

Effects of interferon- τ on cattle persistently infected with bovine viral diarrhea virus

Junko Kohara^{1)*}, Yumiko Nishikura²⁾, Satoru Konnai²⁾,
Motoshi Tajima³⁾ and Misao Onuma²⁾

¹⁾Animal Research Center, Hokkaido Research Organization, Shintoku, Hokkaido 081-0038, Japan

²⁾Laboratory of Infectious Diseases, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan

³⁾Veterinary Teaching Hospital, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan

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Abstract

In this study, the antiviral effects of bovine interferon- τ (boIFN- τ) on bovine viral diarrhea virus (BVDV) were examined *in vitro* and *in vivo*. In the *in vitro* experiments, the replication of cytopathic and non-cytopathic BVDV was inhibited in the bovine cells treated with boIFN- τ . The replication of BVDV was completely suppressed by boIFN- τ at a concentration higher than 10^2 U/ml. In order to examine the effect of boIFN- τ on virus propagation in cattle persistently infected (PI) with non-cytopathic BVDV, boIFN- τ was subcutaneously administered to PI cattle at 10^5 U/kg or 10^6 U/kg body weight 5 times per week for 2 weeks. No physical abnormality such as depression was observed in the cattle during the experiment. The mean BVDV titers in the serum of the PI cattle decreased slightly during the boIFN- τ administration period with the dose of 10^6 U/kg. However, the BVDV titers in the serum returned to the pre-administration level after the final boIFN- τ administration. These results suggest that boIFN- τ demonstrates an anti-BVDV effect, reducing the BVDV level in serum transiently when injected into PI cattle.

Key words: Bovine viral diarrhea virus, Cattle, Interferon- τ , Persistent infection

Introduction

Bovine viral diarrhea virus (BVDV) is distributed worldwide and is responsible for serious economic losses in cattle herds. BVDV causes a variety of disease syndromes in cattle, such as diarrhea, respiratory disease, abortion, congenital

malformation, and fatal mucosal disease^{1,3)}. When a susceptible cow is exposed to non-cytopathic (NCP) BVDV in the first trimester of pregnancy, a newborn calf can be persistently infected (PI) with BVDV and immunotolerant to the infecting virus. In PI cattle, the virus is present throughout the organs and is shed from multiple sites

*Corresponding author: Kohara Junko, Animal Research Center, Hokkaido Research Organization, Shintoku, Hokkaido 081-0038, Japan.

Phone: +81-156-64-5321. Fax: +81-156-64-5349. E-mail: kohara-junko@hro.or.jp

including nasal discharge, urine, milk, and blood. Therefore, PI cattle are a serious infectious source of BVDV for susceptible cattle and there is no effective method to eliminate the virus from PI cattle.

Interferons (IFNs) are cytokines with various biological activities, including inhibition of virus replication and immune activation. IFNs have been widely used in antiviral therapy for humans. On the other hand, clinical use of IFNs for domestic animals has been limited. IFN- τ was first identified as an antiluteolytic protein that is secreted by trophoblasts of ruminants, and it was then classified as a type I IFN on the basis of its amino acid homology with IFN- α ^{4,15)}. Type I IFNs α/β are produced by virus-infected cells within a few hours of viral invasion, accumulate at high level within a few days, and act in the innate immune system before the development of the acquired immune response. Ovine IFN- τ demonstrates antiviral activities against human or feline immunodeficiency virus and human papillomavirus infection *in vitro* similar to IFN- α ^{5,14)}, and bovine IFN- τ inhibits the replication of bovine leukemia virus⁸⁾. Type I IFNs have cross-species reactivity, however, IFNs are species-specific and its activity *in vivo* may be lower than expected when administered to nonhomologous species¹³⁾. Therefore, it is expected that IFN- τ , which is exclusively produced in ruminants, exhibits high antiviral activities in cattle. Ovine IFN- τ also shows less toxicity than human IFN- α ²²⁾. Thus, IFN- τ will be a promising agent for managing BVDV infections in cattle with less IFN-inducing toxic effects.

In this study, we examined the effects of bovine IFN- τ on the replication of BVDV *in vitro* and the reduction of viral load in cattle persistently infected with BVDV.

Materials and Methods

Viruses and virus titration: Japanese reference strains of cytopathic (CP) BVDV, Nose (genotype

1a), KZ-91-CP (genotype 2a), non-cytopathic (NCP) BVDV, No. 12 (genotype 1a), and field isolates, Chitose287 (genotype 1b) and Shimukappu (genotype 2a)^{7,9,11)}, were propagated in bovine fetal muscular (BFM) cells. The cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum free of both BVDV and anti-BVDV antibody.

