



HOKKAIDO UNIVERSITY

Title	Studies on bovine viral diarrhea virus in quality control of veterinary vaccines
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Degree Grantor	北海道大学
Degree Name	博士(獣医学)
Dissertation Number	乙第7070号
Issue Date	2019-03-25
DOI	https://doi.org/10.14943/doctoral.r7070
Doc URL	https://hdl.handle.net/2115/74793
Type	doctoral thesis
File Information	Takashi_KOZASA.pdf



Studies on Bovine Viral Diarrhea Virus in Quality

Control of Veterinary Vaccines

動物用ワクチンの品質管理における

牛ウイルス性下痢ウイルスに関する研究

Takashi KOZASA

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Abbreviations

AKAV:	Akabane virus
BAd7V:	bovine adenovirus 7
BES:	N,N-Bis (2-hydroxyethyl)-2-aminoethanesulfonic acid
BFM:	bovine fetal muscle
BHV-1:	bovine herpesvirus-1
BLV:	bovine leukemia virus
BPI3V:	bovine parainfluenza virus 3
BRSV:	bovine respiratory syncytial virus
BT:	bovine testicle
BVD:	bovine viral diarrhea
BVD-MD:	bovine viral diarrhea-mucosal disease
BVDV:	bovine viral diarrhea virus
BVDV-1:	bovine viral diarrhea virus 1
BVDV-2:	bovine viral diarrhea virus 2
BVDV-3:	bovine viral diarrhea virus 3
CP:	cytopathogenic
CPE:	cytopathic effect
CSF:	classical swine fever
CSFV:	classical swine fever virus
ELISA:	enzyme-linked immunosorbent assay
EMEM:	Eagle's minimum essential medium
END:	exaltation of Newcastle disease virus
END ⁺ :	END-phenomenon-positive
END ⁻ :	END-phenomenon-negative
FBS:	fetal bovine serum

GMT:	geometric mean titer
HA:	hemagglutination
HAU:	HA unit
HI:	hemagglutination inhibition
HS:	horse serum
IBAV:	Ibaraki virus
IBR:	infectious bovine rhinotracheitis
ICTV:	International Committee on Virus Taxonomy
IPMA:	immunoperoxidase monolayer assay
IRF:	interferon regulatory factor
KV:	killed vaccine
MLV:	modified-live vaccine
MD:	mucosal disease
MM:	maintenance medium
NCP:	non-cytopathogenic
NDV:	Newcastle disease virus
PBS:	phosphate-buffered saline
PI:	persistently infected
PMD Act:	Act on Securing Quality, Efficacy and Safety of Products including Pharmaceuticals and Medical Devices
RBC:	red blood cell
RFLP:	restriction fragment length polymorphism
RT-PCR:	reverse transcription-polymerase chain reaction
SDS-PAGE:	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPase:	signal peptidase
TPB:	tryptose phosphate broth

UTR	untranslated region
VMPs	veterinary medicinal products
VSV:	vesicular stomatitis virus

Notes

Contents of the present thesis were published in the following articles.

1. **Kozasa T, Aoki H, Nakajima N, Fukusho A, Ishimaru M, Nakamura S.** 2011. Methods to select suitable fetal bovine serum for use in quality control assays for the detection of adventitious viruses from biological products. *Biologicals*. **39**: 242-248.
Copyright © 2011 The International Alliance for Biologicals. Published by Elsevier Ltd.
2. **Kozasa T, Abe Y, Mitsuhashi K, Tamura T, Aoki H, Ishimaru M, Nakamura S, Okamatsu M, Kida H, Sakoda Y.** 2015. Analysis of a pair of END⁺ and END⁻ viruses derived from the same bovine viral diarrhea virus stock reveals the amino acid determinants in N^{pro} responsible for inhibition of type I interferon production. *J. Vet. Med. Sci.* **77**: 511-518.
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3. **Kozasa T, Torii S, Kameyama, K, Nagai M, Isoda N, Shiokawa M, Aoki H, Okamatsu M, Sekiguchi H, Saito A, Sakoda Y.** 2018. Prevalence of HoBi-like viruses in Japan between 2012 and 2017 based on virological methods and serology. *Jpn. J. Vet. Res.* **66**: 317-324.
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General Introduction

Current regulation on veterinary vaccines

Vaccines are essential for the prevention and control of infectious diseases in humans and animals. Looking back on the history of the control of infectious diseases by vaccines in the veterinary field, rinderpest has been eradicated by the use of vaccines, including lapinized and tissue culture products, worldwide [15], and Japan had been classical swine fever (CSF) free for 26 years by the successful eradication by the use of the vaccine developed in Japan until CSF reoccurred in 2018. Genetically engineered products, including component and vector vaccines, have been gradually increased; however, the importance of classical vaccines remain the same. Classical vaccines include modified-live and killed vaccines (MLV and KV, respectively). Both MLV and KV have advantages and disadvantages. Basically, one dose of MLV is enough to induce cellular and humoral immunity in vaccinated hosts; however, efficacy of MLV is affected by maternal antibodies. In addition, MLV have risks for reversion to virulence of vaccine strains and for contamination of extraneous agents. Inactivation of components makes KV safer than MLV. However, adjuvants are usually needed for KV to induce enough immunity and this may increase the risk of adverse events. Booster vaccination is usually required for KV.

While veterinary vaccines significantly contribute to the prevention and control of infectious diseases, lack of quality, efficacy and safety of these vaccines leads to the unsuccessful control of targeted diseases, the increase of adverse events and even worse to the unexpected and undesired spread of infectious diseases occurred by the contaminated pathogens inside the vaccines. Veterinary vaccines are manufactured by using raw materials of animal origin, and therefore, risks, that extraneous viruses derived from these materials could contaminate them, are always present. In fact, the

contamination of veterinary vaccines by animal pathogens has been reported worldwide (Table 1), and the origins of these pathogens were considered as raw materials used for the production of veterinary vaccines including virus seeds, cells and fetal bovine sera (FBS). Of these viruses, the most frequently involved contaminants are pestiviruses [64]. It was also indicated that emerging pestiviruses, HoBi-like viruses, were introduced into the European continent via contaminated biological products [5, 20].

Veterinary medicinal products (VMPs) including veterinary vaccines are regulated worldwide in each country or region by the relevant competent authorities and regulations. In Japan, marketing approvals in each product by the Minister of Agriculture, Forestry and Fisheries are required to market VMPs in accordance with the Act on Securing Quality, Efficacy and Safety of Products including Pharmaceuticals and Medical Devices (PMD Act). Approvals are granted if the products are efficacious and safe with appropriate quality. The PMD Act regulates each stage of development, manufacturing, importing, retailing and usage of the products. To ensure quality, efficacy and safety of each lot of veterinary vaccines, the PMD Act requests quality control assays in the manufacturing process and for final products by manufacturers. In addition, veterinary vaccines designated by the Minister of Agriculture, Forestry and Fisheries are required to pass the examination by the National Veterinary Assay Laboratory. Quality control assays in final products include sterility test, virus titration test, extraneous virus testing, potency test, and target and/or laboratory animal testing. One of the major targets of quality control assays in veterinary vaccines is bovine pestivirus.

Bovine viral diarrhea virus

Bovine viral diarrhea (BVD) was firstly reported as the new transmissible disease in cattle causing leukopenia, high fever, depression, diarrhea and dehydration, anorexia,

salivation, nasal discharge, gastrointestinal erosions, and hemorrhages in various tissues, in the State of New York, the US in 1946 [23]. In the same year, after the first report of BVD, a similar but more severe disease in cattle causing fever, anorexia, depression, profuse salivation, nasal discharge, gastrointestinal hemorrhages, erosions and ulcers, and severe diarrhea with watery feces that were sometimes mixed with blood, later named as mucosal disease (MD), was reported in Canada [23]. Virus neutralization studies in 1960s revealed that BVD and MD were different disease manifestations caused by the same virus; therefore, the disease was officially known as bovine viral diarrhea-mucosal disease (BVD-MD) [23]. Despite the name of the disease, the causative agent of BVD-MD is still named as bovine viral diarrhea virus (BVDV) and currently the name BVD or BVDV infection is commonly used officially and/or in the literature to show this disease. In late 1980s through 1990s, the new type of BVDV spread in the North America and caused outbreaks of hemorrhagic syndrome (severe thrombocytopenia with hemorrhages), and acute and severe BVD, both with high mortality. These viruses were antigenically and genetically different from classical BVDV; therefore, BVDV was segregated into two groups, BVDV-1 for classical BVDV and BVDV-2 for the new type of BVDV [65, 68].

BVDV belongs to the genus *Pestivirus* of the family *Flaviviridae*. Some Scandinavian countries successfully eradicated BVDV and some countries started national eradication program; however, BVDV are still distributed in many countries worldwide including North America, Europe and Japan where many vaccines against BVD have been developed. BVDV are economically important pathogen in the veterinary fields because it causes a lot of economic losses directly and/or indirectly in the affected herds. In addition to BVDV-1 and BVDV-2, HoBi-like virus, also referred to as BVDV-3, was firstly isolated from FBS of Brazilian origin [79]. Subsequently, HoBi-like viruses have been isolated from FBS of South American

origin and cattle and/or water buffaloes in South America, Southeast Asia and Europe [5]. BVDV-1 and BVDV-2 are isolated in Japan, and vaccines, MLV and KV, were developed and used to control them; however, the prevalence of HoBi-like viruses remains unclear and efficacy against HoBi-like viruses of currently available vaccines containing BVDV-1 and/or BVDV-2 need to be investigated.

Until recently, the genus *Pestivirus* comprised four recognized species by the International Committee on Virus Taxonomy (ICTV), BVDV-1, BVDV-2, classical swine fever virus (CSFV) and Border disease virus. In addition to these recognized species, other pestiviruses, including Giraffe virus, HoBi-like virus, Pronghorn virus and Bungowannah virus, were isolated and proposed to be included in this genus [5, 12]. At its meeting in 2017, the ICTV adopted new classification of the genus *Pestivirus*. According to this, pestiviruses reported until today were proposed to be classified as species shown in Table 2. In the new classification, BVDV-1, BVDV-2 and HoBi-like viruses are classified as species *Pestivirus A*, *Pestivirus B* and *Pestivirus H*, respectively, and these three species are designated as causative agents of BVD/MD [83]. Despite the new names of species, it was determined that virus names could be used as before. In the present thesis, the classical names (BVDV-1, BVDV-2 and HoBi-like viruses) to show each species is used.

Clinical manifestations of BVDV infection ranges from subclinical to overt diseases, including immunosuppression, growth retardation, depression, anorexia, oculonasal discharge, occasionally oral lesions characterized by erosions and ulcerations, diarrhea, and a decrease in milk production in lactating cattle [4]. Transplacental infection with non-cytopathogenic (NCP) BVDV leads to the various manifestations depending on the gestation stage, including abortion, death of fetus, immunotolerance to affected BVDV, malformation and antibody responses in fetus [4]. Immunotolerance leads to the birth of persistent infected (PI) animals. PI animals

spread infectious BVDV in the herd during their lives; therefore, PI animals are the source of BVDV in the herd. Some PI cattle succumb to MD and cytopathogenic (CP) BVDV, generated from and antigenically similar to PI NCP viruses, is isolated in these animals [90].

The genome of pestiviruses is a positive-sense, single-stranded RNA of approximately 12.3 kb in length. The single open reading frame, flanked by 5' and 3' untranslated regions (UTRs), encodes nonstructural and structural proteins, N^{pro}, C, E^{rns}, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B [90]. C, E^{rns}, E1 and E2 are structural proteins and others are nonstructural proteins. N^{pro} is the unique protein for pestiviruses in the family *Flaviviridae* and autocatalytically cleaves itself from the nascent polyprotein to generate the N terminus of C protein. C/E^{rns} site is cleaved by cellular signal peptidase (SPase) followed by signal peptide peptidase. E^{rns}/E1, E1/E2, E2/p7 and p7/NS2 sites are processed by SPase. NS2/NS3 site is cleaved by a protease activity located within NS2. Other downstream sites are processed by the NS3 protease [90].

For the genetic classification of pestiviruses, 5'UTR, N^{pro} and E2 are frequently used. Pestiviruses are segregated into each species by phylogenetic analysis (Fig. 1). Two biotypes of pestiviruses, CP and NCP viruses, are distinguished by their ability to induce a cytopathic effect (CPE) in tissue cultures [50]. It is reported that CPE of CP BVDV disappears when CP BVDV superinfects cells which are already infected with NCP BVDV (homologous interference) [32]. In addition, NCP pestiviruses are divided into two groups based on their ability to induce exaltation of Newcastle disease virus (END) phenomenon, END-phenomenon-positive (END⁺) and END-phenomenon negative (END⁻) viruses. END⁺ viruses subvert host innate immune defenses by preventing type I interferon production *in vitro* and the CPE of superinfected Newcastle disease virus (NDV) is exalted. END⁻ viruses do not enhance NDV, but induce intrinsic

interference against Western equine encephalitis virus and vesicular stomatitis virus (VSV) (Fig. 2) [30, 54]. In the quality control assays of veterinary vaccines in Japan, these biological characters shown by pestiviruses are used in accordance with the regulations. Homologous interference is applied to detect extraneous NCP BVDVs in seed viruses and cell seeds, final products of modified-live bovine vaccines and FBS for the use in the production of MLV. Homologous interference and END phenomenon are used for the titration of NCP BVDVs in vaccines. END phenomenon is also used to detect extraneous CSFVs in modified-live swine vaccines.

Homologous interference is induced by dual mechanism which protects cells by superinfection of homologous BVDV at entry and RNA replication [47]. END phenomenon of pestiviruses is induced by the inhibition of type I interferon production *in vitro* via proteasomal degradation of cellular interferon regulatory factor (IRF)-3. The zinc-binding TRASH motif of pestivirus N^{pro}, including the zinc-binding residues Cys112-Cys134-Asp136-Cys138, is required for IRF-3 binding and for the prevention of type I interferon production [33, 85]. It has been reported for CSFV that amino acid substitutions, C112A/R, C134A, D136N or C138A in N^{pro}, result in the disappearance of this function and that mutation of a single amino acid residue (R112C and N136D) restored the N^{pro} functions of wild-type END⁻ viruses [73, 85, 88]. The past studies indicated that nearly the entire N^{pro} is required in BVDV for the prevention of type I interferon production based on the deletion study of amino acid residues, and substitutions of amino acid residue L8P, E22V or H49L of N^{pro} abolished this function [16, 31, 36]. Until date, however, the basic mechanism by which BVDV inhibits type I interferon production has not been approached through engineering *in vitro*-rescued viruses with mutations on the basis of the differences between a pair of END⁺ and END⁻ viruses. It is important for regulatory authorities to uncover the basic mechanism of END phenomenon to be used for the detection of NCP BVDVs in veterinary vaccines.

As shown above, quality control assays of veterinary vaccines are conducted to confirm that these products are efficacious and safe; they should not contain extraneous viruses. BVDV-1, BVDV-2 and HoBi-like viruses induce economically important disease, and these viruses are also known as the risk factors to contaminate veterinary vaccines via raw materials of animal origin; therefore, regulatory authorities of veterinary vaccines need to pay attention to them. Thus, in this thesis, the author focuses on the quality control of vaccines and targets the quality, efficacy and safety of veterinary vaccines, including BVD vaccines.

This thesis consists of three chapters. In chapter I, methods for the selection of FBS for the use in quality control assays to detect extraneous viruses, including BVDVs, in seed viruses, cell seeds, final products of modified-live bovine vaccines and FBS for use in the production of modified-live veterinary vaccines is described. In chapter II, mechanism of END phenomenon induced by BVDV is demonstrated. In chapter III, prevalence of HoBi-like viruses in Japan and cross-reactivity of available BVD vaccines including BVDV-1 and/or BVDV-2 against HoBi-like viruses in cattle sera collected in Japan are shown.

