponds to positions 2862-3281 in BVDV reference strains. Dots indicate the identity of the nucleotides. The homology % of each PI virus compared to the PI milking cow was shown between parentheses.

Milking cow	1:	CTLANKDITLRTTWRITYKRNRPFPYRQDCVTOKTIGEDLYDCALGGNWTCPGDALQYWG	60
Calf 1	1:		60
Calf 2	1:	I.....	60
Calf 3	1:	T.....	60
Calf 4	1:		60
Calf 5	1:		60
Calf 6	1:		60
Calf 7	1:		60
Calf 8	1:		60
Milking cow	61:	GPVESCWCGHKETKSEGLPHEPTIGCKLKNESGYRQVDETSNRRDGVAIVLSGQVKCKI	120
Calf 1	61:	Y.....L.....	120
Calf 2	61:		120
Calf 3	61:	Y.....L.....	120
Calf 4	61:	Y.....L.....	120
Calf 5	61:		120
Calf 6	61:	R.....	120
Calf 7	61:	R.....	120
Calf 8	61:		120
Milking cow	121:	GDTVVQVIAMDDKLGPMPCR	140
Calf 1	121:		140 (100.0%)
Calf 2	121:		140 (99.3%)
Calf 3	121:	T.....K	140 (96.4%)
Calf 4	121:	T.....	140 (97.7%)
Calf 5	121:		140 (100.0%)
Calf 6	121:		140 (99.3%)
Calf 7	121:		140 (99.3%)
Calf 8	121:		140 (100.0%)

Fig. 4. Comparison of amino acid sequences translated from a part of the E2 region of the BVDV genes from PI animals in the herd.

Comparison of 140 amino acid sequences translated from 420 bp of the E2 region in the PI milking cow and PI calves. Dots indicate the identity of amino acids. The homology % of each PI virus compared to the PI milking cow was shown between parentheses.

DISCUSSION

A dairy herd included a total of 128 cattle that were examined during over 15 months, nine PIs were detected. Prevalence of PI animals in this herd estimated 7.0%. This prevalence was very high compared to those estimated in previous reports estimated 0.5-2.0% (Houe, 1999; Houe and Meyling, 1991; Reinhardt et al., 1990).

All viruses from PI animals in the herd were classified as BVDV1b which is the most prevalent subgenotype in the world (Tajima, 2006) In addition, viral genes from PI animals in the present herd had no specific genetic characters in phylogenetic analysis (data not shown). The 5'UTR is a highly conserved region of the genome and it can be used as a tool of molecular epidemiology of BVDV infection (Luzzago et al., 2001). 100% nucleotide sequences homology in 220 bp in the 5'UTR was found among of viral genes from PI animals in the present herd. Sequence identity of the viral genes from PI animals based on 5'UTR has been previously reported in other cases (Ståhl et al., 2005; Vilček et al., 1999). Using 288 bp nucleotide segment of the 5'UTR, isolated viruses from the same herd had the same nucleotide sequences or differed only in 1-2 nucleotides (Vilček et al., 1999). Furthermore, another study compared 237 nucleotide segment of the 5'UTR and several sets of identical sequences were found within the same herd (Ståhl et al., 2005). Although the E2 region of BVDV is a highly variable region (Donis, 1995), a very high homology was recognized among nucleotide (99-100%) and amino acid (96.4-100%) sequences in 420 bp of E2 region of BVDV PI animals in the present study. The nucleotide variations in the E2 region among viral genes from PI animals in the herd were very small estimated up to 1%. Similar results were reported using 188 bp fragment and nucleotide variations estimated 1.6%,

suggesting that these viruses were having a single common origin (Paton, 1995). Moreover, it was also reported that nucleotide variations reached up to 1.0% in 389 bases of the E2 region within the same herd (Hamers et al., 1998). In the present study, therefore, the identity of the PI viruses was strongly indicated.

The PI milking cow was introduced to the herd 165 days before the first PI calf detection. It was reported that infection of susceptible pregnant cows with ncp BVDV before 150 days of gestation may result in birth of a PI calf, due to the infection occurring prior to the development of immune competence (Baker, 1995; Grooms, 2004; McClurkin et al., 1984; Peterhans et al., 2010; Thurmond, 2005). The PI milking cow was fed in the herd during the period of risk of immunotolerance against BVDV of all PI calves. There was no known contact between this herd and other herds. In addition, there was no history of previous BVDV infection in the herd. The detection of PI calves in the present herd was limited to a period of 4 months and was strongly suspected to be caused by a single origin of infection. It was reported that 70% to 100% of susceptible nonvaccinated cattle becoming infected after PI animal exposure (Fulton et al., 2005). The estimation of antibodies in the present herd could not be performed because all cattle in the herd were vaccinated after the elimination of the PI milking cow before the start of surveillance. It is difficult, therefore, to estimate the spread of BVDV within the herd using antibody titer.

In the present study, after the elimination of the PI milking cow, 14 calves were born within the first 4 months of surveillance. Six of them were identified as PI and 8 calves were normal. These normal calves might be due to insufficient contact with the PI milking cow or presence of sufficient BVDV-specific or cross reacting serum neutralizing antibodies in the dams. In the following 11 months of surveillance, no PI

calves were produced inspite of the presence of the PI milking cow in contact with some dams of these calves during the period of risk of immunotolerance. Moreover, some PI calves were kept in the herd for a period more than two months after birth until elimination (calves 4 and 5). These calves could be a potential source of BVDV infection. However, these calves were fed individually in isolated hatches and had no direct contact with pregnant cows. Dams producing normal calves inspite of exposure to BVDV infection indicated that they were immune as they might be infected with BVDV before pregnancy and seroconversion. The control method for BVDV in the present reported herd was “test and cull”. All PI calves could be detected within a short period. Thus, it was possible to obtain a BVDV-free herd by the examination of newborn calves and newly introduced cows to the herd.