The CP viruses were titrated by the cytopathogenic effect (CPE) method and the NCP viruses were titrated by the immunoperoxidase monolayer assay (IPMA) modified from a previously described method¹⁷⁾. Serial 10-fold dilutions of virus sample were inoculated into 4 wells of BFM cultures in 96-well plates with 0.1 ml of each dilution and the plates were incubated at 37°C in 5% CO₂ for 4 days. The infectivity of CP viruses was determined from the appearance of CPE. In the case of the NCP viruses, the plates were air-dried after removal of the medium, fixed at 75°C for 90 min., and the virus titers was determined by IPMA using monoclonal antibody (MAb) JCU/BVD/CF10 (mouse IgG anti-pestiviruses, TropBio Pty Ltd., Queensland, Australia). The plates were incubated with MAb for 1 hr at room temperature, washed two times in PBS containing 0.05% Tween 20 (PBST), and then treated with horseradish peroxidase (HRP) anti-mouse IgG for 1 hr at room temperature. After washing in PBST, the plates were supplemented with a solution containing 5% AEC solution (20 mg of 3-amino-9-ethylcarbazole dissolved in 5 ml of dimethylsulfoxide), 0.015% H₂O₂ in 50 mM acetate buffer (pH 5.0), and incubated in the dark for more than 30 min. The buffer was then decanted, and the plates were washed and allowed to air-dry. A positive reaction was characterized by the appearance of red cytoplasmic staining in at least 1 cluster of cells.

Interferon- τ : Recombinant bovine interferon- τ (boIFN- τ) was prepared using a silkworm-baculovirus gene expression system¹⁰⁾. The antiviral activity of the boIFN- τ was determined by measuring the inhibition of the CPE of the vesicular stomatitis

virus (VSV) with the Madin-Darby bovine kidney (MDBK) cell line. This antiviral activity assay was calibrated against a human IFN- α reference standard (interferon alpha 2b, human, 2nd International Standard, 1999).

In vitro antiviral activity of boIFN- τ against BVDV: To determine the quantitative antiviral effect of boIFN- τ on BVDV replication in infected cells, BFM cells were cultivated in 24-well tissue culture plates at 37°C for 24 hr and 0.1 ml of different concentrations of boIFN- τ (0–10³ U/ml) was added. The cells were cultivated for 24 hr and then were challenged with 0.1 ml of BVDV fluid containing 10³ TCID₅₀/0.1 ml of infective titers. After 4 days of incubation, each culture supernatant was collected for evaluation of virus replication. Infective titers in the culture were compared to those in control culture without IFN.

Animals and experimental design: Six Holstein PI cattle that were persistently infected with NCP genotype 1 BVDV were used in the experiments (Table 1). The PI cattle were housed under controlled conditions. Three cows (PI-1 - PI-3) were subcutaneously injected with 10⁵ U/kg body weight of boIFN- τ , while three other cows (PI-4 - PI-6) were injected with 10⁶ U/kg body weight of boIFN- τ . The cattle were injected with boIFN- τ for two rounds of daily injections for five days from Day

0 to Day 4 (the first round) and from Day 7 to Day 11 (the second round).

Clinical signs were monitored throughout the experimental period from 7 days before the beginning of the first boIFN- τ administration (Day -7) until 10 days after the end of the second round of boIFN- τ administrations (Day 21). General attitude, appetite, and body temperature were observed.

Measurement of 2'-5'oligoadenylate synthetase activity in serum: 2'-5'oligoadenylate synthetase (OAS) activity in serum of cattle was measured at 0, 3, 6, 9, and 24 hr after the first administration of boIFN- τ using a radioimmunoassay kit (2 5A Kit Eiken, Eiken Chemical, Tokyo, Japan).