Table 1. Examples of extraneous viruses contaminated in veterinary vaccines

Vaccine	Extraneous virus	Country/region	Year of publication	Reference
Infectious bovine rhinotracheitis (IBR)	BVDV	USA	1968	Tamoglia <i>et al.</i> [87]
Marek's disease	Reticuloendotheliosis virus	Japan	1976	Yuasa <i>et al.</i> [97]
Bovine babesiosis and anaplasma	Bovine leukemia virus (BLV)	Australia	1988	Rogers <i>et al.</i> [70]
Pseudorabies (Aujeszky's disease)	Ovine pestivirus	France	1988	Vannier <i>et al.</i> [91]
CSF	BVDV or Border disease virus	Netherlands	1988	Wensvoort <i>et al.</i> [95]
IBR	BVDV	EU	2000	Falcone <i>et al.</i> [26]
Marek's disease	Avian leukosis virus	USA	2006	Fadly <i>et al.</i> [25]
BVD, IBR, Parainfluenza, bovine respiratory syncytial virus (BRSV) infection	BVDV-2	USA	2013	Palomares <i>et al.</i> [63]
Newcastle disease	Fowl adenovirus and chicken infectious anemia virus	China	2018	Su <i>et al.</i> [84]

Table 2. New classification of the genus *Pestivirus* in the family *Flaviviridae*

Species name ^{a)}	Virus name	Report in Japan
<i>Pestivirus A</i>	BVDV-1	Yes (in ruminants)
<i>Pestivirus B</i>	BVDV-2	Yes (in ruminants)
<i>Pestivirus C</i>	CSFV	Yes, once eradicated, reoccurred in 2018 (in pigs and wild boars)
<i>Pestivirus D</i>	Border disease virus	Yes (in pigs)
<i>Pestivirus E</i>	Pronghorn virus	No
<i>Pestivirus F</i>	Bungowannah virus	No
<i>Pestivirus G</i>	Giraffe virus	No
<i>Pestivirus H</i>	HoBi-like virus, BVDV-3, atypical bovine pestivirus	No
<i>Pestivirus I</i>	Aydin-like pestivirus	No
<i>Pestivirus J</i>	Rat pestivirus	No
<i>Pestivirus K</i>	Atypical porcine pestivirus	No

a) Based on the proposal by Smith *et al.* [83]

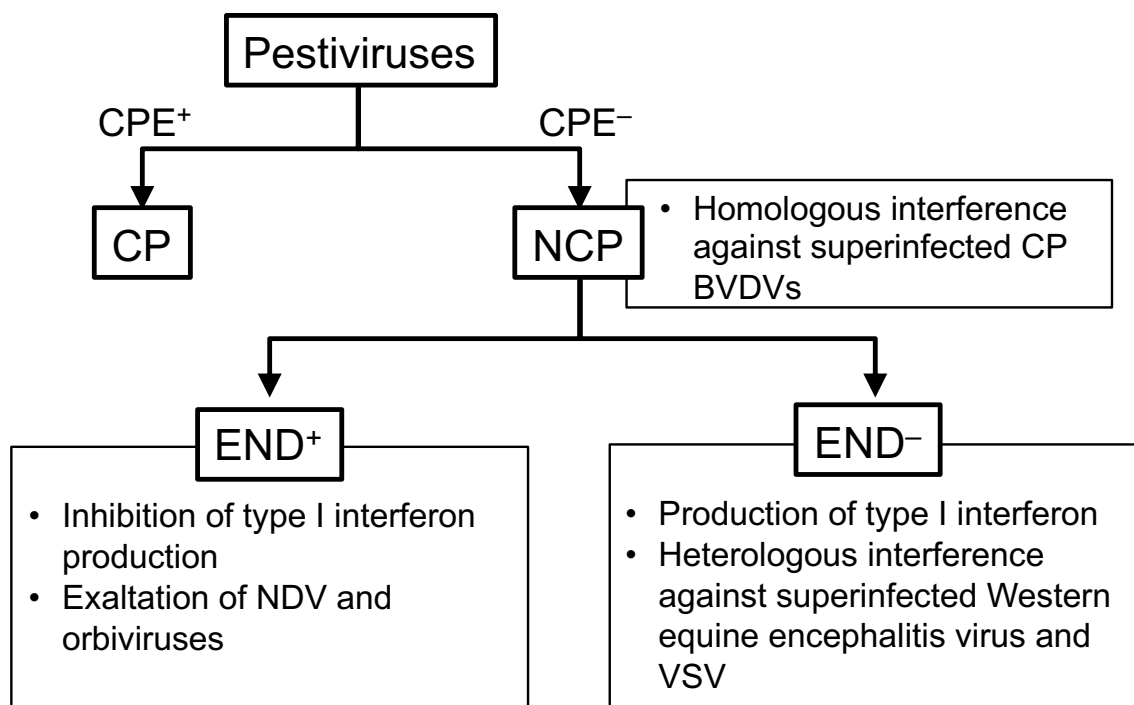


Fig. 1. Biological characterization of pestiviruses *in vitro*.

Pestiviruses are divided into two biotypes (CP and NCP) by their ability to induce a CPE in tissue cultures. NCP pestiviruses comprise END⁺ and END⁻ viruses with/without the ability to induce END phenomenon, respectively.

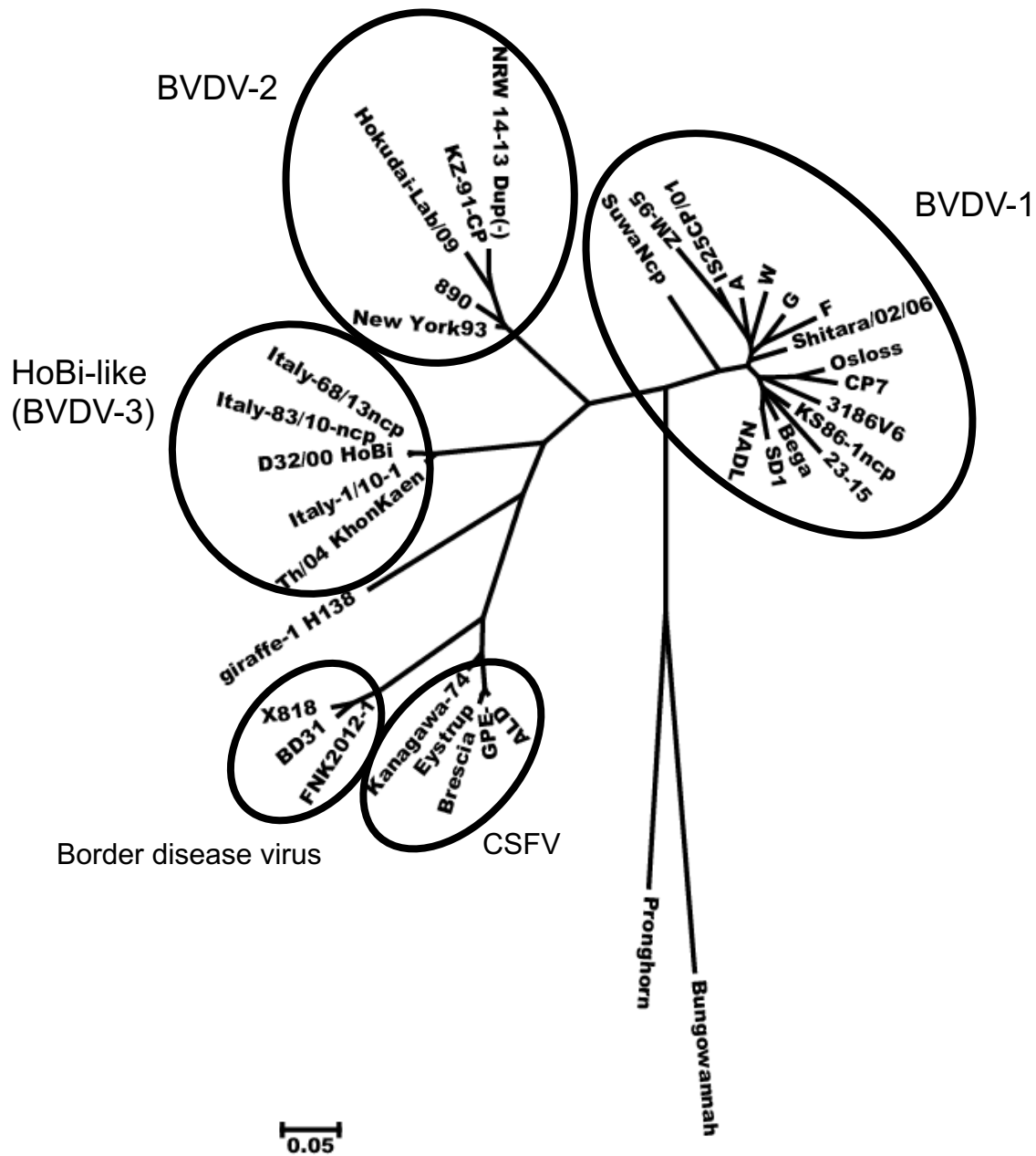


Fig. 2. Phylogenetic analysis of the genus *Pestivirus* of the family *Flaviviridae*.

A phylogenetic tree was constructed based on the partial sequences of 5'UTR by the maximum-likelihood method for 1,000 bootstrap replications using the MEGA 7 software [46]. Sequences were obtained from the DDBJ/EMBL/GenBank databases. Accession numbers of each isolate are as follows: SD1 (M96751), NADL (M31182), Osloss (M96687), CP7 (U63479), Bega (AF049221), 3186V6 (AF298062), W (AF298073), A (AF298064), G (AF298066), 23-15 (AF298059), KS86-1ncp (AB078950), SuwaNcp (KC853440), ZM-95 (AF526381), Shitara/02/06 (LC089876), IS25CP/01 (AB359931), F (AF298065), KZ-91-CP (AB003619), Hokudai-Lab/09 (AB567658), 890 (U18059), NRW 14-13_Dup(-) (HG426485), New York'93 (AF502399), D32/00_'HoBi' (AB871953), Italy-83/10-ncp (JQ612704), Italy-68/13ncp (KJ627179), Th/04_KhonKaen (NC_012812), Italy-1/10-1 (HQ231763), ALD (D49532), GPE⁻ (D49533), Brescia (AF091661), Eystrup (NC_002657), Kanagawa-74 (AB019664), BD31 (U70263), FNK2012-1 (AB846839), X818 (NC_003679), giraffe-1 H138 (NC_003678), Bungowannah (NC_023176) and Pronghorn (NC_024018).

Chapter I

**Methods to select suitable fetal bovine serum for the use in
quality control assays for the detection of extraneous viruses
from biological products**

Introduction

Production of biological products such as vaccines usually requires animal derived materials, such as embryonated hens' eggs, cultured cells and animal sera. Therefore, there are risks that animal pathogens derived from these materials could contaminate the final products. There are several reports that BVDV, BLV and other animal viruses were detected in live animal and human vaccines [19, 25-27, 59, 70, 91, 95]. In many cases, it was considered that extraneous viruses were derived from the cells or bovine sera used in vaccine production or production of the cell seed. It is therefore very important to detect extraneous viruses in vaccines to prevent any contaminated vaccines to be used. This purpose is achieved by quality control assays of vaccines, carried out by the manufacturers and also by regulatory authorities. In Japan, before commercial use, veterinary vaccines including those against viral and bacterial pathogens are assayed by the National Veterinary Assay Laboratory in accordance with Japanese regulations for veterinary biological products, in which the detection of extraneous viruses within live viral vaccines using cell culture techniques is described.

FBS is often used as a supplement in cell culture media and can interfere with quality control assays. Contamination by extraneous agents in materials such as cells and cell culture media may result in a false positive, and contamination of FBS with antibodies against bovine pathogens will result in a false negative as the antibodies in the serum may mask contamination by bovine viruses in vaccines. Therefore, it is very important to check and select suitable FBS for use in quality control assays using cell culture techniques.

Although FBS used in quality control assays can be a major risk factor for providing unreliable results, there are no reports which describe the quality of FBS

based on the standpoints for quality control on vaccines. In this chapter, in order to determine the quality of commercial FBS, pan-pestivirus reverse transcription-polymerase chain reaction (RT-PCR) assays for the detection of the genes of pestiviruses [93], including BVDV, and isolation of infectious BVDV were performed. In addition, the detection of antibodies against core viruses used in bovine vaccines including: BVDV-1 and BVDV-2, by the virus neutralization tests using both serum-dilution and virus-dilution methods, bovine herpesvirus-1 (BHV-1), bovine parainfluenza virus 3 (BPI3V), BRSV, bovine adenovirus 7 (BAd7V), Akabane virus (AKAV) and Ibaraki virus (IBAV) were attempted. Recommended methods to select suitable batches of FBS for the use in quality control assays on vaccines, especially bovine vaccines, will be shown based on the results of these assays.

Materials and Methods

Samples of FBS

Forty-nine samples of commercially available FBS were collected between February 2007 and April 2008 (Table 3). These samples were derived from ten different manufacturers of FBS. Forty-eight of the samples were standard FBS, and the final sample was IgG-stripped FBS. Samples were examined without gamma-irradiation. Before the virus neutralization and hemagglutination inhibition (HI) tests for the detection of antibodies, each serum sample was heat-inactivated.

Cells and Viruses

Bovine testicle (BT) cells, Vero cells and HmLu-1 cells were grown in Eagle's minimum essential medium (EMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.275% tryptose phosphate broth (TPB) (Becton Dickinson, San Jose, CA, USA) and 5% FBS (Mitsubishi Chemical, Tokyo, Japan). It was confirmed that all cells and FBS were free from BVDV and anti-BVDV neutralizing antibodies as well as any other viruses that cause CPE on BT cells and bovine kidney cells.

CP BVDV Nose strain (BVDV-1) [44], BVDV KZ-91-CP strain (BVDV-2) [53] and BHV-1 No.758 strain [56] were grown in BT cells in EMEM containing 2% FBS (maintenance medium; MM) in preparation for the virus neutralization tests. BRSV NMK-7 strain [40] was obtained by inoculating the virus on Vero cells in MM, AKAV JaGAr 39 strain [62] was inoculated on HmLu-1 cells, and IBAV No.2 strain [60, 61] was grown on bovine kidney cells. BPI3V strain BN-CE, which is a strain used in a Japanese live attenuated vaccine, and BAd7V Fukuroi strain [49] were used in the HI tests.

BVDV detection from serum samples

Viral RNA was extracted from 140 µl of serum samples using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The RT-PCR was carried out using a TaKaRa RNA PCR Kit Ver. 3.1 (TaKaRa BIO, Shiga, Japan) according to the manufacturer's protocol and pestivirus-specific primers, 324 and 326 [76, 93], were used. After amplification, the PCR products were resolved on 2% agarose gels by electrophoresis in Tris-borate-EDTA buffer.

Isolation of BVDV was performed using an immunoperoxidase monolayer assay (IPMA). Briefly, 50 µl of each sample was inoculated into 24-well microtiter plates with the BT cell suspension, and the cells were incubated at 37°C/5% CO₂ for five through seven days. Following the observation of CPE in cells, supernatants were collected and inoculated into other wells containing fresh BT cell suspensions. The cells were incubated at 37°C/5% CO₂ for seven days and development of CPE was observed. NCP BVDV was detected by use of an immunoperoxidase staining technique using a pestivirus specific monoclonal antibody (46/1), which binds to the NS3 protein of pestiviruses [43].

Genomic sequencing of isolated BVDV

The 5'UTR and E2 gene of isolated BVDV were amplified and sequenced using primers 324/326 and E2f/E2r [86], respectively. Purification, concentration, sequencing and phylogenetic analyses were carried out as described previously [86, 96].

Detection of neutralizing antibodies against BVDV

Titers of neutralizing antibodies against BVDV-1 Nose strain and BVDV-2 KZ-91-CP strain were examined by the serum-dilution method. A 50 µl volume of two-fold

diluted serum sample and viruses containing 200 TCID₅₀/50 µl were added to 96-well microtiter plates. After incubation at 37°C for 1 hr, BT cells (2 × 10⁵ cells/ml) were added and incubated at 37°C/5% CO₂ for 1 week. In wells where CPE was inhibited, these were designated as antibody positive and titers were recorded as the reciprocal of the highest serum dilution that caused 50% neutralization [13, 38, 48]. Methods are summarized in Table 4.

Virus neutralization test by the virus-dilution method was carried out as shown below. BVDV-1 Nose strain and BVDV-2 KZ-91-CP strain were diluted ten-fold with MM. Each dilution of the viruses was mixed with the same volume of undiluted serum sample or phosphate-buffered saline (PBS) as a negative control. After incubation for 1 hr at 37°C, 100 µl of the virus-sample mixture was inoculated into 48-well microtiter plates that contained a monolayer of BT cells (1 × 10⁵ cells/ml at the time of plating) and adsorbed for 1 hr at 37°C. The inocula were removed and the cells were washed once with PBS. Then, 0.5 ml of MM was added to all the wells and incubated at 37°C/5% CO₂ for seven days and plates were checked for CPE, and the TCID₅₀ of BVDV was measured [57, 78, 81]. If a difference in the TCID₅₀ of BVDV between sample and PBS (neutralization index) was less than or equal to 0.50 (Log₁₀), the sample was confirmed to not contain neutralizing antibodies against BVDV.

Detection of neutralizing antibodies against BHV-1

Viruses containing 100 plaque forming units/100 µl and the same volume of serum samples were mixed and incubated at 37°C for 1 hr, and 100 µl of the virus-sample mixture were inoculated onto 6-well microtiter plates containing monolayers of BT cells. After adsorption at 37°C for 1 hr, the inocula were removed and 1.5% methylcellulose in MM was added. After incubation at 37°C for three days, all the cells were fixed with 100% methanol and stained with 0.2% crystal violet. The numbers of

plaques were counted and presence of neutralizing antibodies was confirmed when the number of viral plaques was reduced by 50%. Methods are summarized in Table 4.

Detection of neutralizing antibodies against BRSV

Two-fold diluted serum samples and the same volume of viruses containing 200 TCID₅₀/0.1 ml were mixed. After incubation at 22°C for 24 hr, 100 µl of virus-serum mixture were inoculated onto Vero cells that were grown as a monolayer in glass tubes, and adsorbed at 37°C for 1 hr. A 0.5 ml volume of MM was added to all tubes and they were incubated at 34°C for 10 days in a rotary culture chamber. The tubes where CPE was inhibited were considered antibody positive and titers were recorded as the reciprocal of the highest serum dilution that caused 50% neutralization. Methods are summarized in Table 4.

Detection of neutralizing antibodies against AKAV and IBAV

Two-fold diluted serum samples and the same volume of viruses containing 200 TCID₅₀/50 µl were mixed. After incubation at 37°C for 1 hr, 100 µl of the virus-serum mixture were inoculated onto HmLu-1 cells that were grown as a monolayer in 96-well microtiter plates and adsorbed at 37°C for 1 hr. A 100 µl volume of MM was added to all the cells and the plates were incubated at 37°C/5% CO₂ for 1 week. Wells where CPE was inhibited were considered antibody positive and titers were recorded as the reciprocal of the highest serum dilution that caused 50% neutralization. Methods are summarized in Table 4.

Detection of HI antibodies against BPI3V

Fifty microliters of two-fold diluted serum samples which were treated with kaolin to remove non-specific hemagglutination (HA)-inhibitors and four HA units

(HAU) of virus were added to 96-well microtiter plates. After incubation at 37°C for 1 hr, 50 µl of 0.3% guinea pig red blood cells (RBC) were added to all the wells and the plates were incubated at 4°C for 4 hr. Wells that exhibited inhibition of HA were considered antibody positive and titers were recorded as the reciprocal of the highest serum dilution that caused inhibition.