In conclusion, in chapter 1, a dairy herd with a high prevalence of PI calves having a single origin of infection in a period of 4 months was described. All PI calves were produced within this herd from non PI dams in natural occurrence. Therefore, the herd condition was suitable to the analysis of maternal immunological status to the persistent infection with BVDV.

SUMMARY

In chapter 1, a natural occurrence of BVDV PI calves was detected. A dairy herd including 50 milking cows and 40 heifers and calves was investigated. High prevalence of BVDV PI calves was detected in this herd. A newborn PI calf was detected in the herd as a clinical case suffering from nervous manifestations. In order to eliminate BVDV from the herd, all cattle in this herd were examined and 2 PIs (a milking cow and a calf) were also detected. During 15 months of surveillance, 36 calves were produced in the herd. Six of them were identified as PI within the first 4 months of the surveillance period. The virus genes detected in these PIs were sequenced and compared to the virus gene of the PI milking cow. A very high homology was recognized among the PIs; 100% nucleotide homology in the 5'UTR and 96.4-100% among amino acid sequences in the E2 region. Based on the epidemiological information and the high sequence homologies in the 5'UTR and E2 region among these PIs, the PI milking cow was strongly suspected to be the origin of BVDV infection into the herd. Prevalence of PI animals in this herd estimated 7.0% during 15 months of surveillance which was very high compared to those estimated in previous reports. In the present study, all PI calves had a single origin of infection and the high prevalence of PIs was recognized in a period of 4 months. All PI calves were produced within this herd from non PI dams in natural occurrence. Therefore, the herd condition was suitable for the analysis of maternal immunological status to the persistent infection with BVDV.

CHAPTER 2

INVESTIGATION OF MATERNAL C-X-C CHEMOKINE RECEPTOR TYPE 4 AND CYTOKINE EXPRESSIONS IN NATURAL OCCURRENCE OF CALVES PERSISTENTLY INFECTED WITH BOVINE VIRAL DIARRHEA VIRUS

INTRODUCTION

BVDV has a unique capacity to cause persistent infections of fetuses exposed within the first 150 days of gestation. Establishment of fetal persistent infection with BVDV involves complex interactions between the maternal, fetal, and placental immune responses (Smirnova et al., 2012). The immunological response to the infection in the pregnant cow could affect the transplacental transmission and persistent infection (Smirnova et al., 2009). Studies concerning C-X-C chemokine receptor type 4 (CXCR4) and PI production were conducted using experimentally-induced infection *in vivo* or examined using *in vitro* approaches (Smirnova et al., 2009; Wiener et al., 2012). Based on a genome-wide microarray analysis of BVDV experimentally infected heifers, CXCR4 was significantly down-regulated in the dams of PI fetuses (Smirnova et al., 2009). On the other hand, in an *in vitro* study (Wiener et al., 2012), BVDV induced up-regulation of CXCR4 in naïve peripheral blood mononuclear cells following culture with serum from pregnant cows infected with ncp BVDV. The down-regulation of maternal blood cell CXCR4 that occurred *in vivo* may require specific immune responses that are difficult to replicate *in vitro* (Wiener et al., 2012). The importance of chemokines in viral persistence, pathogenesis and infection of immune cell subsets has been clearly demonstrated for some members of the *Flaviviridae* other than BVDV such as Dengue virus and Hepatitis C virus, (Chen and Wang, 2002; Gandini et al., 2011; Heydtmann and Adams, 2009; Kang and Shin, 2011; Kelley et al., 2011; Larrubia et al., 2008).

Cytokines might also play a role in increasing susceptibility of transplacental transmission of BVDV and production of PI animals (Baigent et al., 2002; Rhodes et al.,

1999; Smirnova et al., 2009). It was reported that interference with type I IFN and associated pathway for evasion of the innate immune response was related to the mechanism of BVDV persistent infection (Baigent et al., 2002, Smirnova et al., 2009). Moreover, stimulation of a biased Th2 cytokines response in pregnant cows may be of advantage to the virus in establishing persistent infection with BVDV (Rhodes et al., 1999). The involvement of cytokines in the transplacental infection was previously reported in many pathogens as toxoplasmosis, leishmaniasis and neosporosis (Almería et al. 2003; Hunter et al., 1996; Khan et al., 1994; Krishnan et al., 1996; Long and aszler, 2000; Thouvenin et al., 1997).

Alteration of maternal CXCR4 and cytokines expressions has been suspected as closely concerned with the mechanism of production of BVDV PI fetuses as reported in previous experimental studies. In chapter 2, the involvement of maternal CXCR4 and cytokine expressions in the natural occurrence of BVDV PI calves was investigated. A dairy herd with natural occurrence of PI calves within a short period having a single origin of infection was previously reported in chapter 1. CXCR4 and cytokine expressions were investigated in the cows of this herd in order to estimate the maternal immune response.

MATERIALS AND METHODS

Animals and herds

A dairy herd with natural occurrence of high prevalence of BVDV PI animals estimated 7.0% having a single origin of infection was described in chapter 1. The cows of this herd were investigated in chapter 2. Other two dairy herds were also used for comparison. One herd with low prevalence of PI calves included 34 milking cows and 18 heifers and calves. One PI was detected and then surveillance continued for 9 months and no more PI calves were detected. Prevalence of PI animals in this herd estimated 1.5%. Another herd was BVDV-free herd included 27 milking cows and 27 heifers and calves. No PI animals were detected in this herd after repeated BVDV examinations. All cows in this herd had been vaccinated with inactivated BVDV vaccine including both BVDV1 and BVDV2 genotypes.