Measurement of viral load of BVDV in PI cattle: Viral load of BVDV in the serum of PI cattle was determined at the end of the first round of boIFN- τ administration (Day 4), 3 days after the end of the first round of boIFN- τ administration (Day 7), at the end of the second round of boIFN- τ administration (Day 11), and 3 days and 10 days after the end of the second round of boIFN- τ (Day 14 and Day 21). The BVDV in serum was titrated by the IPMA methods described above. To confirm the viral titers in the individual PI cattle were stable before boIFN- τ administration, the virus titers in the serum of each PI cattle was measured

Table 1. PI cattle used for *in vivo* experiment

Animal no.	Age (month)	Isolated BVDV biotype, genotype	BVDV titers in the serum ^a (log ₁₀ TCID ₅₀ /ml)	
Cattle administered with 10 ⁵ U/kg of boIFN-τ				
PI-1	18	NCP, 1a	4.7	0.4
PI-2	43	NCP, 1b	3.9	0.3
PI-3	56	NCP, 1b	4.1	0.3
Cattle administered with 10 ⁶ U/kg of boIFN-τ				
PI-4	18	NCP, 1a	4.7	0.2
PI-5	34	NCP, 1b	4.1	0.4
PI-6	44	NCP, 1b	3.9	0.8

^aBVDV titers were measured three different times prior to the start of the *in vivo* experiment and are expressed as the mean ± S.D.

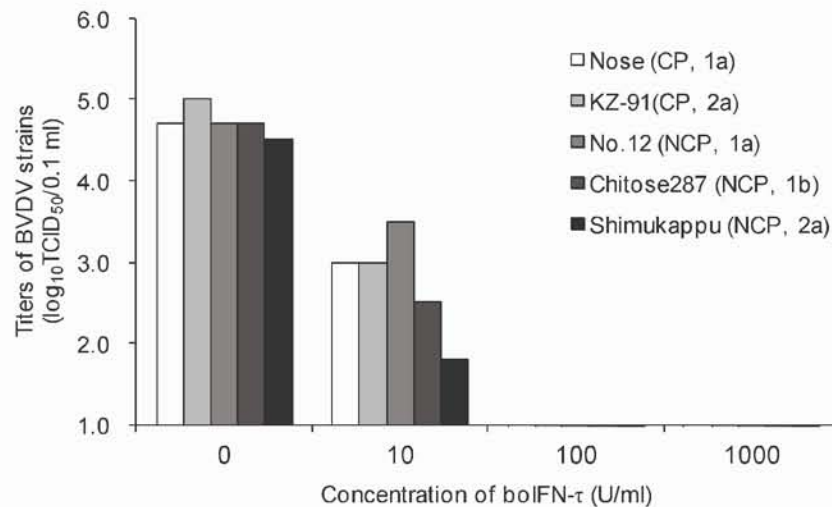


Fig. 1. Titters of BVDV strains in boIFN- τ -treated cells. Bovine fetal muscular cells were treated with boIFN- τ for 24 hr and then challenged separately with BVDV strains. After 4 days of incubation, the viral infective titers were determined. The viral titers are given as \log_{10} TCID₅₀/0.1 ml.

at three different times before the administration (from Day -7 to Day 0). The mean viral titers in the pre-administration period were used as non-boIFN- τ administration control.

Statistical analysis: The two-tailed Student's *t*-test was employed for statistical analyses. Differences between groups were determined to be significant when $P < 0.05$.

Results

In vitro antiviral effects of boIFN- τ on BVDV

The titers of BVDVs, genotype 1 or 2 of either CP or NCP strain, were reduced in BFM cells treated with 10 U/ml boIFN- τ to between 1/16 and 1/500 compared with that in untreated cells (Fig. 1). The replication of all the BVDV strains was completely suppressed at a concentration higher than 10² U/ml for boIFN- τ .

BVDV-infected animals injected with boIFN- τ

In order to examine the *in vivo* effects of boIFN- τ , PI cattle were injected with boIFN- τ . Six and nine hours after the first administration of boIFN- τ , the body temperature of BVDV PI cattle significantly ($P < 0.01$) went up, and then

the temperature went down to the initial level within 24 hr (Figs. 2A and B). During the experimental period, boIFN- τ -injected cattle did not show any physical abnormalities and any adverse effects of IFN toxicity, such as anorexia or depression.

OAS activity in serum and PBMC of PI cattle administered with boIFN- τ

OAS activity has been used in clinical chemistry as a reliable marker of IFN action *in vivo*²¹. We confirmed the biological activity of the boIFN- τ *in vivo* by measuring OAS activity in serum of experimental cattle through 24 hr after the first administration of boIFN- τ (Figs. 2C and D). OAS activity in each cattle was slightly elevated by the boIFN- τ administration. OAS activity in the cattle administered with 10⁵ U/kg boIFN- τ most increased at between 6 and 9 hr, and it stayed higher than level than the initial activity at 24 hr (Fig. 2C). On the other hand, in the cattle administered with 10⁶ U/kg boIFN- τ , the elevation of the activity was more obvious and still increasing at 24 hr (Fig. 2D).