Detection of HI antibodies against BAd7V

A 50 µl volume of two-fold diluted serum samples which were treated with kaolin to remove non-specific HA-inhibitors and 4 HAU of antigen were added to 96-well microtiter plates. After incubation at 4°C overnight, 50 µl of 0.3% goat RBC, which were treated with protamine sulfate, were added to all the wells and the plates were incubated at 4°C for 4 h. Wells where HA was inhibited were determined to be antibody positive and titers were recorded as the reciprocal of the highest serum dilution that caused inhibition.

Results

Detection of pestivirus genes and infectious viruses in serum samples

The pestivirus specific RT-PCRs revealed that 28 of all 49 serum samples (57.1 %) contained portions of pestivirus genes (Table 3). Moreover, infectious BVDV was isolated from two serum samples (Table 3). Two isolates (BVDV/FBS#25 and BVDV/FBS#30) were determined to be BVDV-1 (BVDV/FBS#25: BVDV-1b, BVDV/FBS#30: unclassified BVDV-1) by phylogenetic analysis of the 5' UTR and 420 bp of the E2 gene (data not shown). Sequences of two isolates have been deposited into the DDBJ/EMBL/GenBank databases under the accession numbers AB583194–AB583197.

As shown in Table 3, pestivirus genes were detected in FBS derived from all countries or regions examined in this study. All PCR products from serum samples were examined by PCR-restriction fragment length polymorphism (RFLP) [65] and direct sequencing using the 5' UTR to discriminate between BVDV-1 and BVDV-2, but this was not accomplished.

Detection of neutralizing antibodies against BVDV

Neutralizing antibodies against BVDV-1 were detected in 20 of the 49 samples (40.8%) by the serum-dilution method (Table 5). Neutralizing antibodies against BVDV-2 were detected in 32 of 49 samples (65.3%). In 29 serum samples where antibodies against BVDV-1 were not detected, 18 serum samples contained antibodies against BVDV-2. There were 38 samples (77.6%) that contained neutralizing antibodies against BVDVs. Neutralizing antibodies against BVDVs could not be detected in 11 samples (22.4%) using the serum-dilution method (Table 5).

These 11 samples were examined to detect neutralizing antibodies against

BVDVs by the virus-dilution method. As a result, eight of the 11 serum samples were found to contain neutralizing antibodies against BVDV-1. Moreover, two out of three serum samples that demonstrated a negative result in the detection of neutralizing antibodies against BVDV-1 contained neutralizing antibodies against BVDV-2. Therefore, only one sample remained out of the 49 serum samples that was negative for the presence of neutralizing antibodies against BVDVs, this was the IgG-stripped FBS.

Detection of antibodies against BHV-1, BRSV, AKAV, IBAV, BPI3V and BAd7V

All serum samples were examined to detect neutralizing antibodies against BHV-1, BRSV, AKAV and IBAV as well as HI antibodies against BPI3V. And 11 serum samples, whose neutralizing antibody titers were less than 2 against both BVDV-1 and BVDV-2 by the serum dilution method, were examined to detect HI antibodies against BAd7V. These serum samples were negative for the presence of antibodies against BHV-1, BRSV, IBAV, BPI3V and BAd7V (Table 5). However, neutralizing antibodies against AKAV were detected in 1 sample (Table 5).

Discussion

Assays for detecting extraneous viruses in commercial vaccines often utilize cell culture techniques. Therefore, FBS, which is used as a supplement in cell culture media, may influence the results of these quality control assays. Therefore, in the present chapter, FBS for use in the quality control assays on vaccines was focused and the status of commercial FBS was determined. Collected samples of commercial FBS were examined for the presence of bovine pathogens, including BVDV, which is a common contaminant within vaccines.

BVDV is a common pathogen that contaminates bovine derived materials such as FBS, which is used in cell culture, and also antibodies against BVDV in FBS have often been detected [13, 14, 48, 59, 71]. The high positive rate of genes of pestiviruses in FBS samples was confirmed (Table 3). And as shown in other reports [13, 14, 48, 59, 71] and from results presented here (Table 3), infectious BVDV can be isolated from commercial FBS. These results indicated that the parent cow might be infected with BVDV during pregnancy and that fetuses, which are the actual source of FBS, may be infected with BVDV. Batches of FBS are usually made from the sera of hundreds or thousands of fetuses, therefore various BVDV strains may contaminate FBS. From the PCR-RFLP and direct sequencing results, the PCR products may not be made up of a single strain of BVDV. Both BVDV-1 and BVDV-2 were isolated from commercial FBS [13], and thus there is the possibility that FBS can contain genes for both BVDV-1 and BVDV-2. Results presented in this chapter indicate there is nothing remarkable about FBS from countries or regions that contain pestivirus genes and indicate that there is always the risk that FBS can be contaminated with BVDV. Many reports show that BVDV is distributed worldwide, including countries and regions that are the main sources of FBS, such as North America, Australia, New Zealand and

Europe [3, 66, 92, 96]. It appeared that HoBi-like viruses contaminated FBS derived from Brazil, which was not detected by the pan-pestivirus RT-PCR [79]. These results also suggested that FBS should be gamma-irradiated or filtered, not only for the detection of extraneous viruses but also for ordinary cell cultures to avoid contamination with infectious BVDV. It should be noted that in the research targeted on natural immunity, where cultured cells are commonly used, BVDV contaminated FBS can work as an effector and affect the results.

There are two major methods to detect anti-BVDV antibodies: the virus neutralization test and the enzyme-linked immunosorbent assay (ELISA) [35, 77]. The virus neutralization test is the “gold standard” for antibody detection against BVDV and has been used as a reference test [17, 35, 77]. It is a very specific and sensitive assay though it is cell culture-dependent and takes 5-7 days to perform. On the other hand, the ELISA is independent of cell cultures and gives a test result within a few hours, and it is usually preferred when a large sample throughput is required [17, 77]. ELISAs have been improved to show high specificity and sensitivity when compared with the virus neutralization test [17, 18, 35]. In the present study, the virus neutralization test was selected because of its specificity and sensitivity as an established reference test.

From the results of examination for detection of neutralizing antibodies against BVDV-1 and BVDV-2 by the virus neutralization test (Table 5), it is indicated that fetuses may be infected with BVDV during the gestation period and may produce antibodies against BVDVs. As shown in Table 5, neutralizing antibody titers against BVDV in many FBS samples were relatively low. However, it is significant to consider the presence of extraneous BVDVs in vaccines being neutralized by antibodies inside the FBS, as these lots and batches of FBS are not suitable for use in quality control assays.

As a result of the virus neutralization test by the virus-dilution method, antibodies against BVDVs were detected in 10 of 11 samples, from which antibodies against BVDVs by the serum-dilution method were not detected (Table 5). The only sample that was free from anti-BVDV antibody was the IgG-stripped FBS sample. Because the virus neutralization assays using the virus-dilution method were more sensitive than those using the serum-dilution method, it was believed that the virus-dilution method was suitable for detecting antibodies against BVDVs in FBS which is used in quality control tests for vaccines. These results also suggest that FBS that do not contain antibodies against BVDVs are rare and difficult to obtain.

As challenge viruses for the virus neutralization test, two subgenotypes of BVDV, BVDV-1a and BVDV-2a strains, were used. These two subgenotypes of BVDV are groups of prototypes of BVDV-1 and BVDV-2, respectively, and they are often used as reference strains [29, 69, 77]. However, there are several subgenotypes of BVDV [29, 69, 94] and predominant subgenotypes differ depending on the region [92]. Therefore, it may be necessary to choose challenge viruses belong to the predominant subgenotypes of the country/region where FBS are derived. In addition, the inclusion of HoBi-like virus as a challenge virus should be considered due to the presence of this emerging virus in South America and Europe [5, 12].

From the results of antibody detection against bovine viruses except for BVDV (Table 5), antibodies against BHV-1, BPI3V, BRSV, BAd7V, AKAV and IBAV were detected rarely in commercial FBS, however this does not mean that there is no need to check FBS for antibodies against bovine pathogens except BVDV; BHV-1 and AKAV are well-known viruses that can infect transplacentally and there is one report that antibodies against BRSV are detected in FBS [34]. Since the detection of antibodies against bovine viruses except for BVDV is rare, detection of antibodies against BVDV first leads to the efficient screening of FBS.

Based on the results, recommended methods are outlined in Fig. 3 for selecting appropriate FBS for use in quality control assays for vaccines. First, BVDVs in FBS samples need to be checked by a pan-pestivirus RT-PCR and virus isolation. FBS that is free from infectious BVDV can be used. However, even if infectious BVDV is not isolated, a use of PCR-positive FBS in the quality control assays is not recommended because it means that the pan-pestivirus RT-PCR cannot be applied for detection of isolated viruses from vaccines as long as these FBS are used. Second, FBS should be screened for the presence of neutralizing antibodies against both BVDV-1 and BVDV-2 by the virus neutralization tests using the serum-dilution method. This method can be easily applied and many samples can be simultaneously checked using a 96-well microtiter plate. In case there are too many FBS samples to be tested, it may be useful to screen for the presence of antibodies by the ELISA before performing the virus neutralization test. If FBS samples are limited by the virus neutralization tests using the serum-dilution method, proceeding to the virus neutralization tests using the virus-dilution method, which shows higher sensitivity, is recommended. When it is concluded from these assays that FBS samples do not contain neutralizing antibodies against BVDVs, the presence of antibodies against other bovine viruses should be determined. To avoid contamination with unknown and undetectable pathogens in FBS, the use of gamma-irradiated FBS is recommended.

In conclusion, the use of FBS selected by the methods shown in Fig. 3 is required as long as cell culture techniques are used in the quality control assays. However, as shown in the results, it is difficult to obtain FBS that is completely free from pathological agents and antibodies against these agents, especially BVDV. In the future, it may be necessary to consider FBS production using fetuses from countries where BVDV has been eradicated; use IgG-stripped FBS or other animal sera for tissue culture; develop culture conditions that do not require serum; or introduce highly

sensitive tests to detect BVDV that do not use cell culture techniques, such as detection of pestivirus RNA [2, 93] in the quality control assays after improving the methods. It may be necessary that the minimum requirements and grades of FBS used in both manufacturing of vaccines and quality control assays are internationally ratified.

Summary

Production of biological products, especially vaccines, usually requires materials derived from animals, and there are always risks that animal pathogens derived from these materials could contaminate the final products. Detection of extraneous agents is performed by quality control assays. In these biological assays, animal derived materials are also used and another problem arises, as FBS is used as an ingredient in tissue culture media. FBS contaminated with BVDV or other bovine pathogens, as well as antibodies against these pathogens may lead to false results in quality control assays. In this chapter, in order to determine the actual status of commercial FBS, quality tests on various FBS samples were performed. As a result, in 28 of 49 FBS samples (57.1%), pestivirus genes were detected by pan-pestivirus RT-PCR assay. Furthermore, two samples contained infectious BVDV. Neutralizing antibodies against BVDVs were detected in 48 of 49 samples (97.6%) by the virus neutralization test based on the serum-dilution or virus-dilution methods. Antibodies against other bovine pathogens were detected rarely in these samples. From these results, methods that are focused on detection of BVDV and neutralizing antibodies against BVDVs are recommended to select the suitable FBS for use in quality control assays of veterinary vaccines.

Table 3. Detection of pestivirus genes and infectious viruses from FBS samples

Serum identification	Manufacturer	Origin country or region	RT-PCR	Virus isolation	
				CPE	IPMA
1	A	Canada	-	-	-
2	A	Canada	-	-	-
3	A	Canada	-	-	-
4	A	Canada	+	-	-
5	A	Canada	-	-	-
6	A	Canada	+	-	-
7	A	Canada	+	-	-
8	A	Canada	-	-	-
9	A	Canada	-	-	-
10	A	Canada	-	-	-
11	B	Canada	+	-	-
12	B	Canada	+	-	-
13	C	Canada	+	-	-
14	B	Canada	+	-	-
15	D	Canada	-	-	-
16	D	Canada	+	-	-
17	D	Canada	+	-	-
18	A	USA	+	-	-
19 ^a	A	USA	-	-	-
20	E	North America	-	-	-
21	F	Mexico	+	-	-
22	F	Mexico	+	-	-
23	F	Mexico	+	-	-
24	G	Costa Rica	+	-	-
25	B	Central America	+	-	+
26	B	Central America	+	-	-
27	B	Central America	+	-	-
28	E	France	-	-	-
29	E	France	-	-	-
30	C	France	+	-	+
31	C	France	+	-	-
32	C	France	+	-	-
33	E	France	-	-	-
34	E	France	-	-	-
35	A	Australia	-	-	-
36	A	Australia	-	-	-
37	A	Australia	-	-	-
38	A	Australia	-	-	-
39	A	Australia	-	-	-
40	H	Australia	+	-	-
41	H	Australia	+	-	-
42	H	Australia	+	-	-
43	I	Australia	+	-	-
44	J	Australia	+	-	-
45	E	Australia	-	-	-
46	E	Australia	-	-	-
47	E	Australia	+	-	-
48	B	Australia	+	-	-
49	H	New Zealand	+	-	-

^aIgG-stripped FBS

Table 4. Summary of methods to detect neutralizing antibodies against BVDV, BHV-1, BRSV, AKAV and IBAV

Target antigens	Viral strains for neutralization	Cells	Conditions of neutralization	Conditions of incubation	Methods
BVDV-1	Nose	BT	37°C for 1 hr	37°C for 7 days	Inhibition of CPE
BVDV-2	KZ-91-CP	BT	37°C for 1 hr	37°C for 7 days	Inhibition of CPE
BHV-1	No.758	BT	37°C for 1 hr	37°C for 3 days	Plaque reduction
BRSV	NMK-7	Vero	22°C for 24 hr	34°C for 10 days	Inhibition of CPE
AKAV	JaGAr39	HmLu-1	37°C for 1 hr	37°C for 7 days	Inhibition of CPE
IBAV	No.2	HmLu-1	37°C for 1 hr	37°C for 7 days	Inhibition of CPE

Table 5. Detection of neutralizing or HI antibodies against BVDV, BHV-1, BPI3V, BRSV, BAd7V, AKAV and IBV in FBS samples

Serum No.	Origin country or region	Anti-BVDV				Anti-BHV-1	Anti-BPI3V	Anti-BRSV	Anti-BAd7V	Anti-AKAV	Anti-IBAV
		Serum dilution method		Virus dilution method							
		BVDV-1	BVDV-2	BVDV-1	BVDV-2						
1	Canada	_ ^a	2	NT	NT	_ ^b	_ ^c	_ ^a	NT	_ ^a	_ ^a
2	Canada	-	8	NT	NT	-	-	-	NT	-	-
3	Canada	-	2	NT	NT	-	-	-	NT	-	-
4	Canada	4	_ ^a	NT	NT	-	-	-	NT	-	-
5	Canada	-	2	NT	NT	-	-	-	NT	-	-
6	Canada	-	16	NT	NT	-	-	-	NT	-	-
7	Canada	-	32	NT	NT	-	-	-	NT	-	-
8	Canada	-	32	NT	NT	-	-	-	NT	-	-
9	Canada	-	2	NT	NT	-	-	-	NT	-	-
10	Canada	-	8	NT	NT	-	-	-	NT	-	-
11	Canada	8	16	NT	NT	-	-	-	NT	-	-
12	Canada	32	16	NT	NT	-	-	-	NT	-	-
13	Canada	2	2	NT	NT	-	-	-	NT	-	-
14	Canada	8	8	NT	NT	-	-	-	NT	-	-
15	Canada	-	4	NT	NT	-	-	-	NT	-	-
16	Canada	-	4	NT	NT	-	-	-	NT	-	-
17	Canada	2	8	NT	NT	-	-	-	NT	-	-
18	USA	-	-	1.75	0.75	-	-	-	_ ^d	-	-
19 ^e	USA	-	-	0.25	0.25	-	-	-	-	-	-
20	North America	-	-	0.75	NT	-	-	-	-	-	-
21	Mexico	2	-	NT	NT	-	-	-	NT	-	-
22	Mexico	8	4	NT	NT	-	-	-	NT	-	-
23	Mexico	8	4	NT	NT	-	-	-	NT	-	-
24	Costa Rica	-	-	≥1.75	NT	-	-	-	-	-	-
25	Central America	-	-	1.25	NT	-	-	-	-	-	-
26	Central America	-	2	NT	NT	-	-	-	NT	-	-
27	Central America	-	-	1.25	NT	-	-	-	-	-	-

Table 5. (continued)

Serum No.	Origin country or region	Anti-BVDV				Anti-BHV-1	Anti-BPI3V	Anti-BRSV	Anti-BAd7V	Anti-AKAV	Anti-IBAV
		Serum dilution method		Virus dilution method							
		BVDV-1	BVDV-2	BVDV-1	BVDV-2						
28	France	-	2	NT	NT	-	-	-	NT	-	-
29	France	-	-	≥0.75	NT	-	-	-	-	-	-
30	France	2	-	NT	NT	-	-	-	NT	-	-
31	France	-	-	≥1.75	NT	-	-	-	-	-	-
32	France	2	2	NT	NT	-	-	-	NT	-	-
33	France	4	4	NT	NT	-	-	-	NT	-	-
34	France	-	2	NT	NT	-	-	-	NT	-	-
35	Australia	-	-	0.5	1.0	-	-	-	-	-	-
36	Australia	-	2	NT	NT	-	-	-	NT	-	-
37	Australia	-	2	NT	NT	-	-	-	NT	-	-
38	Australia	-	4	NT	NT	-	-	-	NT	-	-
39	Australia	-	-	±0	1.0	-	-	-	-	-	-
40	Australia	8	2	NT	NT	-	-	-	NT	-	-
41	Australia	4	-	NT	NT	-	-	-	NT	-	-
42	Australia	8	-	NT	NT	-	-	-	NT	-	-
43	Australia	8	4	NT	NT	-	-	-	NT	-	-
44	Australia	4	2	NT	NT	-	-	-	NT	2	-
45	Australia	2	4	NT	NT	-	-	-	NT	-	-
46	Australia	-	-	1.0	NT	-	-	-	-	-	-
47	Australia	-	4	NT	NT	-	-	-	NT	-	-
48	Australia	64	4	NT	NT	-	-	-	NT	-	-
49	New Zealand	16	-	NT	NT	-	-	-	NT	-	-