Blood Samples

Blood with anticoagulant, sodium salt of ethylenediaminetetraacetic acid (EDTA), was collected from 26 cows in the high prevalence herd including 6 dams of PI calves, 34 cows in the low prevalence herd and 25 cows in the BVDV-free herd. These samples included different periods of pregnancy and fetuses were not diagnosed at the sampling time. Blood samples were collected twice from 3 dams of PI calves and 12 dams of non PI calves at about two months interval. Centrifugation of the blood samples at $1,710 \times g$ for 10 minutes was performed to obtain the buffy coat cells.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from leukocytes using QIAamp RNeasy Mini Kit (Qiagen Inc.) according to the manufacturer's instructions. Briefly, 500 μ l of Buffer RLT containing 1% beta mercaptoethanol (β -ME) was added to 15 μ l of the buffy coat cells in the 1.5-ml microcentrifuge tube. The tube was vortexed vigorously for 3 minutes and then briefly centrifuged. The lysate was pipetted directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuged at 16,400 x g for 1 minute. 520 μ l of 70% ethanol was added to the lysate and mixed well by pipetting. Up to 640 μ l of the sample, including any precipitate was transferred to an RNeasy spin column placed in a 2 ml collection tube and centrifuged at 12,100 x g for 1 minute. The flow-through was discarded and the collection tube was reused. Successive aliquot was centrifuged in the same RNeasy spin column. The flow-through was discarded after each centrifugation. 640 μ l of Buffer RW1 was added to the RNeasy spin column and centrifuged for 12,100 x g for 1 minute. The flow-through was discarded and collection tube was reused. 500 μ l of Buffer RPE was added to the RNeasy spin column and centrifuged at 12,100 x g for 1 minute. The RNeasy spin column was placed in a new 2 ml collection tube and centrifuged at 12,100 x g for 1 minute. The RNeasy spin column was placed in a new 1.5 ml collection tube. 50 μ l of nuclease-free water was added directly to the spin column membrane and left at room temperature for 1 minute then centrifuged at 12,100 x g for 1 minute to elute the RNA. The RNA was heated at 96°C for 1 minute and quickly chilled on ice for denaturation. The quantity of extracted RNA was determined spectrophotometrically using

spectrophotometer (Beckman Coulter Inc., Brea, CA, USA) and stored at -80°C until use.

Synthesis of cDNA was carried out using 8 μl of the RNA, MMLV-RT and a random hexamer as described in chapter 1. The cDNA was stored at -30°C until use.

Quantification of CXCR4 and cytokine gene expressions

CXCR4 and cytokine gene expressions including interleukin-4 (IL-4), IL-6, IL-10, IL-12p40, interferon - α (IFN- α), IFN- γ and transforming growth factor- β (TGF- β) of the cows in these herds were estimated using real-time polymerase chain reaction (real-time PCR). Real-time PCR was performed using 7300 real-time PCR systems (Applied Biosystems) using with 5 ng of cDNA in a total of 25 μl reaction mixture including 5 pmol of each primer and 12.5 μl of Power SYBR[®] Green PCR Master Mix (Applied Biosystems). The conditions for real-time PCR were 50°C for 2 minutes, 95°C for 3 minute, 50 cycles (denaturation at 95°C for 30 seconds, annealing at $55 - 65^{\circ}\text{C}$ for 30 seconds according to the primer and extension at 72°C for 60 seconds) and a dissociation step for the confirmation of specific amplification. The details of primers used in real-time PCR were described in Table 1. The results of quantification of each gene were analyzed with the comparative threshold cycle (ΔCt) method and presented as $2^{-\Delta\text{Ct}}$ (Schmittgen and Livak, 2008). ΔCt is the difference between the Ct of a given target and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. The amplification efficiencies of the target genes and GAPDH were approximately equal. Statistical analysis of data was performed using Student's *t*-test and one-way analysis of variance (ANOVA). Differences were considered statistically significant when $P < 0.05$.

Table 4. Details of nucleotide sequences of primers used for real-time PCR

Gene	Primer sequence (5'–3')	Size of amplified product (BP)	Annealing temp. (°C)	Reference or accession number
CXCR4	F: TATCGTCCATGCTACCAACA R: GAGTCGATGCTGATCCCAAT	377	55	NM_174301
IL-4	F: TGCCCCAAAGAACACAACCTG R: TTTAGCCTTTCCAAGAGGTC	200	55	Riollet et al., 2001
IL-6	F: TCC AGA ACG AGT ATG AGG R: CAT CCG AAT AGC TCT CAG	236	55	Konnai et al., 2003
IL-10	F: TGCTGGATGACTTTAAGG R: AGGGCAGAAAGCGATGACA	186	55	Konnai et al., 2003
IL-12p40	F: AGGTCGTGGTAGAAGCTGTG R: CCTTGTGGCATGTGACTTTG	275	65	Riollet et al., 2001
IFN-α	F: GAAGGCTCAAGCCATCTCTG R: CCAGGTGTGTGTCAGTCCTT	365	60	E00135
IFN-γ	F: ATAACCAGGTCATTCAAGG R: ATTCTGACTTCTCTTCCGCT	218	55	Konnai et al., 2003
TGF-β	F: AGAGAGGAAATAGAGGGCTT R: ATGAATCCACTTCCAGCCCA	306	55	Compton et al., 2009
GAPDH	F: GCGTGAACCACGAGAAGTATAA R: CCCTCCACGATGCCAAAGT	120	55	Robinson et al., 2007