BVDV titers in the serum of PI cattle administered with boIFN- τ

The antiviral effect of boIFN- τ on BVDV in PI cattle is shown in Fig. 3. With the dosage of

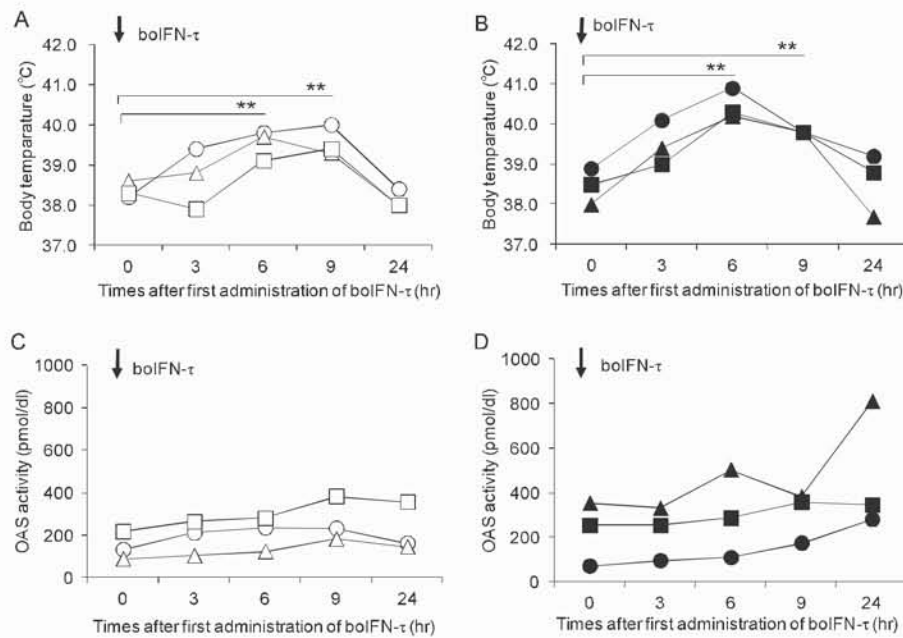


Fig. 2. Body temperature (A and B) and OAS activity (C and D) in serum of PI cattle after first administration of bolFN- τ . A and C; PI cattle (○PI-1, △PI-2 and □PI-3) administered with 10^5 U/kg of bolFN- τ . B and D; PI cattle (●PI-4, ▲PI-5 and ■PI-6) administered with 10^6 U/kg of bolFN- τ . The periods in which a significant difference ($**P < 0.01$) was observed are indicated with asterisks.