^aNeutralizing antibody titer <2^bNeutralizing antibody titer <1^cHI antibody titer <4^dHI antibody titer <10^eIgG-stripped FBS

NT: not tested

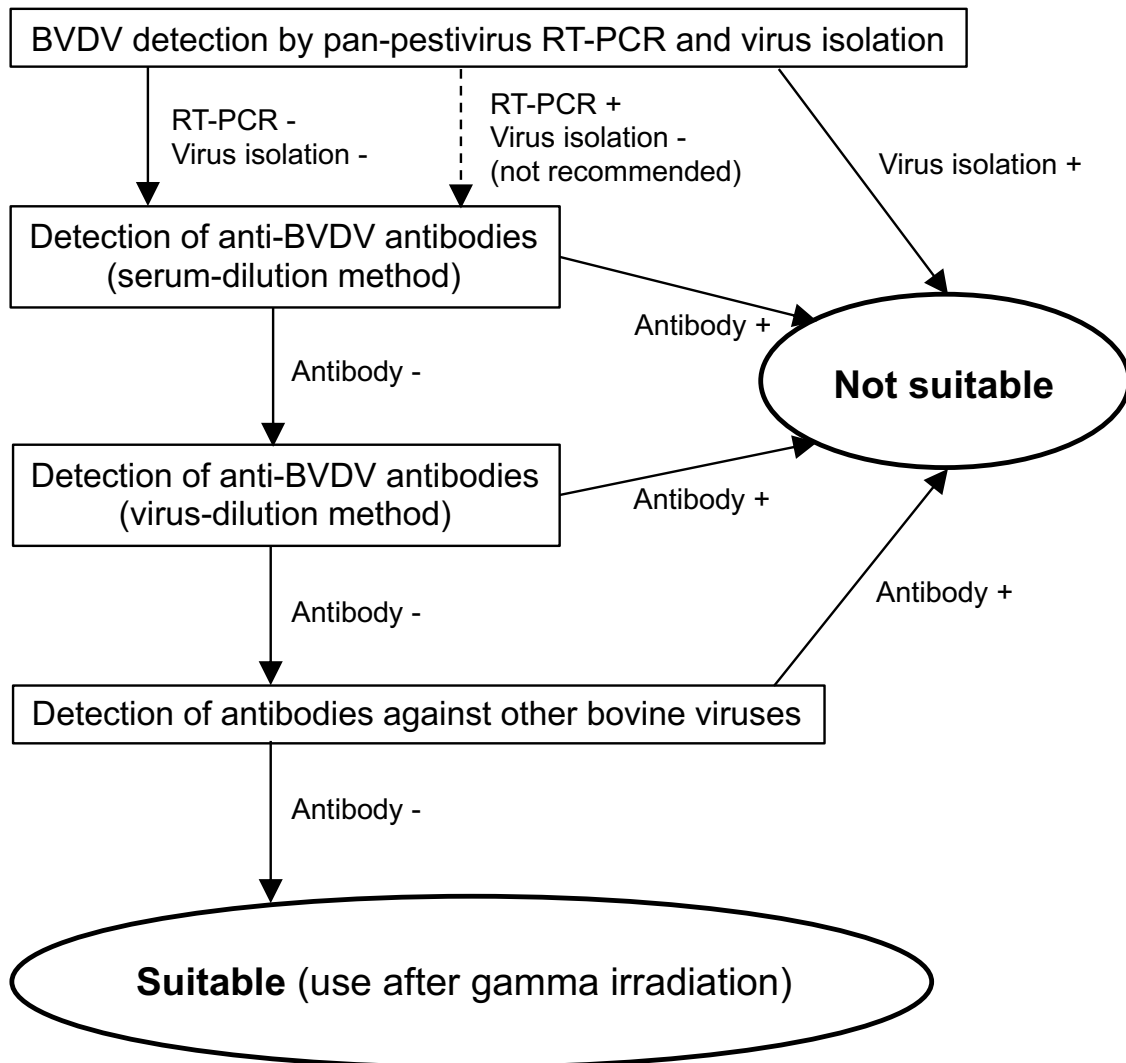


Fig. 3. Selection of suitable FBS for use in quality control assays for the detection of extraneous viruses from biological products.

Samples without infectious viruses can proceed to antibody detection. RT-PCR positive samples are not recommended for further analysis. Then, detection of anti-BVDV neutralizing antibodies followed by antibodies against other bovine viruses is recommended. Samples without antibodies against BVDVs and other bovine viruses are considered as suitable. To avoid contamination with unknown/undetectable viruses, FBS should be gamma irradiated before use in quality control assays.

Chapter II

Analysis of a pair of END⁺ and END⁻ viruses derived from the same bovine viral diarrhea virus stock reveals the amino acid determinants in N^{pro} responsible for inhibition of type I interferon production

Introduction

Pestiviruses have been reported to inhibit the production of type I interferon in infected cells *in vitro* in coordination with the property of viral protein N^{pro} [11, 28, 36, 80]. Subsequently, NDV and some orbiviruses replicate efficiently and induce distinguishable CPE in cells infected with pestiviruses [45, 55]. The END phenomenon is a well-known property of BVDV and CSFV that has been used as a biological marker of vaccine virus and for the titration purpose. A pair of END⁺ and END⁻ viruses can be cloned from the same NCP virus stock using reverse plaque formation techniques [30, 54]. END⁻ viruses do not enhance NDV, but induce intrinsic interference against Western equine encephalitis virus and VSV [30, 54]. One of the END⁻ viruses, the GPE⁻ strain of CSFV, attenuated and cloned from the virulent ALD strain [82], is a Japanese live vaccine strain against CSF that contributed to the eradication of CSF in Japan.

It is well documented that END⁺ pestiviruses subvert host innate immune defenses by preventing the production of type I interferon. This occurs via the proteasomal degradation of cellular IRF-3 in infected cells through its interaction with viral autoprotease N^{pro} [11, 28, 36, 80]. N^{pro} is a unique protein in pestiviruses that autocatalytically cleaves itself from the nascent polyprotein to generate the N terminus of the viral C protein [74]. N^{pro} has two domains: a catalytic N-terminal domain and a C-terminal domain containing a zinc-binding TRASH motif. The zinc-binding TRASH motif, which includes the zinc-binding residues Cys112-Cys134-Asp136-Cys138, is required for IRF-3 binding and for the prevention of type I interferon production [33, 85].

Recent studies have shown that N^{pro} of CSFV interacts with IRF-7 and dampens the production of type I interferon in plasmacytoid dendritic cells [28]. It has also been

reported for CSFV that deletion of up to 19 amino acids from the N terminus of N^{pro} does not abolish its capacity to inhibit the production of type I interferon [33, 73] and that amino acid substitutions C112A/R, C134A, D136N or C138A in N^{pro} result in the disappearance of this function [73, 85]. In contrast, Ruggli *et al.* and Tamura *et al.* succeeded in restoring the N^{pro} functions of wild-type END⁻ viruses, the Ames-END⁻ and GPE⁻ strains of CSFV, by mutating a single amino acid residue (R112C and N136D, respectively) using reverse genetics techniques [73, 88].

N^{pro} of both CP BVDV (e.g., the NADL strain) and NCP BVDV (e.g., the pe515 strain) blocks type I interferon production *in vitro* via proteasomal degradation of cellular IRF-3 [16, 36]. It has been reported for BVDV that nearly the entire N^{pro} is required for the prevention of type I interferon production because removal of 30 residues from the N terminus or the removal of 24 or 88 residues from the C terminus abolishes this function [16, 36]. Deletion mutants expressing residues 1–69 or 70–168 of N^{pro} also lack the ability to inhibit the production of type I interferon [16]. In addition, the previous reports revealed that substitutions of amino acid residue L8P, E22V or H49L of N^{pro} abolish its capacity to function as an interferon antagonist [16, 31]. Until date, however, the basic mechanism by which BVDV inhibits type I interferon production has not been approached through engineering *in vitro*-rescued viruses with mutations on the basis of the differences between a pair of END⁺ and END⁻ viruses.

In Chapter II, to take advantage of the differences between a wild-type END⁻ virus and its END⁺ virus pair, a pair of END⁺ and END⁻ viruses was cloned from a viral stock of the BVDV-2 GBK strain. Following this, the amino acid residue which involves in the inhibition of type I interferon production is shown by determining and comparing the complete genome sequence of both viruses as well as investigating mutant viruses generated from the END⁺ virus using reverse genetics techniques.

Materials and Methods

Cells and viruses

BT cells were grown in EMEM (Nissui Pharmaceutical) supplemented with 0.295% TPB (Becton Dickinson) and 5% FBS (Mitsubishi Chemical). Bovine kidney cell line MDBK-HS [43] and porcine kidney cell line SK-L [75] were grown in EMEM supplemented with 0.295% TPB, 10% horse serum (HS) (Life Technologies, Carlsbad, CA, USA) and 10mM N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) (Sigma-Aldrich, St. Louis, MO, USA). Bovine fetal muscle (BFM) cells were grown in EMEM supplemented with 0.295% TPB, 5% FBS, 5% HS and 10mM BES. Cells were confirmed to be free from BVDV, and FBS was confirmed to be free from both BVDV and anti-BVDV neutralizing antibodies as shown in Chapter I.

The BVDV GBK strain is an extraneous BVDV-2 isolated from cells of the bovine kidney cell line GBK [37]. The BVDV GBK strain was grown in BT cells. BVDV GBK_E⁺ and GBK_E⁻ strains were grown in MDBK-HS cells. The NDV Miyadera strain was propagated in 10-day-old embryonated hens' eggs. The New Jersey serotype strain of VSV was grown in SK-L cells.

Cloning of a pair of END⁺ and END⁻ viruses from viral stocks

A reverse plaque formation technique [30] was used to obtain a pair of END⁺ and END⁻ viruses from viral stocks as described previously [54]. Viruses were used after three rounds of limited dilution.

Sequencing

BVDV GBK_E⁺ and GBK_E⁻ strains, the full-length cDNA clones and the *in vitro*-rescued viruses were completely sequenced as described previously [89]. In brief,

the nucleotide sequences of cDNA clones and PCR fragments from viral RNA were determined using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and a 3130 Genetic Analyzer or a 3500 Genetic Analyzer (Life Technologies) according to the manufacturer's protocol. Sequencing data were analyzed using GENETYX version 10 software (GENETYX, Tokyo, Japan).

Plasmid constructs

The cDNA fragments from the GBK_E⁺ strain, obtained by RT-PCR, were cloned into plasmid pCR[®]-Blunt II-TOPO[®] (Life Technologies) using the Zero Blunt[®] TOPO[®] Cloning Kit (Life Technologies). The cDNA sequence was flanked by a modified T7 promoter sequence at the 5' end and a *Pst* I restriction site at the 3' end. Subclones were assembled into a full-length cDNA clone, termed pGBK_E⁺, by replacing the CSFV genome of the full-length cDNA clone of the CSFV Alfort187-1 strain pA187-1 [72] with the genome of the GBK_E⁺ strain using appropriate restriction enzymes and the In-Fusion HD Cloning Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. The full-length cDNA clone pGBK_E⁺ was transformed and propagated in competent cell Stbl3 cells (Life Technologies) and purified using the QIAGEN Plasmid Plus Midi Kit (QIAGEN) according to the manufacturer's protocol.

Three full-length cDNA clones with combinations of the four amino acid substitutions D136G, I2623V, D3148G and D3502Y were constructed in the pGBK_E⁺ backbone using the QuickChange Lightning Multi Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) and the In-Fusion HD Cloning Kit. The plasmid pGBK_E⁺/D136G has a single amino acid substitution at position 136 of GBK_E⁺, whereas the plasmids pGBK_E⁺/I2623V; D3148G; D3502Y and pGBK_E⁺/D136G; I2623V; D3148G; D3502Y have multiple amino acid substitutions at positions 2623, 3148 and 3502 of GBK_E⁺ and at positions 136, 2623, 3148 and 3502 of GBK_E⁺,

respectively.

Full-length PCR amplification, in vitro RNA transcription, transfection and viral recovery

The cDNA-derived viruses were rescued as described previously [89] with some modifications. The full-length genome amplification strategy [67] was employed to obtain a full-length PCR amplicon for *in vitro* RNA transcription. The cDNA clones were amplified using primers 5GBKE⁺_T7 (5'- taa tac gac tca cta ta GTA TAC GAG ATT AGC TAA AGT ACT CG -3', T7 promoter sequence underlined) and 3GBKE⁺ (5'- GGG GCT GTT AGA GGC ATC CTC TAG TC -3') with AccuPrime Taq DNA polymerase High Fidelity (Life Technologies). Viral RNA was transcribed *in vitro* from the purified full-length PCR amplicon using the MEGAscript T7 Kit (Life Technologies), the remaining PCR amplicon was digested using DNase (Life Technologies), and the viral RNA was then purified with a MicroSpin S-400 column (GE Healthcare, Buckinghamshire, UK). MDBK-HS cells (3×10^6 cells) were transfected with 10 µg of viral RNA in a 0.4 cm cuvette using the Gene Pulser Xcell Electroporation system (Bio-Rad, Hercules, CA, USA) set at 180 V and 950 µF. The cells were incubated at 37°C in 5% CO₂ for 3 days, and the supernatants were then transferred onto naive MDBK-HS cells to obtain infectious viruses.

The viruses were named according to the plasmid from which they were rescued, replacing “p” with “v” in the nomenclature. The cDNA-derived viruses generated in the present study are shown in Fig. 4.

END assay and measurements of type I interferon production

The END assay was conducted using BFM cells as described previously [39]. In brief, BFM cells grown in 96-well plates were infected with BVDV and incubated for

5 days at 37°C in 5% CO₂. After removal of the supernatants, the cells were superinfected with 1 HAU/ml of the NDV Miyadera strain. The END phenomenon was regarded as positive, if strong CPE was observed in NDV-infected cells.

Measurements of type I interferon production in BVDV-infected cells were performed using a previously described plaque reduction method with VSV as a challenge virus [55], with some modifications. In brief, the supernatants from BVDV-infected cells were inactivated by exposure to UV light (254nm) in the UV Crosslinker (ATTO Corporation, Tokyo, Japan) under the condition of 500 mJ/cm². Viral inactivation of samples was confirmed using indirect FA techniques with anti-BVDV NS3 monoclonal antibody 46/1 [43] after inoculation of samples onto MDBK-HS cells and incubation for 2 days at 37°C in 5% CO₂. Then, monolayers of SK-L cells in 12-well plates were inoculated with 1 ml of a four-fold dilution of UV-inactivated supernatant and incubated for 24 hr at 37°C in 5% CO₂. The supernatants were removed, and the cells were inoculated with VSV. Interferon titers were expressed as reciprocals of dilutions that reduced the number of challenge viral plaques by 50%.

Detection of IRF-3 in BVDV-infected cells by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

SDS-PAGE and western blotting were performed as described previously [42]. BFM cells were infected with BVDV (multiplicity of infection of 1) in six-well plates and incubated for 5 days at 37°C in 5% CO₂. Lysates of cells were separated with SDS-PAGE. After transferring the proteins from the gels to the Immobilon-P Transfer Membrane (Millipore, Billerica, MA, USA), the membranes were treated with anti-human IRF-3 rabbit polyclonal antibodies (GeneTex, Hsinchu City, Taiwan), goat anti-rabbit IgG-HRP conjugate (Bio-Rad) and Immobilon Western Detection Reagents (Millipore), in that order. The membranes were read using Lumi Vision PRO (Aishin

Seiki, Kariya, Japan), and specific bands for IRF-3 were detected.

Results

Cloning of a pair of END⁺/ END⁻ viruses from the BVDV GBK strain and determining their whole genome sequences

A pair of END⁺ and END⁻ viruses (GBK_E⁺ and GBK_E⁻, respectively) was cloned from the BVDV GBK strain by means of reverse plaque formation techniques and limited dilution. The genomes of GBK_E⁺ and GBK_E⁻ viruses were both 12,284 nucleotides in length and encoded 3,897 deduced amino acids. Comparison of the complete genome sequences of both viruses revealed only six nucleotide and four amino acid differences (D136G, I2623V, D3148G and D3502Y, the numbers refer to the amino acid position in GBK_E⁺) between the two viruses (Table 6). The complete genome sequences of the GBK_E⁺ and GBK_E⁻ strains were deposited in the DDBJ/EMBL/GenBank databases under accession numbers AB894423 and AB894424, respectively.

Generation and characterization of in vitro-rescued viruses

The infectious BVDV vGBK_E⁺ was successfully rescued by electroporation of MDBK-HS cells with viral RNA transcribed *in vitro* from a full-length PCR amplicon obtained from a cDNA clone pGBK_E⁺. The complete sequence of vGBK_E⁺ was entirely identical to that of GBK_E⁺. The mutant viruses vGBK_E⁺/D136G, vGBK_E⁺/I2623V; D3148G; D3502Y and vGBK_E⁺/D136G; I2623V; D3148G; D3502Y were also recovered from full-length cDNA clones. Sequencing of the complete genomes of these three viruses confirmed the mutations at the desired amino acid positions and demonstrated the lack of any other mutations in comparison with the parental GBK_E⁺ virus.