RESULTS

CXCR4 and cytokine gene expressions in the high prevalence herd:

CXCR4 and cytokine gene expressions in the dams of PI calves and non PI calves in the herd were estimated. No significant differences were observed between both of the dams (Fig. 5). In spite of these non significant differences, there was tendency of IL-6, IL-10 and IFN- γ expressions to be higher in the dams of PI calves than the dams of non PI calves. Also, there was tendency of IL-12, IFN- α and TGF- β expressions to be lower in the dams of PI calves than the dams of non PI calves (Fig. 5). IL-4 expression was very low in both of the dams. The blood samples of the dams of PI calves and non PI calves included different status of pregnancy while CXCR4 expression might be variable during pregnancy. Therefore, the CXCR4 expression of these dams was estimated according to the pregnancy period. CXCR4 expressions in the dams of PI calves and non PI calves showed no specific tendency (Fig. 6). To investigate changes in CXCR4 expression in a cow during pregnancy, CXCR4 expression was tested twice in the same cow at different periods of pregnancy in each of dams of PI calves and non PI calves. There was no specific tendency in both dams (Fig. 7).

Comparison of CXCR4 and cytokine gene expressions among herds:

Because CXCR4 expression in the dams of PI calves was not significantly different from the dams of non PI calves in the high prevalence herd, the expression of CXCR4 in dams of the high prevalence herd was compared to those of a low prevalence herd and BVDV-free herd. As shown in Figure 8, CXCR4 expressions in

the PI-producing herds including the high prevalence and low prevalence herds were significantly lower than the BVDV-free herd. CXCR4 expressions in the high prevalence and the low prevalence herds were similar. There were no significant differences in cytokine expressions among herds except for IL-10. IL-10 was significantly higher in the high prevalence herd and the BVDV-free herd than the low prevalence herd. In the high prevalence herd, IL-6, IL-12, IFN- γ and IFN- α had tendency to be higher and TGF- β had tendency to be lower. The cytokine profile was summarized in Table 5. These results indicated some bias toward Th2 cytokines including IL-10 and IL-6 in the high prevalence herd and the BVDV-free herd.

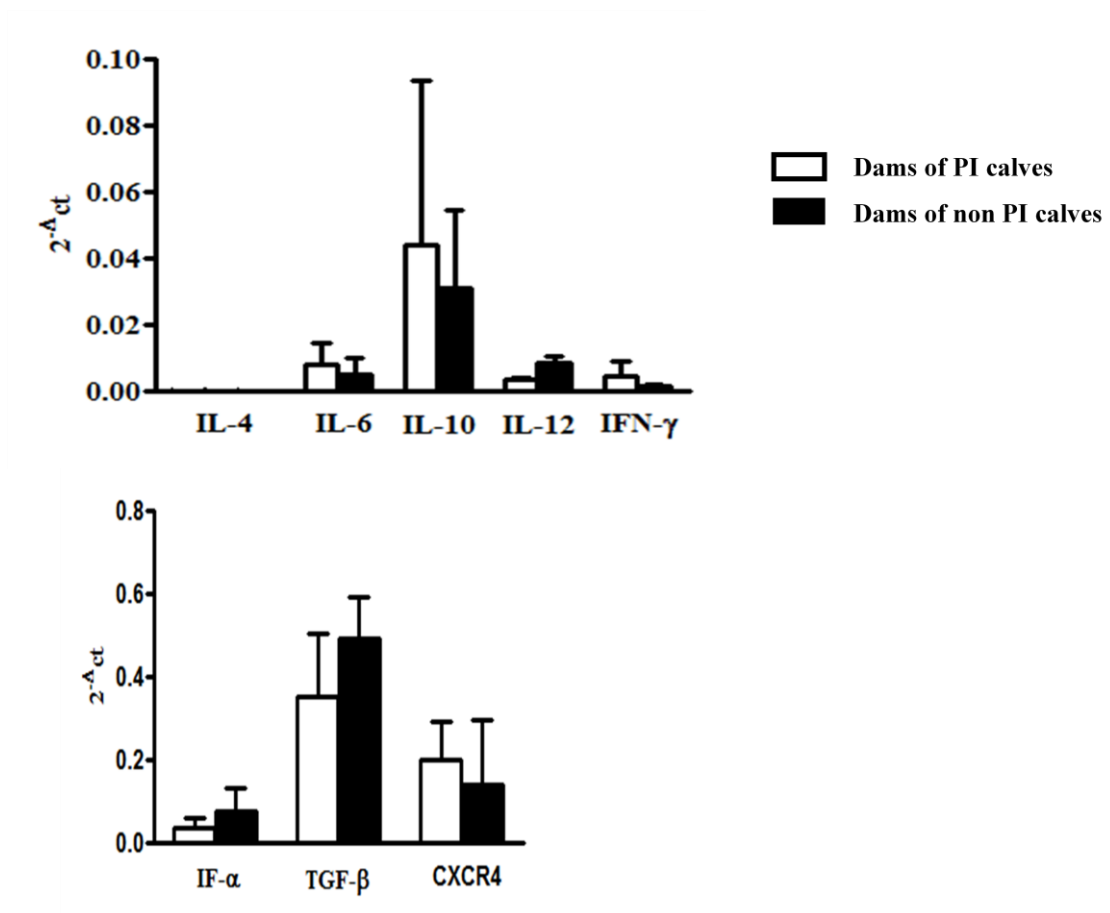


Fig. 5. CXCR4 and cytokine gene expression in the high prevalence herd. CXCR4 expression and cytokine expressions including IL-4, IL-6, IL-10, IL-12, IFN-α, IFN-γ and TGF-β in 6 dams of PI calves (open column) and 20 dams of non PI calves (closed column). Expression values were calculated using comparative Ct method ($2^{-\Delta Ct}$). Bars indicated standard deviation. Data were analyzed using Student's *t*-test.