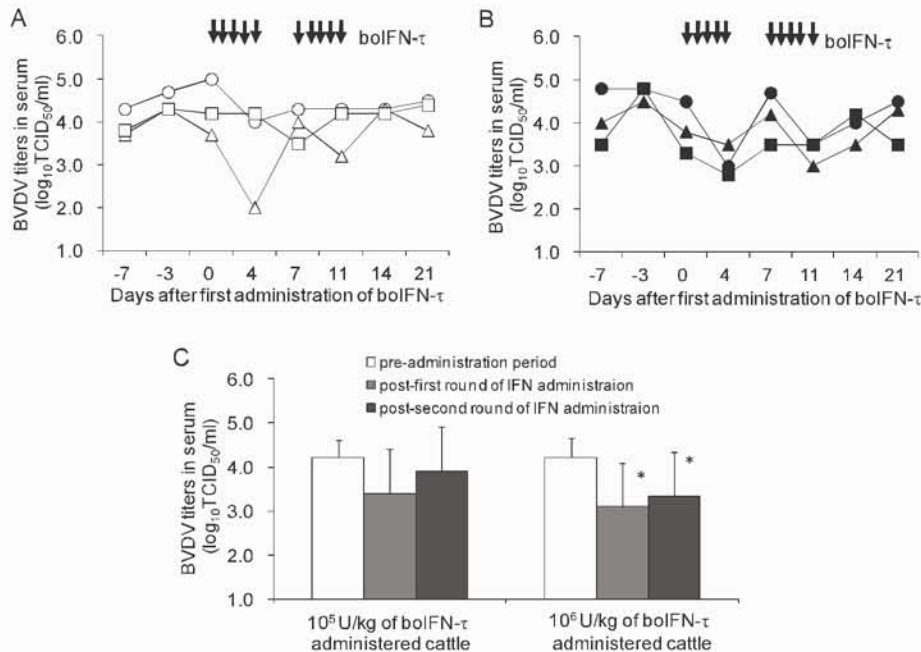


Fig. 3. BVDV titers in serum of PI cattle administered with bolFN- τ for two rounds of 5-day-daily (first round, from Day 0 to Day 4; second round, from Day 7 to Day 11, indicated by arrows). A; BVDV titers in PI cattle (○PI-1, △PI-2 and □PI-3) administered with 10^5 U/kg of bolFN- τ . B; BVDV titers in PI cattle (●PI-4, ▲PI-5 and ■PI-6) administered with 10^6 U/kg of bolFN- τ . C; Mean BVDV titers in the pre-administration period (from Day -7 to Day 0), at the post-first round of administration (Day 4) and at the post-second round of administration (Day 11). Asterisks represent significance relative to pre-administration period. $*P < 0.05$.

10^5 U/kg body weight of boIFN- τ , BVDV titers in the serum of two of three cows (PI-1 and PI-2) decreased during the first round of boIFN- τ administration and BVDV titers in the serum of one of three cows (PI-2) decreased during the second round of boIFN- τ . However, the BVDV titers returned to the pre-administration level at three days after the end of both first and second rounds of boIFN- τ administration (Day 7 and Day 14) (Fig. 3A). With the dosage of 10^6 U/kg body weight of boIFN- τ , BVDV titers in the serum of all the three cows (PI-4, PI-5 and PI-6) decreased during the first round of the administration, followed by a return to the pre-administration level at three days after the end of the first round of the administration (Day 7). The BVDV titers of two of three cows (PI-4 and PI-5) decreased during the second round, but returned to the pre-administration level at 10 days after the end of the second round (Day 21) (Fig. 3B). Administration with 10^6 U/kg boIFN- τ was more effective than 10^5 U/kg boIFN- τ in decreasing the degree of BVDV titer in serum of PI cattle and showed a longer duration of effectiveness (Fig. 3C). Mean BVDV titers in the cattle, both at the post-first and post-second rounds of the administration (Day 4 and Day 11), were significantly less than the titer in the pre-administration period (from Day -7 to Day 0).

Discussion

We firstly showed that boIFN- τ exhibits antiviral activity against BVDV *in vitro*. For all the BVDVs tested, their replication was inhibited by boIFN- τ of higher than 10^2 U/ml (Fig. 1). This finding indicates that the level of antiviral activity of boIFN- τ against BVDV is similar to or higher than that of IFN- $\alpha^{12,20}$ and that boIFN- τ will might suppress the virus shedding from PI cattle.

In the experiments *in vivo* followed, the PI cattle injected with boIFN- τ showed transient fever after the first administration of boIFN- τ (Figs. 2A and B). It reflects the biological activity

of boIFN- τ in animals. Apart from transient fever, boIFN- τ did not induce any severe side effects even when it was administered to PI cattle at high dose. OAS activity in the serum of individual cattle tends to increase by the IFN- τ administration (Figs. 2C and D). However, the elevation of the activity was not statistically significant. On the other hand, we demonstrated that BVDV in PI cattle reduced by administration of IFN- τ (Fig. 3). The difference in antiviral efficiency observed between 10^5 U/kg and 10^6 U/kg might reflect the dose-dependent antiviral effect by IFN- $\tau^{8,14}$.

The anti-viral effect of boIFN- τ in PI cattle was transient and weak, and the viral load returned to the initial level when the administration of boIFN- τ stopped (Fig. 3). One of the reasons that causes unsustainable antiviral state might be insufficient activity of OAS *in vivo*. OAS is up-regulated in response to viral infection and IFN induction, resulting in degradation of viral RNA and inhibition of virus replication. OAS induced by IFN is responsible for the resistance of cells against viral infection. Like other type I IFNs, IFN- τ is shown to be an effective inducer of OAS, and the antiviral activity of IFN- τ is considered to be mainly regulated through OAS^{6,16}. Therefore, significant induction of OAS activity and the subsequent induction of endogenous type I IFNs will be necessary to exert antiviral effects of IFN- τ *in vivo*. The weak response in PI cattle to exogenously injected IFN- τ might also be due to negative feedback systems that modulate the response to persistent viral stimulus of a persisting viremia and prevent hyperstimulation of IFNs. Infection of NCP BVDV to bovine fetuses during the first 120 days of gestation can cause the birth of PI calves that are immunotolerant to BVDV. In PI cattle, the infected NCP BVDV is not recognized as a foreign antigen, and the immunotolerance is specific to the infecting NCP strain of BVDV. Interestingly, NCP BVDV does not induce endogenous type I IFN production in its host cells. Since type I IFNs play an important role in antiviral defense of the innate immune system,

suppression of the signaling pathway of IFNs will be essential to establish persistent infection. For maintenance of persistent infection, BVDV is resistant to type I IFN and evade of the innate immune system^{2,18,19}. Therefore, it might be difficult to demonstrate the effect of antiviral drugs in the cattle persistently infected with BVDV.