To investigate the biological properties of the mutant viruses, BFM cells were

inoculated with both the parental GBK_E⁺ and the *in vitro*-rescued vGBK_E⁺ viruses. The results revealed that both GBK_E⁺ and vGBK_E⁺ were NCP (data not shown) and exhibited the END phenomenon (END⁺) (Figs. 4 and 5). In addition, titration of both viruses in MDBK-HS cells revealed that vGBK_E⁺ exhibited the same growth characteristics as wild-type GBK_E⁺ (data not shown). Moreover, compared with the wild-type GBK_E⁺ strain, other *in vitro*-rescued viruses grew equally in MDBK-HS cells (data not shown).

Identification of the amino acid determinants responsible for inhibition of type I interferon production

As expected, the mutant viruses vGBK_E⁺/D136G, vGBK_E⁺/I2623V; D3148G; D3502Y and vGBK_E⁺/D136G; I2623V; D3148G; D3502Y remained NCP (data not shown). An END assay revealed that the vGBK_E⁺/D136G and vGBK_E⁺/D136G; I2623V; D3148G; D3502Y viruses were changed to END⁻; BFM cells infected with these viruses did not exhibit CPE after NDV superinfection. In contrast, the vGBK_E⁺/I2623V; D3148G; D3502Y virus remained END⁺; BFM cells infected with this virus exhibited clear CPE after superinfection with NDV, as observed in BFM cells infected with GBK_E⁺ (Figs. 4 and 5).

The amount of type I interferon in supernatants from BFM cells infected with the parent or one of the mutant viruses GBK_E⁺, GBK_E⁻, vGBK_E⁺, vGBK_E⁺/D136G, vGBK_E⁺/I2623V; D3148G; D3502Y, or vGBK_E⁺/D136G; I2623V; D3148G; D3502Y was measured in SK-L cells. Secretion of type I interferon was inhibited in BFM cells infected with GBK_E⁺, vGBK_E⁺ or the virus carrying the mutations without N^{pro} (vGBK_E⁺/I2623V; D3148G; D3502Y), which is in consistent with the results from the END assay. Cells infected with GBK_E⁻ or viruses carrying mutations in N^{pro} (vGBK_E⁺/D136G and vGBK_E⁺/D136G; I2623V; D3148G; D3502Y) did induce type

I interferon (Table 7).

These results clearly indicate that the aspartic acid of N^{pro} (at amino acid position 136 of the GBK_E⁺ strain) is the key amino acid residue that determines the capacity to inhibit the production of type I interferon and induce the END phenomenon.

Detection of IRF-3 in cells infected with the parental or mutant viruses

To investigate the expression levels of IRF-3 in BVDV-infected cells, IRF-3 in the lysates of BFM cells infected with GBK_E⁺, GBK_E⁻, vGBK_E⁺ or one of the three mutant viruses was detected by western blotting. IRF-3 was not detected in the lysates of cells infected with GBK_E⁺, vGBK_E⁺ or vGBK_E⁺/I2623V; D3148G; D3502Y, whereas clear bands of IRF-3 appeared in the lysates of cells infected with the END-GBK_E⁻ virus or one of the N^{pro} mutant viruses (vGBK_E⁺/D136G and vGBK_E⁺/D136G; I2623V; D3148G; D3502Y) (Fig. 6).

Comparison of amino acid sequences of zinc-binding TRASH motif in N^{pro}

The amino acid sequences of the zinc-binding TRASH motifs in the N^{pro} proteins from various BVDV strains deposited in the DDBJ/EMBL/GenBank databases were compared with those of the GBK_E⁺ and GBK_E⁻ strains. At least one strain per subgenotype [1a–1o (except for 1l) and 2a–2c] [41, 52] was chosen in the present study. As a result, BVDV strains except for the G strain, contain amino acid residues Cys112-Cys134-Asp136-Cys138 in the zinc-binding TRASH motif of N^{pro}, as observed in GBK_E⁺ (Fig. 7).

Discussion

Both BVDV and CSFV inhibit the production of type I interferon *in vitro* through proteasomal degradation of cellular IRF-3 [11, 28, 36, 80]. Cells infected with these (END⁺) viruses exhibit strong CPE after superinfection with NDV or some orbiviruses [45, 55]. One of the two domains of pestivirus N^{pro}, the C-terminal domain containing a zinc-binding TRASH motif consisting of Cys112-Cys134-Asp136-Cys138, is required for IRF-3 binding and prevention of type I interferon production [33, 85]. For CSFV, it has been reported that mutations at amino acid positions 112, 134, 136 or 138 in N^{pro} of END⁺ viruses abolish the inhibition of type I interferon production, while mutations at amino acid positions 112 or 136 in N^{pro} of END⁻ viruses restore this function [73, 85, 88]. For BVDV, previous studies have revealed that amino acid substitutions L8P, E22V, or H49L in N^{pro} abolish its capacity to interfere with type I interferon production [16, 31]. However, there were no studies with BVDV that had approached the basic mechanism of the inhibition of type I interferon production using the amino acid differences between a pair of END⁺ and END⁻ viruses as well as viruses genetically engineered on the basis of these differences. Pestiviruses are quasispecies; single strains consist of populations with various characteristics, such as END⁺ and END⁻ [51]. A pair of END⁺ and END⁻ viruses (GBK_E⁺ and GBK_E⁻) was isolated from the bovine kidney cell line GBK using reverse plaque formation techniques [30, 54]. Determination of complete genome sequences of these viruses revealed that there were only four amino acid differences between them (Table 6). To clarify the molecular mechanism of the inhibition of type I interferon production and the END phenomenon, a full-length cDNA clone of GBK_E⁺ (pGBK_E⁺) as well as three other full-length cDNA clones with single (D136G) or multiple (I2623V; D3148G; D3502Y and D136G; I2623V; D3148G; D3502Y) mutations were generated. Four viruses were rescued from

these full-length cDNA clones (Fig. 4), and their properties were investigated.

The END assay and the interferon bioassay of vGBK_E⁺ and three mutant viruses revealed that vGBK_E⁺ and vGBK_E⁺/I2623V; D3148G; D3502Y exhibited the END phenomenon and inhibited the production of type I interferon, whereas the N^{pro} mutant viruses vGBK_E⁺/D136G and vGBK_E⁺/D136G; I2623V; D3148G; D3502Y were END⁻ and did not inhibit the production of type I interferon (Figs. 4 and 5, Table 7). This result demonstrates that the single aspartic acid residue in the zinc-binding TRASH motif of N^{pro}, which occurs at amino acid position 136 of the genome of the GBK_E⁺ virus, is the key to determine viral capacity to inhibit type I interferon production and display the END phenomenon. Because it has been reported that the N^{pro} proteins of both BVDV and CSFV inhibit the production of type I interferon by proteasomal degradation of cellular IRF-3 [11, 28, 36, 80], whether N^{pro} of wild-type GBK_E⁺, wild-type GBK_E⁻, vGBK_E⁺ and the three mutant viruses reduced the amount of cellular IRF-3 in infected cells was assessed by western blotting. An apparent reduction in cellular IRF-3 was observed in cells infected with wild-type GBK_E⁺, vGBK_E⁺ or vGBK_E⁺/I2623V; D3148G; D3502Y, whereas wild-type GBK_E⁻ and mutant viruses with mutations in N^{pro} showed no reduction in cellular IRF-3 (Fig. 6). Therefore, the results indicate that the inhibition of type I interferon production and the END phenomenon displayed by GBK_E⁺ occurs by the degradation of cellular IRF-3 caused by combining with the zinc-binding TRASH motif. Furthermore, this function was abolished by mutation of N^{pro} at amino acid position 136. Comparison of vGBK_E⁺/D136G; I2623V; D3148G; D3502Y (the same amino acid sequence as GBK_E⁻) with vGBK_E⁺/I2623V; D3148G; D3502Y revealed that the function of N^{pro} was restored by a single G136D mutation in N^{pro} of the GBK_E⁻ virus.

In the present study, a comparison of the amino acid sequences of GBK_E⁺, GBK_E⁻ and viruses from DDBJ/EMBL/GenBank databases revealed that amino acid

residues Cys112-Cys134-Asp136-Cys138 in the zinc-binding TRASH motif of N^{pro} were well conserved among field BVDV isolates (Fig. 7). It is reported that these four residues are required for the inhibition of type I interferon production [33, 85]. Taken together, the above-mentioned results suggest that these field isolates inhibit the type I interferon production in infected cells, although there remains a possibility that amino acid residues other than those of TRASH motif contribute to this phenomenon. The G strain contains the amino acid residue Glu136 in the TRASH motif of N^{pro} (Fig. 7), and this may affect the structure of N^{pro} and result in the production of type I interferon *in vitro*. It was reported that 35 out of 45 (77.8%) field isolates of BVDV in Japan contained END⁺ virus as the predominant virus population compared with END⁻ virus and seven isolates (15.6%) contained similar titers of END⁺ and END⁻ viruses, whereas two isolates (4.4%) contained only END⁻ virus [58]. Therefore, further studies are needed to investigate how quasispecies of BVDV (END⁺ and END⁻) contribute *in vivo*.

In conclusion, the results shown here indicate that a single mutation in the TRASH motif at amino acid position 136 of BVDV N^{pro} abolishes its interaction with bovine IRF-3 and halts the degradation of IRF-3. Moreover, the mutation of the amino acid residue at position 136 of GBK_E⁻ restores its function as an interferon antagonist, as shown for CSFV. However, how N^{pro} interacts with IRF-3 and the nature of the cascade after interaction with N^{pro} and IRF-3 are hardly understood. In addition, it is unknown how the inhibition of type I interferon production contributes to the viral infection strategy when cells are infected with BVDV *in vivo*. Therefore, an additional study is also required to reveal the fundamental mechanism by which pestiviruses inhibit the production of type I interferon and how this mechanism functions *in vivo*.

Summary

The END phenomenon is induced by the inhibition of type I interferon production in pestivirus-infected cells *in vitro*, via proteasomal degradation of cellular IRF-3 with the property of the viral autoprotease protein N^{pro}. Reportedly, the amino acid residues in the zinc-binding TRASH motif of N^{pro} determine the difference in characteristics between END⁺ and END⁻ CSFVs. However, the basic mechanism underlying this function in BVDV has not been elucidated from the genomic differences between END⁺ and END⁻ viruses using reverse genetics till date. In the present study, comparison of complete genome sequences of a pair of END⁺ and END⁻ viruses isolated from the same virus stock revealed that there were only four amino acid substitutions (D136G, I2623V, D3148G and D3502Y) between two viruses. Based on these differences, viruses with and without mutations at these positions were generated using reverse genetics. The END assay, measurements of induced type I interferon and IRF-3 detection in cells infected with these viruses revealed that the aspartic acid at amino acid position 136 in the zinc-binding TRASH motif of N^{pro} was required to inhibit the production of type I interferon via the degradation of cellular IRF-3, consistently with CSFV.

Table 6. Differences of amino acid sequences in the genome of GBK_E⁺ and GBK_E⁻

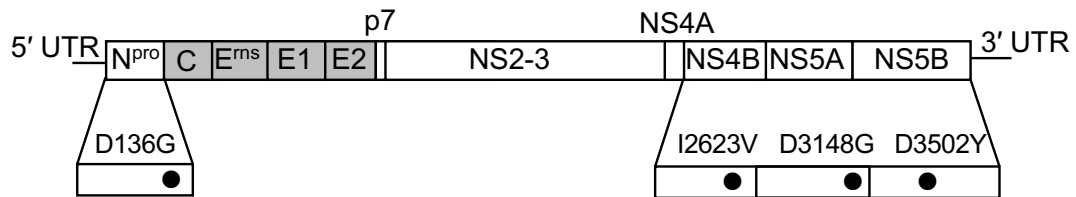
Viral protein	N ^{pro}	NS4B	NS5A	NS5B
Amino acid position	136	2,623	3,148	3,502
GBK_E ⁺	D ^{a)}	I	D	D
GBK_E ⁻	G	V	G	Y

^aD: Aspartic acid, I: Isoleucine, G: Glycine, V: Valine, Y: Tyrosine

Table 7. Measurements of type I interferon in cells infected with wild-type and *in vitro*-rescued viruses

Viruses	Type I interferon
GBK_E ⁺	<4
vGBK_E ⁺	<4
vGBK_E ⁺ /D136G	4
vGBK_E ⁺ /I2623V; D3148G; D3502Y	<4
vGBK_E ⁺ /D136G; I2623V; D3148G; D3502Y	4
GBK_E ⁻	4

A



B

	136	2623	3148	3502	END
vGBK_E ⁺	D	I	D	D	+
vGBK_E ⁺ /D136G	G	I	D	D	-
vGBK_E ⁺ /I2623V; D3148G; D3502Y	D	V	G	Y	+
vGBK_E ⁺ /D136G; I2623V; D3148G; D3502Y	G	V	G	Y	-

Fig. 4. Schematic representation of the genomic differences between GBK_E⁺ virus and GBK_E⁻ virus, and mutant viruses derived from cDNA clones generated in the present study.

(A) Four amino acid substitutions, D136G, I2623V, D3148G and D3502Y, were found in the GBK_E⁻ virus in comparison with the GBK_E⁺ virus. (B) Three recombinant viruses with combinations of amino acid substitutions were generated in the GBK_E⁺ backbone by site-directed mutagenesis and In-Fusion techniques. The white and gray boxes indicate the nonstructural and structural proteins, respectively. The results of the END assay are shown on the right side.

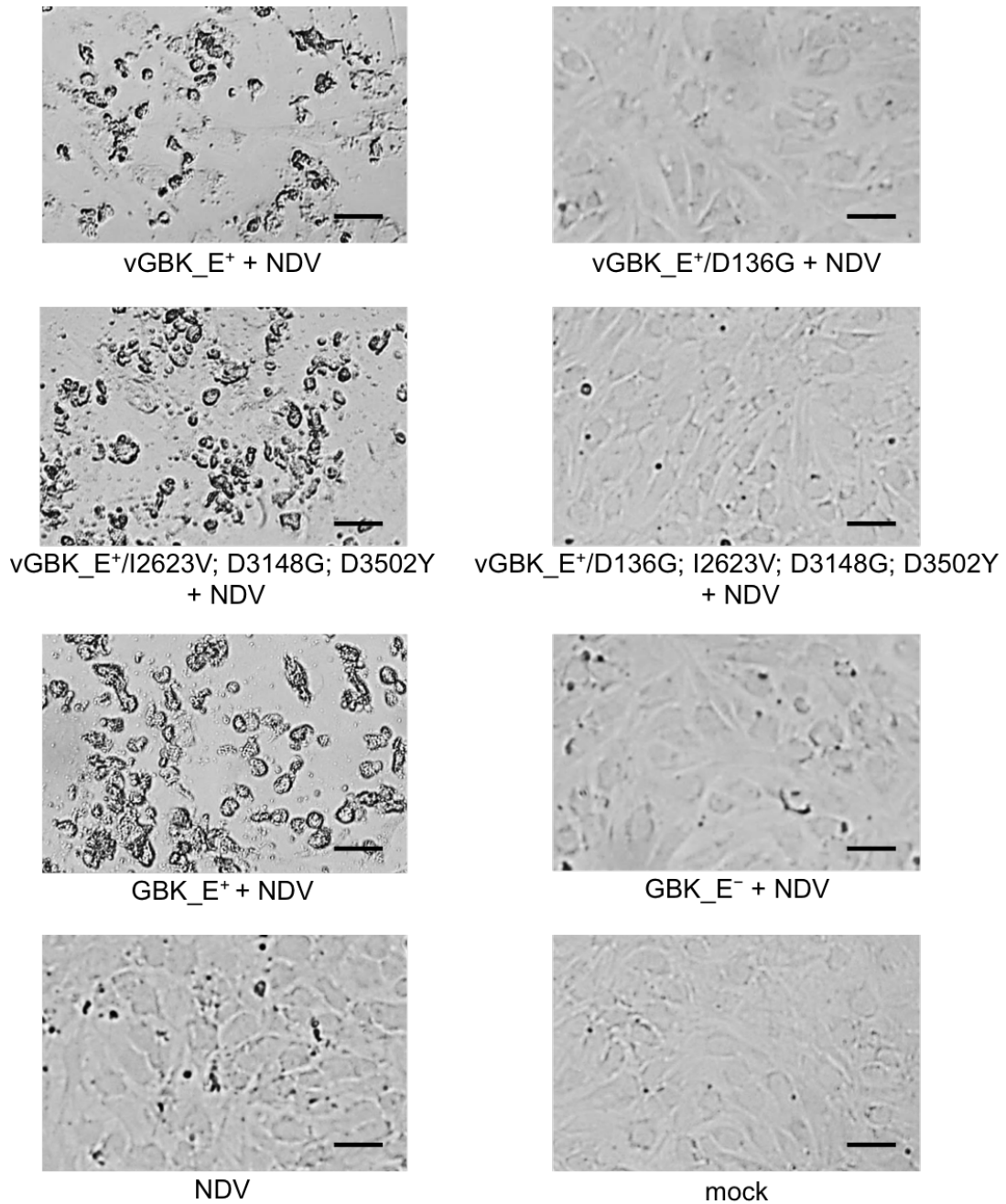


Fig. 5. Results of END assays.

BFM cells infected with GBK_E⁺, GBK_E⁻ or the *in vitro*-rescued viruses were superinfected with NDV. Cells infected with GBK_E⁺, vGBK_E⁺ or vGBK_E⁺/I2623V; D3148G; D3502Y exhibited distinguishable CPE after superinfection with NDV (END⁺), whereas those infected with GBK_E⁻, vGBK_E⁺/D136G or vGBK_E⁺/D136G; I2623V; D3148G; D3502Y did not exhibit CPE (END⁻). Scale bar, 5 μm.