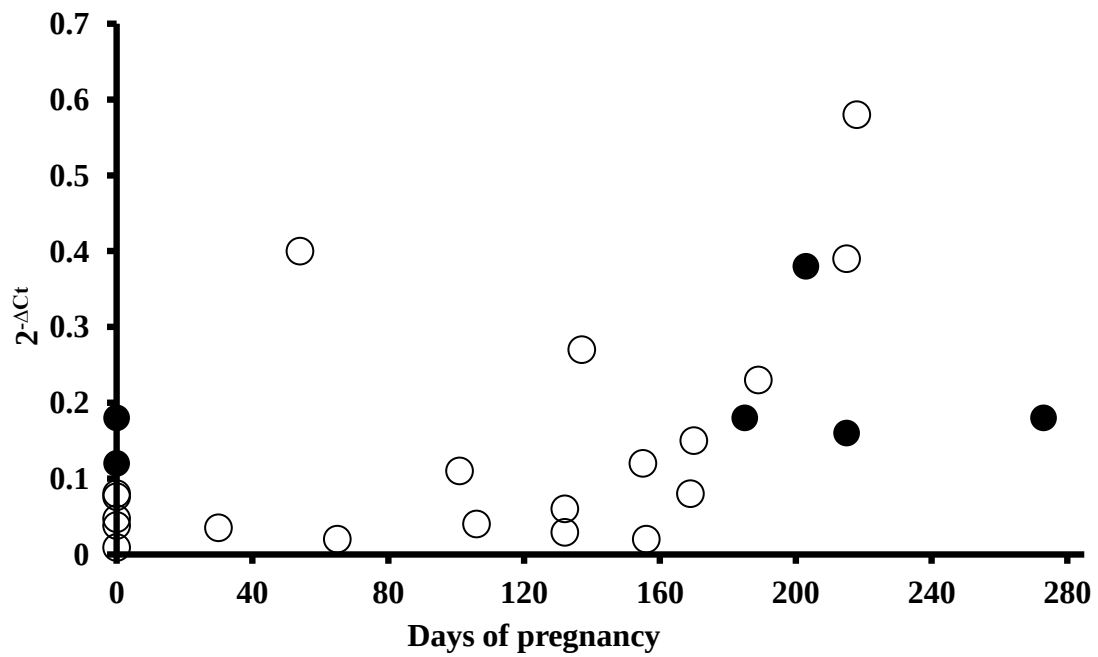


Fig. 6. CXCR4 expression in the cows with different status of pregnancy in the high prevalence herd.

CXCR4 expression in 6 dams of PI calves (●) and 20 dams of non PI calves (○).

Expression values were calculated using comparative Ct method ($2^{-\Delta C_t}$).

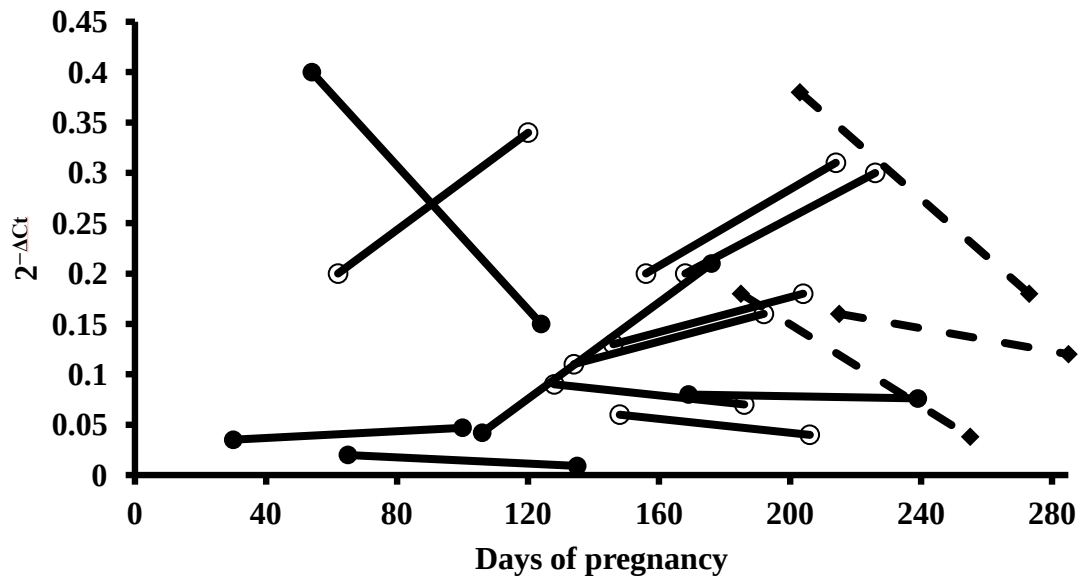


Fig.7. Changes in CXCR4 expression during pregnancy in the same cow. CXCR4 expression was measured twice within about two months interval in the same cow in the high prevalence and low prevalence herds. These cows included 3 dams of PI calves (◆-----◆) and 5 dams of non PI calves in the high prevalence herd (●————●) and 7 dams of non PI calves in the low prevalence herd (○————○). Expression values were calculated using comparative Ct method ($2^{-\Delta C_t}$).