In this study, boIFN- τ demonstrated antiviral activity against BVDV *in vitro*, and injection of boIFN- τ to PI cattle resulted in reduction of serum virus load without IFN side effects. Our findings suggest that boIFN- τ might be used for controlling of BVDV infection in cattle. For example, if boIFN- τ could inhibit virus propagation in acutely infected healthy cattle, boIFN- τ will be possible to prevent BVDV-induced immunosuppression and bovine respiratory disease complex, or transplacental infection. And boIFN- τ will be possible to reduce the risk of developing fatal mucosal disease in PI cattle. To use IFN- τ for clinical purposes in animals including the therapies for acute viral diseases or opportunistic infections, further investigation is necessary to determine if the antiviral effects of IFN- τ are extendable to other viral infections and if IFN- τ can modulate the immune system in animals.

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References

- 1) Baker, J. C. 1995. The clinical manifestations of bovine viral diarrhea infection. *Vet. Clin. North Am. Food Anim. Pract.*, **11**: 425 445.
- 2) Charleston, B., Fray, M. D., Baigent, S., Carr, B. V. and Morrison, W. I. 2001. Establishment of persistent infection with non-cytopathic bovine viral diarrhea virus in cattle is associated with a failure to induce type 1 interferon. *J. Gen. Virol.*, **82**: 1893 1897.
- 3) Houe, H. 1999. Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. *Vet. Microbiol.*, **64**: 89 107.
- 4) Imakawa, K., Anthony R. V., Kazemi, M., Marotti, K. R., Polites, H. G. and Roberts, R. M. 1987. Interferon-like sequence of ovine trophoblast protein secreted by embryonic trophectoderm. *Nature*, **330**: 377 379.
- 5) Johnson, J. A., Hochkeppel, H. K. and Gangemi, J. D. 1999. IFN-tau exhibits potent suppression of human papillomavirus E6/E7 oncoprotein expression. *J. Interferon Cytokine Res.*, **19**: 1107 1116.
- 6) Johnson, G. A., Stewart, M. D., Gray, C. A., Choi, Y., Burghardt, R. C., Yu-Lee, L., Bazer, F. W. and Spencer, T. E. 2001. Effects of the estrous cycle, pregnancy, and interferon tau on 2', 5'-oligoadenylate synthetase expression in the ovine uterus. *Biol. Reprod.*, **64**: 1392 1399.
- 7) Kodama, K., Sasaki, N., Fukuyama, S., Izumida, A. and Ishii, F. 1974. Studies on cytopathogenic bovine viral diarrhea virus recovery, identification, and properties of the isolated virus. *Bull. Nippon Vet. Zootech. Coll.*, **23**: 51 60.
- 8) Kohara, J. and Yokomizo, Y. 2007. *In vitro* and *in vivo* effects of recombinant bovine interferon- τ on bovine leukemia virus. *J. Vet. Med. Sci.*, **69**: 15 19.
- 9) Nagai, M., Sato, M., Nagano, H., Pang, H., Kong, X., Murakami, T., Ozawa, T. and Akashi, H. 1998. Nucleotide sequence homology to bovine viral diarrhea virus 2 (BVDV 2) in the 5' untranslated region of BVDVs from cattle with mucosal disease or persistent infection in Japan. *Vet. Microbiol.*, **60**: 271 276.
- 10) Nagaya, H., Kanaya, T., Kaki, H., Tobita, Y., Takahashi, M., Takahashi, H., Yokomizo, Y. and Inumaru, S. 2004. Establishment of a large-scale purification procedure for purified recombinant bovine interferon- τ produced by a silkworm-baculovirus gene expression system. *J. Vet. Med. Sci.*, **66**: 1395 1401.
- 11) Omori, T., Inaba, Y., Morimoto, T., Tanaka, Y., Kurogi, H. and Matsumoto, M. 1967. Bovine diarrhea virus. I. Isolation of non-cytopathogenic