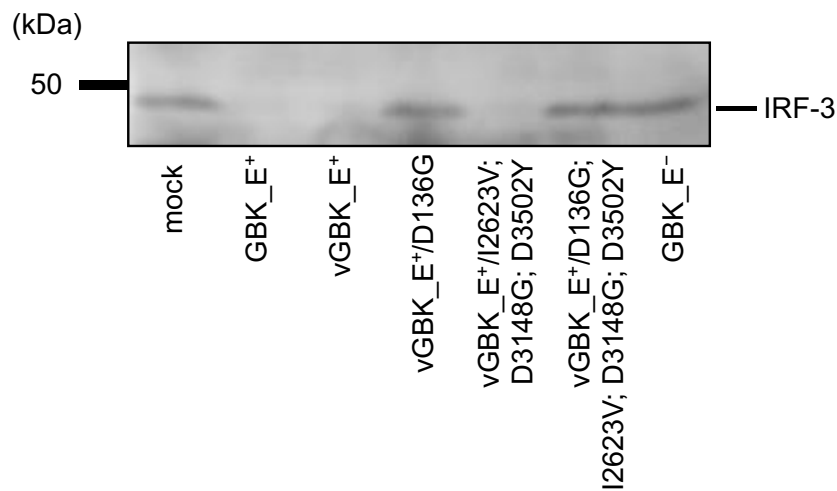


Fig. 6. Detection of IRF-3 in BFM cells infected with parent or mutant viruses. IRF-3 was not detected in western blots of the lysates of cells infected with GBK_E⁺, vGBK_E⁺ or vGBK_E⁺/I2623V; D3148G; D3502Y, whereas clear bands of IRF-3 appeared in western blots of the lysates of cells infected with GBK_E⁻, vGBK_E⁺/D136G or vGBK_E⁺/D136G; I2623V; D3148G; D3502Y.

Sub-genotype	Strain	Zinc-binding TRASH motif			
		112		134	136
2a	GBK_E ⁺	<u>M</u> <u>C</u> ETTRRVGR	VTGSDGKLYH	IYI <u>C</u> IDG <u>C</u> IL	
2a	GBK_E ⁻				G.....
1a	NADL	K.I..		..V..	...I
1a	SD-1	K.I..			...T
1b	Osloss	I..		..V..	...I
1b	CP7	K.I..	SR..	..V..	...I
1c	Bega	K.I..		..V..	...I
1d	721	K.I..	T.....	..V..	
1d	F	K.I..	T.....	..V..	
1e	3186V6	K.I..		..V..	
1f	W	K....		..V..	
1g	A	K.I..		..FV..	
1h	G	K.I..	R..	..V..	E...I
1i	23-15	...I.K.I..		..V.V..	...VM
1j	KS86-1ncp	...I...I..		..V..	...VV
1k	SuwaNcp	K.I..		M.V..	...M
1m	ZM95	K.I..		..V..	
1n	Shitara/02/06	...V.K....	R..	..V.V..	
1o	IS25CP/01	K.I..		..V..	
2a	890	I..			...V.
2b	Hokudai-Lab/09	...V.....	N....	..V..	...V.
2c	NRW 14-13_Dup(-)	I..	N..	..V..	

Fig. 7. Comparison of amino acid sequences of the zinc-binding TRASH motifs in N^{pro}.

The amino acid sequences of the zinc-binding TRASH motifs in the N^{pro} proteins of various BVDV strains deposited in the DDBJ/EMBL/GenBank databases were compared with those of GBK_E⁺ and GBK_E⁻. At least one strain per subgenotype [1a–1o (except for 1l) and 2a–2c] was chosen. The cysteines (Cs) at positions 112, 134 and 138 of the zinc-binding TRASH motif are highlighted in bold and underlined. The amino acid residue at position 136 is boxed in gray. The accession numbers of strains from the DDBJ/EMBL/GenBank are as follows: NADL (M31182), SD-1 (M96751), Osloss (M96687), CP7 (U63479), Bega (AF049221), 721 (AF144463), F (AF287284), 3186V6 (AF287282), W (AF287290), A (AF287283), G (AF287285), 23-15 (AF287279), KS86-1ncp (AB078950), SuwaNcp (KC853440), ZM-95 (AF526381), Shitara/02/06 (AB359930), IS25CP/01 (AB359931), 890 (U18059), Hokudai-Lab/09 (AB567658) and NRW 14-13_Dup(-) (HG426485).

Chapter III

Prevalence of HoBi-like viruses in Japan between 2012 and 2017 based on virological methods and serology

Introduction

The HoBi-like virus strain D32/00_‘HoBi’ was first isolated from contaminated FBS, which was originated in Brazil and used as a supplement in cell culture media [79]. Subsequently, HoBi-like viruses have been isolated from FBS of South American origin or found as contaminants in laboratory-cultured cells worldwide [5]. Genetic similarities of HoBi-like viruses isolated in Italy and South America suggest that HoBi-like viruses have been introduced into European countries via contaminated vaccines or FBS [5, 20]. These facts pose a serious concern about the safety of biological products (e.g., vaccines) manufactured using raw materials of animal origin, particularly FBS.

HoBi-like viruses were also isolated from cattle and/or water buffaloes in South America, Southeast Asia and Europe [5, 12]. When cattle were infected with HoBi-like viruses either naturally or experimentally, the animals exhibited manifestations same as those of BVDV infection, ranging from subclinical to overt diseases, including growth retardation, gastroenteric disease, respiratory disease, reproductive disorders, increased mortality among young stock and persistent infection [5, 8, 12]. MD-like syndrome in PI cattle was also reported to have been caused by HoBi-like viruses in Italy and Brazil [5, 21], and a pair of CP and NCP viruses was isolated in Italian case [21]. Therefore, cases of HoBi-like virus infection may be misdiagnosed with other infections, including BVDV infection, based on only these manifestations.

Infections of pestiviruses are diagnosed by the detection of viruses, viral genes and/or specific antibodies. The 5'UTR, which is well-conserved in pestivirus genomes, is the major target for the genetic detection and classification of pestiviruses [93], but panpestivirus RT-PCR developed by Vilcek *et al.* [93] may fail to detect HoBi-like viruses due to the presence of the nucleotide mismatches between viral and primer

sequences [9, 79]. This fact posed a concern with respect to the diagnostic methods for the detection of pestiviruses, including HoBi-like viruses. Reactivity of monoclonal antibodies against BVDV-1 and BVDV-2 with HoBi-like viruses indicated that antigen/antibody detection methods for extant BVDVs, including virus isolation, antigen-capture ELISA and virus neutralization tests, could be useful for the detection of HoBi-like virus infections [8].

Antigenically and genetically, HoBi-like viruses are related to BVDVs; however, rabbit hyperimmune sera against BVDV-1 or BVDV-2 showed limited cross-reactivity with HoBi-like viruses [79]. Evaluating the cross-antibody responses between BVDVs and HoBi-like viruses in ruminants, the sera of animals immunized with BVDV-1 and BVDV-2 demonstrated low neutralizing antibody titers against HoBi-like viruses [6, 22]. Limited cross-neutralization responses of extant BVDVs against HoBi-like viruses raised the question on the efficacy of available BVD vaccines. Meanwhile, MLV or KV containing BVDV-1 and BVDV-2 induced neutralizing antibodies against HoBi-like viruses in 34%–68% of vaccinated cattle, and the 2–4-fold differences in geometric mean titers (GMTs) of neutralizing antibodies were observed between BVDV-1/BVDV-2 and HoBi-like viruses [10]. A seroprevalence study using 2,000 cattle sera collected in the US showed a positive rate of 84.9% for neutralizing antibodies against HoBi-like viruses (with 13.8% demonstrating titers of 1,024 or above), with no evidence of HoBi-like viruses in the country [7]. These results indicated the cross-reactivity of BVDV-1 and BVDV-2 against HoBi-like viruses in cattle. BVD vaccines are used worldwide to control BVDV infection, except in the regions where BVDV has been eradicated. To control HoBi-like viruses, the efficacy of current BVD vaccines should be evaluated for all products.

In Japan, there is no report on the isolation of HoBi-like viruses to date and the prevalence of HoBi-like viruses remains unclear. Although MLV containing both

BVDV-1 and BVDV-2 or BVDV-1 alone and KV containing both BVDV-1 and BVDV-2 are approved by the Minister of Agriculture, Forestry and Fisheries, the cross-reactivity of these vaccines against HoBi-like viruses has not yet been evaluated. In this chapter, HoBi-like virus infections in cattle in Japan was investigated by the detection of HoBi-like viruses and neutralizing antibodies against HoBi-like viruses from serum samples. The cross-reactivity to HoBi-like viruses in cattle immunized with currently available BVD vaccines was also evaluated.

Materials and Methods

Cells and viruses

BFM and BT cells were grown as shown in Chapter II. Cells were confirmed to be free from BVDVs, and FBS (Japan Bioserum, Hiroshima, Japan) supplemented in cell culture media was confirmed to be free from BVDVs and anti-BVDV neutralizing antibodies by the method shown in Chapter I as well as HoBi-like viruses by RT-PCR [9].

CP BVDV-1 Nose strain [44], BVDV-2 KZ-91-CP and KZ-91-NCP strains [53] and NCP HoBi-like D32/00_‘HoBi’ strain [79] were propagated in BT or BFM cells. BVDV-2 KZ-91-CP and KZ-91-NCP strains were a pair of CP and NCP viruses, respectively, and showed similar antigenicity.

Antisera

Ruminant antisera against BVDV-1, BVDV-2 and HoBi-like viruses used in the present study were obtained by immunization of each viral strains (Table 8) to cows or sheep. Antiserum against BVDV-1 Nose strain was prepared by inoculating a calf with an intravenous injection of 2 ml of virus suspension containing approximately $10^{6.8}$ TCID₅₀ of virus. Sera were obtained 7 weeks after inoculation. Antiserum against BVDV-2 KZ-91-CP strain was prepared in sheep in the previous study [53]. For antiserum against HoBi-like D32/00_‘HoBi’ strain, 5 ml of virus suspension containing approximately $10^{6.0}$ TCID₅₀ of virus was inoculated in a cow intranasally or intravenously at 4-week intervals. Sera were obtained 4 days after the second inoculation. Antisera were stored at -20°C and heat inactivated at 56°C for 30 min before use.

Serum samples

A total of 495 bovine serum samples were collected between 2012 and 2017 from four prefectures in Japan as follows: 200 samples from Hokkaido between 2012 and 2014, 64 samples from Hokkaido in 2016, 137 samples from Yamanashi in 2016–2017, 22 samples from Toyama in 2017 and 72 samples from Nagasaki in 2015. Sera collected between 2015 and 2017 included those from cattle immunized with one dose of MLV containing BVDV-1 alone, one dose of MLV containing BVDV-1 and BVDV-2, one dose each of MLV and KV containing BVDV-1 and BVDV-2 or three doses or more of KV containing BVDV-1 and BVDV-2, or from unimmunized cattle against BVDVs. All the vaccines contained other viral antigens, including BHV-1, BPI3V and BRSV. These sera were collected within 1 year since the last BVD vaccination. All sera were stored at -20°C and heat inactivated at 56°C for 30 min before use in the neutralization assay.

Virus genome detection and virus isolation

Total RNA was extracted from pooled samples (5 sera/sample) derived from 183 sera collected in 2012–2014 and from 295 individual sera collected in 2015–2017 using QIAamp Viral RNA Mini Kit with a robotic workstation QIAcube (QIAGEN) in accordance with the manufacturer's instructions. Panpestivirus and HoBi-like specific RT-PCR was performed using 324–326 [93] and N2–R5 [9] primers, respectively, with PrimeScript One Step RT-PCR Kit Ver. 2 (TaKaRa BIO) following the manufacturer's protocol. Virus isolation using cattle sera collected in 2015–2017 was performed using an IPMA. In brief, 50 μl of each sample was inoculated into 24-well microtiter plates with BT cell suspension, and the cells were incubated at 37°C in 5% CO_2 for 5 days. Following the observation of CPE, NCP pestiviruses were detected using an immunoperoxidase staining technique with a pestivirus NS3 specific monoclonal antibody (46/1) [43].

Virus neutralization Assay

Neutralizing antibody titers of antisera and 49 cattle sera collected in Hokkaido between 2012 and 2014 (vaccination history unknown) were measured against CP BVDV-1 Nose, NCP BVDV-2 KZ-91-NCP and NCP HoBi-like D32/00_‘HoBi’ strains in BFM cells as previously reported [1]. Neutralizing antibodies in cattle sera with/without BVD vaccination history were evaluated against CP BVDV-1 Nose, CP BVDV-2 KZ-91-CP and NCP HoBi-like D32/00_‘HoBi’ strains in BT cells as shown in Chapter I using the method of potency testing in quality control assays of BVD vaccines in Japan with modifications. In brief, a 50 µl volume of 2-fold diluted serum sample and an equal volume of viruses containing 200 TCID₅₀/0.1 ml were added to 96-well microtiter plates. After incubation at 37°C for 1 hr, BT cells were added and incubated at 37°C in 5% CO₂ for 5 days. Viruses were detected by observing CPE or IPMA for CP and NCP viruses, respectively. In wells where virus growth was inhibited, these were designated as antibody positive, and titers were recorded as the reciprocal of the highest serum dilution that caused 50% neutralization.

Statistical analysis

Neutralizing antibody titers of cattle sera collected in Hokkaido between 2012 and 2014 were analyzed using the Mann–Whitney’s U-test. In addition, neutralizing antibody titers of cattle with/without BVD vaccination history were analyzed using the Kruskal–Wallis nonparametric test followed by the Steel–Dwass nonparametric multiple comparison test. $P < 0.05$ was considered significant. All statistical analyses were performed using Microsoft Excel (Microsoft Corp, Redmond, WA, USA) with Statcel4 add-ins (OMS, Tokyo, Japan).

Results

Cross-neutralization assays with ruminant antisera

Neutralizing antibody titers of anti-BVDV-1, -BVDV-2 and -HoBi-like virus sera against homologous species were 1,024, 256 and 256, respectively (Table 8). The 32- to 64-fold differences in neutralizing antibody titers were observed between BVDV-1/BVDV-2 and HoBi-like viruses (Table 8).

Cross-neutralization assays with cattle sera

Neutralizing antibody titers against HoBi-like viruses in 49 cattle sera collected in two districts of Hokkaido between 2012 and 2014 ranged from <2 to 4,096. These titers were equal to or less than those against BVDV-1 and/or BVDV-2. GMTs of neutralizing antibodies against BVDV-1, BVDV-2 and HoBi-like viruses were 259.6, 39.6 and 79.1, respectively (Table 9). Neutralizing antibody titers against BVDV-1 and HoBi-like viruses were significantly higher in District 1 than District 2 ($P < 0.05$ and $P < 0.01$, respectively).

Neutralizing antibody titers against HoBi-like viruses in cattle sera without BVD vaccination history were <2 or 2 (Table 10). In cattle immunized with one dose of MLV containing BVDV-1 alone, neutralizing antibody titers against BVDV-1 (GMT = 329.4) were higher than those against BVDV-2 (GMT = 6.6) and HoBi-like viruses (GMT = 5.8). GMTs of neutralizing antibody titers against BVDV-1 and BVDV-2 in cattle immunized with MLV, KV or a combination of MLV and KV, all of which contained both BVDV-1 and BVDV-2, were high and ranged from 115.4 to 374.8. Although GMTs of neutralizing antibodies (30.2–42.2) in these cattle against HoBi-like viruses were lower than those against BVDV-1 and BVDV-2, these titers were 5 to 7 times higher than those induced by one dose of MLV containing BVDV-1 alone ($P < 0.01$).

The results of individual cattle sera are provided in Tables 11 and 12.

Virus detection from cattle sera

Neither genes nor infectious viruses were detected in any of the samples (Table 13).

Discussion

A previous study reported that rabbit hyperimmune sera against BVDV-1 or BVDV-2 demonstrated limited cross-neutralization responses against HoBi-like viruses [79]. In the present study, the cross-reactivity of ruminant antisera immunized with BVDV-1 or BVDV-2 isolated in Japan or a prototype of HoBi-like viruses was examined. As predicted, antisera demonstrated low cross-reactivity against heterologous species, resulting in differences in neutralizing antibody titers of not less than 32 times between BVDV-1/BVDV-2 and HoBi-like virus (Table 8). These results support a previous report that despite antigenic relationship, HoBi-like viruses are antigenically distinct from both BVDV species [6].

Cross-neutralization assays in cattle sera collected in Hokkaido between 2012 and 2014 indicated cross-reactivity of BVDV-1 and BVDV-2 against HoBi-like viruses (Tables 9 and 11). A difference in GMTs of neutralizing antibodies against three species between two districts of Hokkaido may be caused by differences in prevalence of BVDVs and BVD vaccination history in the region.

To investigate the cause of high antibody titers against HoBi-like viruses in cattle, serum samples from cattle with/without BVD vaccination history were evaluated by cross-neutralization assays, suggesting that cattle immunized with MLV and/or KV containing both BVDV-1 and BVDV-2 demonstrated higher titers of neutralizing antibodies against HoBi-like viruses than those immunized with MLV containing BVDV-1 alone, although titers were 3–10 times lower than those of homologous species (Table 10). GMTs of cattle sera collected in 2012–2014 against HoBi-like viruses (Table 9) were equivalent to or higher than those of sera vaccinated with BVDV-1 and BVDV-2 (Table 10). These high titers were considered to be induced by vaccination and/or natural infection with both BVDV-1 and BVDV-2. In the US, the

difference in GMTs of neutralizing antibodies against HoBi-like viruses in cattle immunized with MLV/KV containing both BVDV species was within 2–4 times compared with those against BVDV-1 and BVDV-2 [10]. Significantly higher neutralizing antibody titers were demonstrated against HoBi-like viruses in cattle vaccinated with MLV compared with KV, suggesting that MLV is more effective in preventing HoBi-like virus infections [10]. Neutralizing antibody titers of the MLV group in the present study were higher than those of the KV group, but the differences were not statistically significant. In the US study, positive rates of neutralizing antibodies against HoBi-like viruses in vaccinated cattle were 34%–68% when titers of 10 or above were designated as antibody positive [10]. The present study shows that 80%–90% of cattle vaccinated with both BVDV-1 and BVDV-2 demonstrated neutralizing antibody titers of 16 or above to HoBi-like viruses (Table 12); therefore, BVDV strains used in Japanese vaccines are indicated to induce neutralizing antibodies against HoBi-like viruses more efficiently. The control of BVDVs in Japan is based on the slaughter of PI animals and vaccination, suggesting that these strategies are effective in controlling diseases caused by HoBi-like viruses if vaccines containing BVDV-1 and BVDV-2 are used. However, additional studies are required to evaluate whether these vaccines can prevent transplacental infection in pregnant cows.