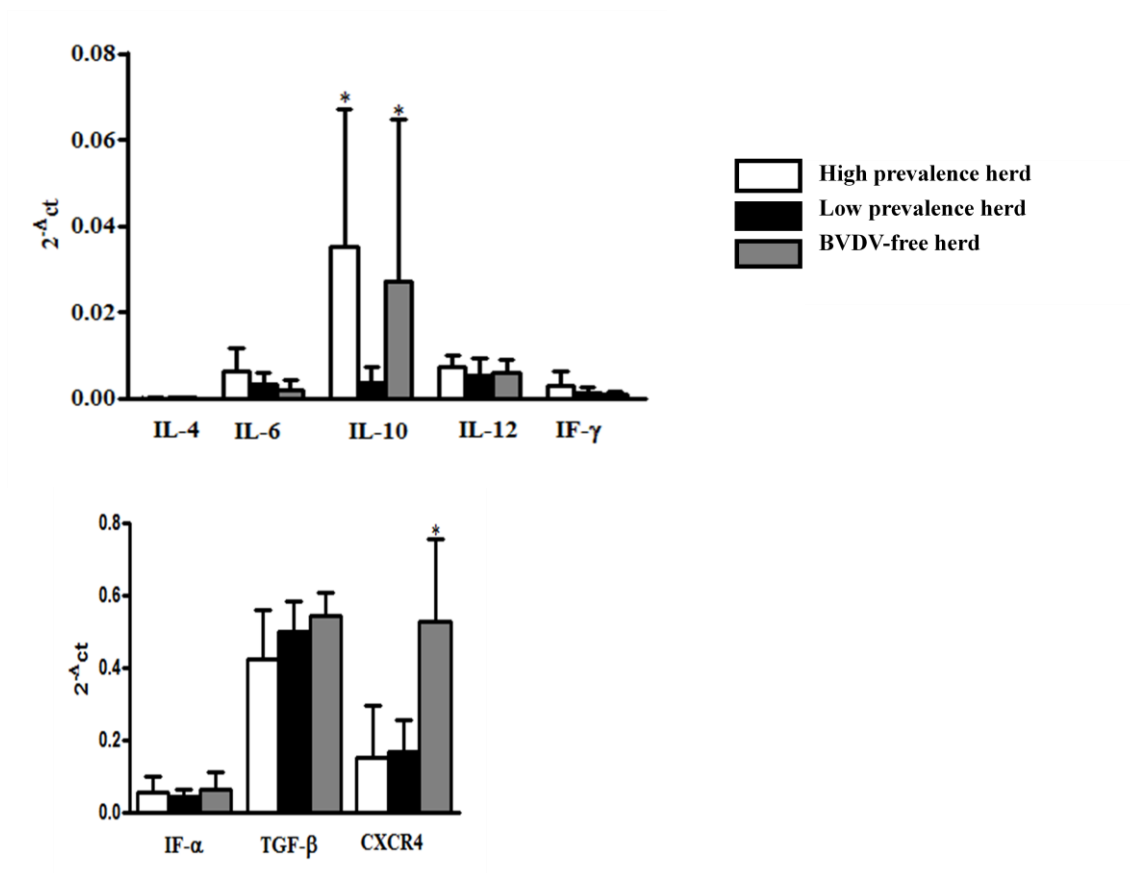


Fig. 8. Comparison of CXCR4 and cytokine gene expressions among herds.

CXCR4 expression and cytokine expressions including IL-4, IL-6, IL-10, IL-12, IFN-α, IFN-γ and TGF-β of 26, 34 and 25 cows in the high prevalence, low prevalence and the BVDV-free herd respectively. Expression values were calculated using comparative Ct method ($2^{-\Delta C_t}$). Asterisk (*) indicated the significant higher difference ($P < 0.05$) to other columns within the same gene. Bars indicated standard deviation. Data were analyzed using one-way analysis of variance (ANOVA).

Table 5. Summary of CXCR4 and cytokine gene expressions

	CXCR4	IL-4	IL-6	IL-10	IL-12	IFN- α	IFN- γ	TGF- β
High prevalence herd	+	–	+	++	+	+	+	++
Low prevalence herd	+	–	+	+	+	+	–	++
BVDV-free herd	++	–	+	++	+	+	–	++

(–: not expressed, +: expressed, ++: highly expressed)

DISCUSSION

In chapter 2, the involvement of maternal CXCR4 and cytokine gene expressions in the natural occurrence of PI calves was investigated. CXCR4 expression in the dams of PI calves was not significantly different from the dams of non PI calves in the high prevalence herd. This was not corresponding to a previous study which reported that CXCR4 was significantly down-regulated in blood of experimentally infected dams carrying PI fetuses (Smirnova et al., 2009). In the comparison among herds, CXCR4 expressions in PI-producing herds were significantly lower than the BVDV-free herd. This result suggests that the cows in PI-producing herd had down-regulated CXCR4 expression whatever they were dams of PI or not. It was reported in an *in vitro* study (Wiener et al., 2012) that BVDV induced up-regulation of CXCR4 in naïve peripheral blood mononuclear cells following culture with serum from pregnant cows infected with ncp BVDV. However, the down-regulation of maternal blood cell CXCR4 that occurred *in vivo* may require specific immune responses that are difficult to replicate *in vitro* (Wiener et al., 2012).