Same as BVDVs, HoBi-like viruses are one of the risk factors for the contamination of biological products (e.g., antisera and vaccines) via raw materials of animal origin [6]. A previous study indicated that a possible introduction of HoBi-like viruses into European countries was caused via biological products [5, 20]. In Japan, FBS used for the production of live veterinary vaccines and final products of bovine live vaccines have been tested under relevant regulations for BVDVs using the *in vitro* assays since the 1980s; these assays include the detection of extraneous NCP BVDVs based on the interference of superinfected CP BVDV in cells infected with NCP

BVDVs [32]. HoBi-like viruses can be detected by this interference assay (data not shown); thus, the current regulation on veterinary vaccines seems to prevent the introduction of HoBi-like viruses into Japan. Although the areas for sample collection and the number of samples were limited in the present study, no HoBi-like viruses were detected (Table 13). In addition, neutralizing antibody titers against HoBi-like viruses in cattle sera collected in the present study were equivalent to or lower than those against BVDV-1 and/or BVDV-2 (Tables 11 and 12). These results show no evidence of presence of HoBi-like viruses in Japan; however, continuous monitoring of HoBi-like viruses in the field and biological products is needed.

Cattle in Japan induce neutralizing antibodies against HoBi-like viruses with no evidence of HoBi-like virus prevalence in the field. Higher antibody titers are demonstrated in cattle vaccinated with BVDV-1 and BVDV-2. These results indicate that relatively high titers of neutralizing antibodies against HoBi-like viruses are induced when cattle are infected (naturally or vaccinated) with both BVDV-1 and BVDV-2 without infection with HoBi-like viruses. The results shown here also indicate that diagnostic laboratories should consider possible cross-reaction against HoBi-like viruses by extant BVDVs in the field. Recent studies revealed the antigenic diversity of Brazilian isolates of HoBi-like viruses [24]; therefore, further studies are needed to investigate the cross-reactivity of vaccines against antigenically distinct HoBi-like viruses.

In conclusion, the results present no evidence of HoBi-like viruses in Japan, and cross-reactivity against HoBi-like viruses was seen in cattle immunized within 1 year with Japanese vaccines containing both BVDV-1 and BVDV-2. Continuous vaccination with both BVDV-1 and BVDV-2 may contribute to the control of HoBi-like viruses in Japan, though it is important to prevent HoBi-like virus introduction into Japan.

Summary

HoBi-like virus, also referred to as BVDV-3 or atypical bovine pestivirus, is one of the emerging pestiviruses. HoBi-like viruses have been isolated from FBS of South American origin and cattle and/or water buffaloes in South America, Southeast Asia and Europe, but their prevalence in Japan remains unknown. The purpose of this study was to investigate the prevalence of HoBi-like viruses in Japan and evaluate the immune response induced by BVD vaccines available in Japan. No HoBi-like viruses were detected by RT-PCR and virus isolation in cattle sera collected in Japan between 2012 and 2017. Nevertheless, neutralizing antibody titers against HoBi-like viruses ranged from <2 to 4,096; these titers were equivalent to or lower than those against BVDV-1 and/or BVDV-2. Cattle immunized with MLV and/or KV containing both BVDV-1 and BVDV-2 induced statistically higher titers of neutralizing antibodies against HoBi-like viruses than those immunized with MLV containing BVDV-1 alone, indicating cross-reactivity to HoBi-like viruses in cattle infected (naturally or vaccinated) with both BVDV-1 and BVDV-2. These results suggest that continuous vaccination with both BVDV-1 and BVDV-2 contributes to the control of HoBi-like viruses in Japan.

Table 8. Neutralizing antibody titers of ruminant antisera against BVDV-1, BVDV-2 and HoBi-like viruses

Antisera against ^{a)}	Virus strain		
	BVDV-1 (Nose strain)	BVDV-2 (KZ-91-NCP strain)	HoBi-like virus (D32/00_'HoBi' strain)
BVDV-1 (Nose strain)	1,024	16	32
BVDV-2 (KZ-91-CP strain)	32	256	8
HoBi-like virus (D32/00_'HoBi' strain)	4	8	256

^aTiters against homologous strains are shown in bold

Table 9. Neutralizing antibody titers of cattle sera collected in Hokkaido between 2012 and 2014

District	Number of samples	GMT (range) of neutralizing antibodies		
		Anti-BVDV-1 (Nose strain)	Anti-BVDV-2 (KZ-91-NCP strain)	Anti-HoBi-like virus (D32/00_ 'HoBi' strain)
1	26	498.5 (<2–4,096)*	81.4 (<2–4,096)	176.3 (<2–4,096)**
2	23	124.2 (<2–2,048)	17.5 (<2–4,096)	32.0 (<2–512)
Total	49	259.6 (<2–4,096)	39.6 (<2–4,096)	79.1 (<2–4,096)

* $P < 0.05$, ** $P < 0.01$ (both vs. District 2)

Table 10. Neutralizing antibody titers of field cattle sera with/without BVD vaccination history

Vaccination history	Number of samples	GMT (range) of neutralizing antibodies		
		anti-BVDV-1 (Nose strain)	anti-BVDV-2 (KZ-91-CP strain)	anti-HoBi-like virus (D32/00_'HoBi' strain)
BVDV-1 MLV (one dose)	22	329.4 (32–2,048)	6.6 (<2–64)	5.8 (<2–32)
BVDV-1, 2 MLV (one dose)	20	174.9 (2–2,048)	374.8 (64–2,048)	34.3 (<2–512)
BVDV-1, 2 MLV (one dose) + BVDV-1, 2 KV (one dose)	20	115.4 (2–2,048)	326.3 (64–1,024)	42.2 (4–128)
BVDV-1, 2 KV (three doses or more)	24 ^{a)}	209.1 (4–2,048)	128.0 (8–1,024)	30.2 (2–256)
None	46	1.0 (<2)	1.0 (<2)	1.0 (<2–2)

^aIncludes 4 samples from cattle immunized additionally with one dose of BVDV-1 MLV

Table 11. Neutralizing antibody titers of cattle sera collected in Hokkaido between 2012 and 2014

Sample No.	District	Date of birth	Date of sample collection	Neutralizing antibody titer		
				anti-BVDV-1 (Nose strain)	anti-BVDV-2 (KZ-91-NCP strain)	anti-HoBi-like virus (D32/00_'HoBi' strain)
1	1	4/8/2012	9/11/2013	<2	<2	<2
2		4/22/2012	9/11/2013	128	16	64
3		9/25/2012	9/24/2013	4	2	<2
4		4/2/2013	11/11/2013	<2	<2	<2
5		11/4/2009	11/15/2013	128	2	<2
6		3/31/2003	11/15/2013	512	<2	256
7		9/26/2004	10/1/2012	1,024	8	512
8		1/7/2008	6/20/2012	1,024	512	256
9		6/18/2008	6/20/2012	1,024	512	256
10		2/11/2003	5/8/2012	1,024	8	256
11		7/24/2009	5/15/2012	128	64	64
12		3/9/2011	10/16/2013	256	32	64
13		1/28/2003	10/17/2013	4,096	4,096	2,048
14		11/28/2008	10/17/2013	4,096	64	2,048
15		8/19/2007	10/18/2013	512	8	256
16		8/31/2007	10/18/2013	2,048	512	512
17		7/13/2003	10/18/2013	4,096	4,096	2,048
18		5/14/2006	10/18/2013	2,048	1,024	1,024
19		10/8/2006	10/18/2013	4,096	256	256
20		11/15/2005	10/18/2013	4,096	4,096	4,096
21		4/14/1999	10/18/2013	4,096	4,096	1,024
22		10/8/2007	10/18/2013	2,048	256	1,024
23		5/3/2006	9/18/2013	256	32	64
24		4/5/2007	9/18/2013	512	64	128
25		3/20/2002	11/27/2012	1,024	4,096	4,096
26		7/13/2005	11/27/2012	4,096	512	1,024
27	2	5/20/2009	4/26/2013	128	<2	128
28		8/4/2010	5/9/2013	512	16	64
29		11/5/2006	6/4/2013	512	1,024	512
30		5/12/2009	6/24/2013	256	16	128
31		9/18/2007	7/5/2013	256	4	64
32		10/17/2010	7/16/2013	<2	<2	<2
33		8/23/2006	7/30/2013	512	64	128
34		8/2/2009	9/2/2013	<2	<2	<2
35		9/20/2004	10/15/2013	512	<2	128
36		2/13/2010	1/20/2014	<2	<2	<2
37		8/25/2007	5/10/2013	512	128	128
38		7/13/2010	6/4/2013	512	8	<2
39		12/31/2008	9/18/2013	<2	<2	<2
40		7/1/2007	9/18/2013	512	64	256
41		4/11/2006	9/24/2013	2,048	64	256
42		8/26/1998	9/24/2013	2,048	256	512
43		8/14/2010	9/24/2013	512	128	512
44		5/22/2008	6/25/2013	512	128	64
45		4/10/2010	6/24/2013	128	4,096	128
46		1/5/2000	6/6/2013	1,024	16	64
47		11/22/2006	7/1/2013	128	8	<2
48		4/5/2011	7/1/2013	<2	<2	<2
49		10/20/2009	6/27/2013	1,024	64	64

Table 12. Neutralizing antibody titers of cattle sera with/without BVD vaccination history

Sample No.	Vaccination history	Date of birth	Date of last BVD vaccination	Date of sample collection	Neutralizing antibody titer		
					anti-BVDV-1 (Nose strain)	anti-BVDV-2 (KZ-91-CP strain)	anti-HoBi-like virus (D32/00_'HoBi' strain)
1	BVDV-1 MLV (one dose)	4/20/2016	8/12/2016	3/16/2017	64	16	8
2		5/15/2016	8/12/2016	3/16/2017	1,024	8	32
3		5/25/2016	8/12/2016	3/16/2017	128	16	4
4		5/31/2016	8/12/2016	3/16/2017	256	4	16
5		6/12/2016	10/18/2016	3/16/2017	512	4	2
6		6/3/2016	10/18/2016	3/16/2017	512	32	32
7		7/5/2016	10/18/2016	3/16/2017	2,048	8	4
8		6/28/2016	10/18/2016	3/16/2017	256	4	4
9		7/8/2016	11/17/2016	3/16/2017	256	16	4
10		7/27/2016	11/17/2016	3/16/2017	512	2	16
11		8/1/2016	11/17/2016	3/16/2017	2,048	64	32
12		8/7/2016	11/17/2016	3/16/2017	1,024	8	8
13		8/24/2016	1/17/2017	3/16/2017	512	8	8
14		8/28/2016	1/17/2017	3/16/2017	512	4	8
15		8/31/2016	1/17/2017	3/16/2017	1,024	4	4
16		9/20/2016	1/17/2017	3/16/2017	1,024	8	16
17		10/15/2016	1/17/2017	3/16/2017	512	8	8
18		10/27/2016	2/17/2017	3/16/2017	64	4	2
19		11/12/2016	2/17/2017	3/16/2017	256	32	4
20		11/17/2016	2/17/2017	3/16/2017	32	2	<2
21		12/1/2016	2/17/2017	3/16/2017	64	<2	<2
22		10/22/2016	2/17/2017	3/16/2017	64	<2	<2
23	BVDV-1, 2 MLV (one dose)	8/8/2015	4/11/2016	10/13/2016	2,048	512	128
24		8/9/2015	4/11/2016	10/13/2016	1,024	256	32
25		8/12/2015	4/11/2016	10/13/2016	256	512	32
26		8/15/2015	4/11/2016	10/13/2016	2,048	512	512
27		8/15/2015	4/11/2016	10/13/2016	512	512	32
28		9/3/2015	4/11/2016	10/13/2016	512	512	64
29		10/8/2015	4/11/2016	10/13/2016	512	64	16
30		10/17/2015	4/11/2016	10/13/2016	512	512	128
31		11/13/2015	4/11/2016	10/13/2016	512	2,048	256
32		1/9/2016	4/11/2016	10/13/2016	256	256	32
33		10/19/2015	4/7/2016	10/14/2016	512	1,024	128
34		11/4/2015	4/7/2016	10/14/2016	32	64	<2
35		11/10/2015	4/7/2016	10/14/2016	512	512	64
36		11/26/2015	4/7/2016	10/14/2016	16	256	16
37		11/28/2015	4/7/2016	10/14/2016	2	256	8
38		12/3/2015	4/7/2016	10/14/2016	512	1,024	64
39		12/21/2015	4/7/2016	10/14/2016	8	256	8
40		12/23/2015	4/7/2016	10/14/2016	512	2,048	64
41		1/20/2016	4/7/2016	10/14/2016	2	64	4
42		2/8/2016	4/7/2016	10/14/2016	64	256	16

Table 12. (continued)

Sample No.	Vaccination history	Date of birth	Date of last BVD vaccination	Date of sample collection	Neutralizing antibody titer		
					anti-BVDV-1 (Nose strain)	anti-BVDV-2 (KZ-91-CP strain)	anti-HoBi-like virus (D32/00_'HoBi' strain)
43	BVDV-1, 2 MLV (one dose) + BVDV-1, 2 KV (one dose)	5/13/2015	4/11/2016	10/13/2016	512	512	32
44		5/14/2015	4/11/2016	10/13/2016	512	1,024	128
45		5/15/2015	4/11/2016	10/13/2016	1,024	256	128
46		5/20/2015	4/11/2016	10/13/2016	1,024	512	128
47		5/23/2015	4/11/2016	10/13/2016	512	256	16
48		6/20/2015	4/11/2016	10/13/2016	64	64	4
49		6/22/2015	4/11/2016	10/13/2016	2,048	1,024	64
50		6/24/2015	4/11/2016	10/13/2016	64	512	64
51		6/27/2015	4/11/2016	10/13/2016	256	512	64
52		7/15/2015	4/11/2016	10/13/2016	16	512	128
53		4/9/2015	4/7/2016	10/14/2016	256	512	32
54		6/17/2015	4/7/2016	10/14/2016	128	256	32
55		7/13/2015	4/7/2016	10/14/2016	64	256	64
56		7/14/2015	4/7/2016	10/14/2016	128	256	64
57		7/23/2015	4/7/2016	10/14/2016	256	128	64
58		8/3/2015	4/7/2016	10/14/2016	256	512	32
59		8/6/2015	4/7/2016	10/14/2016	128	512	128
60		8/9/2015	4/7/2016	10/14/2016	4	256	32
61		8/13/2015	4/7/2016	10/14/2016	2	128	4
62		8/26/2015	4/7/2016	10/14/2016	2	128	16
63	BVDV-1,2 KV(three doses or more) ^{a)}	6/19/2008	10/26/2015	10/19/2016	2,048	1,024	256
64		4/2/2009	10/26/2015	10/19/2016	256	256	128
65		5/4/2009	10/26/2015	10/19/2016	256	128	32
66		9/15/2009	10/26/2015	10/19/2016	512	256	64
67		5/23/2011	10/26/2015	10/19/2016	512	16	16
68		10/20/2011	10/26/2015	10/19/2016	1,024	512	128
69		8/12/2009	10/26/2015	10/19/2016	512	256	64
70		3/31/2009	10/26/2015	10/19/2016	256	512	32
71		8/15/2007	10/26/2015	10/19/2016	128	128	32
72		7/20/2003	10/26/2015	10/19/2016	512	256	32
73		7/13/2004	10/26/2015	10/19/2016	512	256	16
74		9/3/2010	10/26/2015	10/19/2016	1,024	512	256
75		11/14/2011	11/2/2015	10/19/2016	64	128	16
76		2/14/2012	11/2/2015	10/19/2016	128	128	64
77		3/8/2013	11/2/2015	10/19/2016	128	512	32
78		7/29/2013	11/2/2015	10/19/2016	512	64	32
79		12/5/2013	11/2/2015	10/19/2016	8	8	2
80		10/15/2014	11/7/2015	10/19/2016	16	64	8
81		1/23/2011	11/6/2015	10/19/2016	512	256	32
82		3/1/2011	11/6/2015	10/19/2016	1,024	128	32
83		6/7/2011	11/6/2015	10/19/2016	256	32	32
84		8/28/2011	11/6/2015	10/19/2016	128	64	32
85		1/3/2012	11/6/2015	10/19/2016	64	64	8
86		6/30/2013	11/6/2015	10/19/2016	4	8	2

Table 12. (continued)