Cytokines might also play a role in increasing susceptibility of transplacental transmission of BVDV and production of PI animals (Rhodes et al., 1999; Smirnova et al., 2009). It was reported that Th2-biased cytokines may be of advantage to the virus in establishing persistent infections *in utero* (Rhodes et al., 1999). This report might not be coincided with our results as there was a bias toward Th2 cytokines observed in both dams of PI calves and non PI calves in the high prevalence herd. Moreover, in the comparison among herds, a bias toward Th2 cytokines was indicated in the high prevalence herd and the BVDV-free herd. These findings indicated that not only Th2-biased cytokines were involved in the persistent infection with BVDV but also other

factors might be included. Previous studies indicated a relationship between the high expression of IL-10 and the transplacental infection in other diseases such as Leishmania infection in mice and neospora caninum in cattle (Almería et al. 2003; Krishnan et al., 1996). In the present study, no significant differences in IL-10 gene expression between the dams of PI calves and non PI calves in the high prevalence herd. On the other hand, significant higher expression of IL-10 was observed in the cows of the high prevalence herd and the BVDV-free herd compared to the low prevalence herd. These findings indicated a combination of low expression of CXCR4 and high expression of IL-10 in the high prevalence herd. IL-10 and CXCR4 expressions in the high prevalence herd showed no significant differences between dams of PI calves and non PI calves. These findings indicated that both dams had an equal risk for PI production in spite of production of non PI calves. Other factors might also be responsible for birth of non PI calves. In the low prevalence herd, CXCR4 expression was similar to the high prevalence herd. However, IL-10 expression was significantly lower than the high prevalence herd and the BVDV-free herd. This situation might influence the prevalence of PI calves. More detailed profiling of cytokines would be needed.

The relationship between type I IFN and the mechanism of persistent infection with BVDV was previously reported (Baigent et al., 2002; Smirnova et al., 2009). Other reports stated that the type I IFN was associated with expression of CXCR4 (Serra et al., 2008; Wiener et al., 2012). In contrast to these reports, the results obtained in this study indicated no significant differences of IFN- α between the dams of PI calves and non PI calves in the high prevalence herd. Furthermore, IFN- α expression showed no

significant differences among herds. Further studies are necessary to clarify the involvement of IFN- α in the persistent infection and its relationship with CXCR4.

It was reported that maternal CXCR4 was up-regulated in sheep during early pregnancy due to the implantation and placentation (Ashley et al., 2011). Additionally, it was previously indicated that CXCR4 expression was up-regulated in cows carrying normal fetuses in the period between 75 and 160 days of pregnancy (Smirnova et al., 2009). This variation in the degree of CXCR4 expression during pregnancy was estimated in the present study because cows within the same herd were in different periods of pregnancy. In the present study, the changes in CXCR4 expression estimated during pregnancy in the same cow had no specific tendency. Moreover, the change in cytokine expressions estimated during pregnancy in the same cow had no specific tendency (data not shown). It was not clear that this tendency depend on the influence of BVDV infection or not. Further investigations should be necessary to clarify that possibility.

In conclusion, chapter 2, the involvement of maternal CXCR4 and cytokine gene expressions in the natural occurrence of persistent infection with BVDV was investigated. A dairy herd with high prevalence of PI calves having a single origin of infection was used for this investigation. The combination of low expression of CXCR4 and high expression of IL-10 might be closely concerned with some bias for production of PI animals and increase the prevalence of PI calves.

SUMMARY

In chapter 2, the involvement of maternal CXCR4 and cytokine gene expressions in the natural occurrence of PI production was investigated. The dairy herd with high prevalence of PI calves within a short period having a single origin of infection reported in chapter 1 was used. There were no significant differences in CXCR4 and cytokine gene expressions between the dams of PI calves and the dams of non PI calves in the herd. In the comparison among the herds, CXCR4 expressions in the PI-producing herds were significantly lower than the BVDV-free herd. Moreover, CXCR4 expressions in the high prevalence herd and the low prevalence herd were similar. These findings among herds corresponded with the previously reported experimental production of persistent infection with BVDV in cows. Based on the cytokine profile of these herds, IL-10 was significantly higher in the high prevalence herd and the BVDV-free herd. The combination of low expression of CXCR4 and high expression of IL-10 might be closely concerned with some bias for the production of PI calves.

GENERAL CONCLUSION

Bovine viral diarrhea virus (BVDV) infection has a significant impact on both dairy and beef cattle producers worldwide. Animals persistently infected (PI) with BVDV serve as a continuous source of the virus due to life-long shedding. Early detection and elimination of PI animals are important for the control of BVDV. In addition, the prevention of PI animal production should be considered. In order to prevent PI animal production, the mechanism of persistent infection should be thoroughly investigated. Currently, there is no examination method to detect cows carrying PI fetuses under field conditions prior to birth of the PI calf. It was reported in experimental fetal infections that the maternal immune response has been suspected as closely concerned to the PI calf production. Trials for the detection of dams carrying PI fetuses using the maternal immune response were performed using experimental fetal infection. For the control of BVDV infection, more detailed analyses of the maternal immune response under the natural occurrence of PI calves should be estimated. Thus, the aim of this thesis was to investigate involvement of maternal immune response in the natural occurrence of persistent infection with BVDV and analyze the possible mechanism of PI calf production.

In chapter 1, a dairy herd including 50 milking cows and 40 heifers and calves was selected from natural cases of BVDV-contaminated herds. This herd was detected with high prevalence of BVDV PI calves. Nine PI animals including a milking cow and 8 newborn calves were detected in the herd within 4 months. Prevalence of PI animals in this herd was estimated 7.0%, which was very high compared to that estimated in previous reports. All newborn PI calves were strongly suspected to have a single origin

of infection as estimated from the homology of the virus genes. Moreover, all PI calves were produced from non PI dams. Therefore, this herd was a worthy case to study production of PI calves in a natural occurrence.

In chapter 2, in order to estimate the maternal immune response to the natural occurrence of PI calves, the high prevalence herd investigated in chapter 1 was used as an experimental herd. C-X-C chemokine receptor type 4 (CXCR4) expression and cytokine expressions including interleukin-4 (IL-4), IL-6, IL-10, IL-12p40, interferon - α (IFN- α), IFN- γ and transforming growth factor- β (TGF- β) in the cows of this herd were investigated. There were no significant differences in CXCR4 and cytokine expressions between the dams of PI calves and the dams of non PI calves in the herd. In the comparison among the herds, CXCR4 expressions in the PI-producing herds were significantly lower than the BVDV-free herd. The level of CXCR4 expression in the high prevalence herd was similar to that in the low prevalence herd. Based on the cytokine profiles, the high prevalence herd and the BVDV-free herd had almost same immunological responses. IL-10 was significantly higher in the high prevalence herd and the BVDV-free herd than the low prevalence herd.

In this thesis, maternal CXCR4 and cytokine expressions were investigated in the dams of the herd with high prevalence of PI calves. CXCR4 expressions in the high prevalence herd showed no significant differences between dams of PI calves and non PI calves. These findings indicated that both dams had an equal risk for PI production inspite of the production of non PI calves. CXCR4 expressions in both of the dams in this herd were significantly lower than the BVDV-free herd.

It might be possible to predict the susceptibility of the herd to the transplacental persistent infection based on the maternal immune response. The combination of low expression of CXCR4 and high expression of IL-10 might be closely concerned with some bias for the production of PI calves.

JAPANESE SUMMARY (要 旨)

牛ウイルス性下痢ウイルス(BVDV)感染症は、世界各地で乳肉生産に甚大な被害を及ぼしている感染症である。BVDV 持続感染 (PI) 牛は、終生ウイルスを排出し続けて牛群内に感染源として潜伏し続ける。PI 牛の早期摘発淘汰は、BVDV 対策において重要である。さらに、その際には PI 牛の産出を阻止することも、考慮しておかなければならない。PI 牛の産出を阻止するためには、持続感染機序の解明が必要である。現在、PI 胎子を妊娠している母牛を摘発する検査法は、確立されていない。感染実験条件下では、母牛の免疫応答能が PI 子牛産出に密接に関与していることが示唆されている。母牛の免疫応答状態から PI 胎子妊娠牛を摘発する試みは、実験感染条件下ではなされてきている。BVDV を制圧するためには、自然感染状態下での PI 胎子妊娠牛の摘発法確立が望まれる。本研究では、BVDV 自然感染例における母牛の免疫状態を検査し、PI 子牛産出に関与する免疫応答を検討した。

第 1 章では、検査対象となる牛群を選定した。選定された牛群は、泌乳牛約 50 頭、子牛育成牛約 40 頭を飼養する酪農家で、9 頭の PI 牛が摘発され、清浄化が確認されるまでの PI 牛発生率は 7%であった。この牛群における PI 牛の発生率は既報の報告よりかなり高かった。摘発された PI 牛は、泌乳牛 1 頭、子牛 8 頭であった。泌乳 PI 牛は導入牛で、8 頭の PI 子牛はすべてこの泌乳 PI 牛が感染源であったことが、ウイルス遺伝子の相同性解析から確認された。また、PI 子牛の母牛はすべて PI 牛ではなく、摘発された泌乳 PI 牛を感染源とする急性感染による PI 成立であった。したがって、この牛群の母牛の免疫応答状態を解析することは、PI 成立機序の解明に役立つと考えられた。

第 2 章では、第 1 章で選定した PI 牛高率自然発生牛群(高発生牛群)における母牛の免疫状態を解析した。PI 牛産生に関与するとされている C-X-C ケモカイン受容体 4 型 (CXCR4) およびインターロイキン 4 (IL-4)、IL-6、IL-10、IL-12p40、インターフェロン α (IFN- α)、IFN- γ 、トランスフォーミング増殖因子 β (TGF- β)

などのサイトカイン遺伝子発現量を定量した。高発生牛群内の PI 子牛を産出した母牛と正常子牛の母牛との間に、CXCR4 およびサイトカインの遺伝子発現量に有意差は認められなかった。PI 牛は摘発されたが 1 頭のみであった牛群 (低発生牛群) および PI 牛が摘発されなかった牛群 (フリー牛群) の牛と比較すると、発生率の高低に関係なく PI 牛発生牛群では、CXCR4 の遺伝子発現量は有意に低かった。高発生牛群とフリー牛群においては、IL-10 の遺伝子発現量が低発生牛群より有意に高かった。高発生牛群とフリー牛群は、サイトカインプロファイルはほぼ同様であった。

以上のとおり本研究においては、高発生牛群における母牛の CXCR4 およびサイトカインの遺伝子発現量を検討した。高発生牛群内の PI 子牛を産出した母牛と正常子牛を産出した母牛との間に、CXCR4 発現量の有意差は認められなかった。しかしながら、フリー牛群の牛と比較すると、発生牛群の牛の CXCR4 発現量は有意に低かった。これは、発生牛群で飼養されている牛は PI を産出する危険性を有していることを示唆している。サイトカインプロファイルでは、高発生牛群の方が低発生牛群より IL-10 の発現量が有意に高かった。この免疫状態の違いが、PI 子牛発生率の違いに何らかの影響を及ぼしていることが考えられた。すなわち、PI 牛発生牛群において、CXCR4 の発現率が低く IL-10 の発現量が高い牛群では、PI 子牛が産出されてくる危険性が高いと推測できることが判明した。

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