Sample No.	Vaccination history	Date of birth	Date of last BVD vaccination	Date of sample collection	Neutralizing antibody titer		
					anti-BVDV-1 (Nose strain)	anti-BVDV-2 (KZ-91-CP strain)	anti-HoBi-like virus (D32/00_'HoBi' strain)
87	none	3/31/2012		5/19/2015	<2	<2	<2
88		6/16/2012		5/19/2015	<2	<2	<2
89		1/8/2009		5/19/2015	<2	<2	<2
90		1/30/2012		5/19/2015	<2	<2	<2
91		1/10/2011		5/19/2015	<2	<2	<2
92		9/3/2011		5/19/2015	<2	<2	<2
93		8/4/2008		5/19/2015	<2	<2	<2
94		1/28/2009		5/19/2015	<2	<2	<2
95		12/28/2012		5/19/2015	<2	<2	<2
96		9/8/2012		5/19/2015	<2	<2	<2
97		3/1/2006		5/19/2015	<2	<2	<2
98		9/1/2009		5/19/2015	<2	<2	<2
99		1/17/2009		5/19/2015	<2	<2	<2
100		9/22/2012		5/19/2015	<2	<2	<2
101		12/19/2011		5/19/2015	<2	<2	<2
102		1/17/2013		5/19/2015	<2	<2	<2
103		9/8/2008		5/19/2015	<2	<2	<2
104		6/20/2011		5/19/2015	<2	<2	<2
105		9/7/2012		5/19/2015	<2	<2	<2
106		11/24/2002		5/19/2015	<2	<2	<2
107		4/12/2013		5/19/2015	<2	<2	<2
108		8/13/2012		5/19/2015	<2	<2	<2
109		6/7/2010		5/19/2015	<2	<2	<2
110		7/10/2011		5/19/2015	<2	<2	<2
111		9/27/2011		5/19/2015	<2	<2	2
112		9/18/2010		5/19/2015	<2	<2	<2
113		10/19/2012		5/19/2015	<2	<2	<2
114		9/24/2010		5/19/2015	<2	<2	<2
115		1/9/2008		5/19/2015	<2	<2	<2
116		7/14/2010		5/19/2015	<2	<2	<2
117		12/21/2005		5/19/2015	<2	<2	<2
118		7/20/2006		5/19/2015	<2	<2	<2
119		7/14/2006		5/19/2015	<2	<2	<2
120		4/29/2005		5/19/2015	<2	<2	<2
121		1/1/2013		5/19/2015	<2	<2	<2
122		4/13/2008		4/22/2015	<2	<2	<2
123		12/27/2010		4/22/2015	<2	<2	<2
124		3/8/2013		4/22/2015	<2	<2	<2
125		8/20/2013		4/22/2015	<2	<2	<2
126		12/29/2013		4/22/2015	<2	<2	<2
127		10/24/2014		4/22/2015	<2	<2	<2
128		1/8/2014		4/22/2015	<2	<2	<2
129		1/9/2014		4/22/2015	<2	<2	<2
130		1/21/2014		4/22/2015	<2	<2	<2
131		7/11/2014		4/22/2015	<2	<2	<2
132		9/27/2014		4/22/2015	<2	<2	<2

^aIncludes 4 samples from cattle immunized additionally with one dose of BVDV-1 MLV

Table 13. HoBi-like virus detection from cattle sera collected in 2012–2017

Prefecture	Year	Number of samples	Number of tested samples	RT-PCR positive		Virus isolation positive
				BVDV-1, 2	HoBi-like virus	
Hokkaido	2012–2014	200	183 ^{a)}	0	0	NT ^{b)}
Hokkaido	2016	64	64	0	0	0
Yamanashi	2016–2017	137	137	0	0	0
Toyama	2017	22	22	0	0	0
Nagasaki	2015	72	72	0	0	0
Total		495	478	0	0	0

^aRT-PCR was performed using pool samples of each 5 sera, ^bNot tested

Conclusion

Vaccines are important tools for the prevention and control of infectious diseases. Lack of quality, efficacy and safety of vaccines leads to unsuccessful control of targeted diseases and to spread the unexpected and undesired infectious diseases by contaminated viruses. The PMD Act ensures the quality, efficacy and safety of VMPs including veterinary vaccines in Japan. Quality control assays in process control and final products contribute to assure the products are efficacious and safe with appropriate quality.

BVDV belongs to the genus *Pestivirus* within the family *Flaviviridae*. BVDV is an economically important pathogen of cattle. In addition, BVDV is known as contaminants in veterinary vaccines via raw materials of animal origin which are used during the manufacturing of the products. From the regulatory perspective, the author focused on BVDV with respect to the quality control of veterinary vaccines.

Cell culture techniques are used in the quality control assays including virus titration, potency testing which detects neutralizing antibodies in vaccinated animals and extraneous virus testing in vaccines. Animal derived products, particularly FBS, are required as a supplement in growth media for cell culture; therefore, these products may affect the results of these assays. In chapter I, quality of commercial batches of FBS, which will be used for the quality control assays, including extraneous virus testing in live bovine vaccines and FBS for use in the production of MLV, and assays for BVD vaccines, were investigated. As a result, 28 (57.1%) out of 49 FBS samples contained pestivirus genes, and neutralizing antibodies against BVDVs were detected in 48 samples (97.6%). Except one sample which contained neutralizing antibodies against AKAV, these samples did not contain infectious agents which show CPEs in

bovine cells and antibodies against other bovine viruses. High positive rate of pestivirus genes and/or neutralizing antibodies against BVDVs leads to the following selecting methods of FBS for the use in quality control assays, which is focused on BVDVs. Detection of pestiviruses in FBS should be firstly conducted and only products which do not contain infectious viruses can proceed to the next step. Then, neutralizing antibody titers against both BVDV-1 and BVDV-2 should be measured followed by the testing of antibodies against other bovine viruses. To avoid contamination with unknown and undetectable agents, FBS should be used after the gamma irradiation.

END phenomenon has been utilized for the purpose of detecting BVDVs and CSFVs in Japan. END phenomenon is a well-known property of pestiviruses which leads to the exaltation of CPE of superinfected NDV *in vitro*. This phenomenon is caused by the prevention of type I interferon production in cells infected with pestiviruses. For the titration of BVDVs in the quality control assays of BVD vaccines, homologous interference or END methods were designated as a protocol in the regulation. To ensure the validity of testing methods, it is important to reveal the mechanism of END phenomenon in BVDVs. In chapter II, the basic mechanism underlying in the END phenomenon was revealed by determining the key amino acid by means of reverse genetics of a pair of END⁺ and END⁻ viruses (GBK_E⁺ and GBK_E⁻ strains, respectively), which was derived from the same viral stock of BVDV-2 strain contaminated in cell culture. There were 4 differences in amino acids between GBK_E⁺ and GBK_E⁻ strains (D136G, I2623V, D3148G and D3502Y). Asp136 in zinc-binding TRASH motif in N^{pro} was revealed to be the amino acid determinant for END phenomenon and prevention of type I interferon production via proteasomal degradation of IRF-3. Amino acids in zinc-binding TRASH motif in N^{pro} (Cys112-Cys134-Asp136-Cys138) were conserved among BVDVs deposited in the DNA databases; therefore, END⁺ viruses were indicated as major viruses in the field.

BVD was firstly reported in the US in 1946. Outbreaks of highly pathogenic BVDVs which caused thrombocytopenia or severe and acute BVD in North America occurred in late 1980s through 1990s led to the designation of these new type of BVDVs as BVDV-2 based on the antigenic and genomic differences with classical BVDV (BVDV-1). HoBi-like virus (BVDV-3) was firstly reported in 2004 as a contaminant in FBS of Brazilian origin. Since then, HoBi-like viruses have been isolated from FBS of South American origin and cattle and/or water buffaloes in South America, Southeast Asia and Europe. HoBi-like viruses are considered as a risk agent to contaminate veterinary vaccines via FBS. In addition, detection of HoBi-like viruses posed a question on the efficacy of BVD vaccines. In chapter III, prevalence of HoBi-like viruses and cross-reactivity of HoBi-like viruses in cattle vaccinated with commercially available BVD vaccines in Japan were investigated. There was no evidence of HoBi-like viruses in cattle sera collected in Japan between 2012 and 2017, but neutralizing antibodies against HoBi-like viruses ranged from <2 to 4,096. These high titers were indicated to be induced by infection (naturally or vaccinated) with both BVDV-1 and BVDV-2. Therefore, continuous administration of vaccines containing both BVDV-1 and BVDV-2 is considered to contribute to the control of HoBi-like viruses in Japan. HoBi-like viruses show the homologous interference *in vitro* against superinfected CP BVDV; therefore, current quality control assays of MLV are working to prevent the distribution of veterinary vaccines contaminated with HoBi-like viruses.

The outcomes of these researches can be applied for the future quality control assays of veterinary vaccines. They also show the validity of current quality control assays in Japan; therefore, results shown in the present thesis contributes to ensure the quality, efficacy and safety of veterinary vaccines. Harmonization of technical requirements for the registration of VMPs is on-going worldwide, including biologicals quality monitoring such as extraneous virus testing. By the globalization of vaccine

distribution, multi-national companies distribute the same vaccines into many markets. That makes the quality control issues world-wide scale. Therefore, international harmonization on control of BVDVs in vaccines are required to protect animals from unexpected and undesired diseases caused by extraneous BVDVs.

Acknowledgements

The author would like to acknowledge Prof. K. Ohashi, Department of Disease Control, Faculty of Veterinary Medicine, Hokkaido University, for his valuable advice, suggestion, encouragement and detailed review of this manuscript.

The author is extremely grateful to Prof. Y. Sakoda, Department of Disease Control, Faculty of Veterinary Medicine, Hokkaido University, for reviewing the manuscript, his valuable advice, suggestion and encouragement throughout this study. Great appreciation is extended to Prof. H. Kariwa, Department of Preventive Veterinary Medicine, and Dr. S. Konnai, Department of Disease Control, Faculty of Veterinary Medicine, Hokkaido University, for reviewing the manuscript.

The author thanks Emer. Prof. M. Onuma, Hokkaido University, and Dr. S. Nakamura, National Veterinary Assay Laboratory, Ministry of Agriculture, Forestry and Fisheries, for providing an opportunity to start a research on bovine viral diarrhea virus at their laboratories in Hokkaido University and National Veterinary Assay Laboratory, respectively. The author also thanks Dr. H. Aoki, Nippon Veterinary and Life Science University, for his valuable advice, suggestion and encouragement throughout this study.

The author thanks Dr. N. Ruggli, Institute of Virology and Immunology, Switzerland, for providing the full-length CSFV cDNA clone pA187-1; Dr. H. Schirrmeier, Friedrich-Loeffler-Institut, Germany, for providing the HoBi-like virus D32/00_‘HoBi’ strain; the prefectural government of Hokkaido, Hokkaido Veterinary Medical Association Nemuro Branch and Animal Hygiene Service Centers in

Yamanashi, Toyama and Nagasaki prefectures for providing cattle sera.

The author thanks research collaborators, including Prof. H. Kida and Dr. N. Isoda, Research Center for Zoonosis Control, Hokkaido University; Dr. M. Okamatsu, Department of Disease Control, Faculty of Veterinary Medicine, Hokkaido University; Dr. K. Kameyama, National Institute of Animal Health, National Agriculture and Food Research Organization; Dr. M. Nagai, School of Veterinary Medicine, Azabu University; Emer. Prof. A. Fukusho and Dr. M. Shiokawa, Nippon Veterinary and Life Science University.

The author greatly appreciates all colleagues of National Veterinary Assay Laboratory, especially Drs. T. Takahashi, K. Oishi, T. Shimazaki, M. Ishimaru, A. Saito, H. Sekiguchi and N. Nakajima. The author also thanks Drs. Y. Abe, K. Mitsuhashi, T. Tamura and S. Torii, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University.

Finally, the author thanks his family for their continuous support.

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Summary in Japanese（和文要旨）

ワクチンは、感染症の制御のために重要なツールである。ワクチンの品質、有効性及び安全性の低下は、対象とする感染症の制御に悪影響を与え、さらに、ワクチン中の迷入ウイルスは新たな感染症の発生を引き起こすことになる。我が国において、ワクチンを含む動物用医薬品は、医薬品、医療機器等の品質、有効性及び安全性の確保等に関する法律に基づき、その品質、有効性及び安全性の確保が行われている。製造ロットごとの管理は、製造工程中の試験や最終製品の試験といった品質検査によって行われている。

フラビウイルス科ペスチウイルス属の牛ウイルス性下痢ウイルス（BVDV）は、ウシに感染して、経済的に重要な牛ウイルス性下痢・粘膜病（BVD-MD）を引き起こすことに加え、牛胎子血清（FBS）等の動物由来成分を介したワクチンの迷入因子として重要である。そこで本研究では、BVDVに着目して、動物用ワクチンの品質管理の観点から詳細な検討を行った。

動物用ワクチンの品質検査において、ウイルス含有量や中和抗体価を測定する有効性試験、生ワクチン中の迷入ウイルスの有無を確認する試験等に細胞培養技術が使用される。細胞培養には、FBSをはじめとする動物由来成分が必要であり、試験結果は動物由来成分の品質に影響を受けることが考えられる。そこで、第 I 章では、動物用ワクチンの品質検査（特に生ワクチンの製造用 FBS やウシ用生ワクチンの迷入ウイルス否定試験および BVD-MD ワクチンの検査）に用いる市販 FBS の品質について検討した。その結果、国内で流通していた 49 検体のうち、28 検体（57.1%）からペスチウイルスの遺伝子を検出し、48 検体（97.6%）からは BVDV に対する中和抗体を検出した。一方で、アカバネウイルスに対する抗体が検出された 1 検体を除き、ウシの細胞に CPE を引き起こすウイルスや他のウシのウイルス種に対する抗体は検出されなかった。そこで、市販 FBS における BVDV や BVDV 抗体の汚染率の高さから、BVDV を中心に

した FBS の選択方法を提案した。すなわち、市販 FBS のロットチェックにおいては、まずペスチウイルスの検出を行い、感染性ウイルスを含まないものについて、BVDV に対する中和抗体価を測定し、陰性のものを選択する。念のために他の主要なウイルスに対する抗体を測定するとともに、未知のウイルスによる汚染をさけるため、ガンマ線照射を行ってから FBS を使用することである。

国内で古くから用いられているペスチウイルス検出法の 1 つに END (Exaltation of Newcastle Disease virus) 法がある。END 法はペスチウイルスの機能である感染培養細胞におけるニューカッスル病ウイルスの増強作用を利用したものであり、感染培養細胞における 1 型インターフェロンの抑制がその分子機構であるとされる。国内において BVD-MD ワクチンの含有量試験における BVDV 検出法として干渉法または END 法が規定されており、規制当局として、試験法の妥当性を説明するためにもその原理を明らかにしておくことが必要である。そこで、第 II 章では、BVDV における END 現象の分子基盤を解明するため、培養細胞に迷入していた 1 つの BVDV ストック (GBK 株) から単離された END 現象陽性株 (GBK_E⁺株) および同現象陰性株 (GBK_E⁻株) を活用し、リバーシジェネティクス法により、GBK 株における END 現象に関わっている BVDV のタンパクを同定した。GBK_E⁺株および GBK_E⁻株の間には 4 箇所 (D136G、I2623V、D3148G および D3502Y) のアミノ酸の違いのみが存在し、このうち、ウイルスの非構造タンパク質である N^{pro} の 136 番目のアミノ酸 (アスパラギン酸) が END 現象、細胞の IRF-3 の分解を介した 1 型インターフェロンの産生抑制に必要であることを発見した。同部位を含む Cys112-Cys134-Asp136-Cys138 は N^{pro} において亜鉛結合性 TRASH モチーフを形成している。そこで、遺伝子データベースに登録されている各遺伝子亜型から代表的な株を選択し、同領域の遺伝子情報を解析した結果、1 株を除き、GBK_E⁺株と同様のアミノ酸構成であることを確認したことから、野外において END 現象陽性株が主流であると考えられた。

BVD-MD は 1946 年に米国で初めて報告され、現在の 1 型 (BVDV-1) が病原ウイルスとして同定された。その後、1980 年代後半から 1990 年代にかけて北米で血小板減少症等を引き起こす 2 型 (BVDV-2) が流行し、日本を始め各国で両ウイルス型に対する対策が行われてきた。2004 年にブラジル産の FBS から新たに HoBi-like ウイルス (BVDV-3) が報告された。HoBi-like ウイルスは南米産の FBS や南米、南アジア、ヨーロッパのウシ (一部に水牛を含む。) から分離され、ワクチンへの新たな迷入因子として着目された。また、既存 BVD-MD ワクチンの有効性への懸念が示された。そこで第 III 章では、国内で飼育されているウシにおける HoBi-like ウイルスの感染状況と、既存 BVD-MD ワクチンを接種した牛における交差反応の有無を検討した。2012 年から 2017 年にかけて採取された国内のウシの血液から HoBi-like ウイルスは検出されず、国内に HoBi-like ウイルスが侵入した形跡は確認されなかった。一方で、ウシ血液中の中和抗体価は <2 倍から 4,096 倍であった。BVDV-1 および BVDV-2 の両成分を含むワクチンを接種したウシにおいて強い交差反応が認められたことから、この高い抗体価は BVDV-1 および BVDV-2 感染による交差が考えられた。これらのことから、継続的に BVDV-1 および BVDV-2 を含むワクチンを接種することは HoBi-like ウイルスの防御にも効果的であると考えられた。また、HoBi-like ウイルスは感染した培養細胞において CPE を起こす BVDV を干渉することから、現在のワクチンの品質検査が HoBi-like ウイルス対策に効果的に機能していることが確認された。

以上の研究成果は、今後の動物用ワクチンの品質検査に活用できるものであり、さらに、既存の試験方法の妥当性を説明するものであるため、動物用ワクチンの品質、有効性および安全性の確保に貢献するものと思われる。現在、動物用医薬品の承認申請等に必要な技術的要件の世界的な調和が行われており、動物用ワクチンの迷入ウイルス否定試験等、生物学的製剤の検査法もその焦点の 1 つである。昨今、グローバル化が進み、多国籍メーカーが各国に同じワク

チンを流通させる事例が増えており、品質管理上の問題点は世界共通となっている。ワクチン中の迷入 BVDV による動物への被害を防ぐため、国際協調での BVDV 対策が求められる。