Isolation and analysis of herpesviruses from wild animals

野生動物由来ヘルペスウイルスの分離及び解析

The United Graduate School of Veterinary Science,
Yamaguchi University

Keita Ishijima
March, 2019

1. GENERAL INTRODUCTION

1.1. History of identification of herpesviruses

The oldest description of herpesvirus infection was confirmed in A.D. 100. It was the description of herpesvirus lesion by Herodotus (Mettler, 1947). The oldest descriptions of herpesvirus infection in animals were considered to be the report of Aujeszky's disease in 1902 (Aujeszky, 1902) and the report of Marek's disease in 1907 (Marek, 1907).

The first isolation of herpesvirus is considered the report of experimental infection of human herpesvirus 1 (HHV-1) from human lesions to rabbit corneas (Loewenstein, 1919). Following this report, cultivation and titration of HHV-1 using mice was reported in 1932 (Saddington, 1932). Virus growth in cell culture was demonstrated in 1949 (Enders *et al*., 1949). After that, cultivation of several kinds of herpesviruses using cell cultures were reported (Sanders et al., 1953). The electron microscope was put to practical use in the 1930's. Electron microscope and cultivation of virus using cell culture system made it possible to identify a partial virus (Green, 1965). In the 1950's and 1960's, identification of several herpesviruses using immunofluorescence were reported (Biegeleisen *et al*., 1959; Albrecht *et al*., 1963; Schipper and Chow, 1968; Stevens and Watkins, 1969).

Sanger et al. (1977) and Maxam et al. (1977) developed basics of DNA sequencing methods. Since then, nucleotide sequences of viruses have been reported. DNA sequence data of herpesviral genome have been reported since 1980 (McKnight, 1980). In 1984, the complete genome sequence of human herpesvirus 4 was reported (Baer *et al*., 1984). Until 1990's, nucleotide sequence data of a number of herpesviruses had been accumulated. The family *Herpesviridae* are further classified into three subfamilies, *Alpha*-, *Beta*-, and *Gammaherpesvirinae*, according to the biological features. Molecular phylogenetic analysis based on nucleotide sequences of

herpesviruses indicated that the classification of herpesviruses was consist with genetic evolution of herpesviruses (McGeoch and Cook, 1994; McGeoch *et al*., 1995).

Van Devanter et al. (1996) designed consensus primers for amplification of DNA of various kinds of herpesviruses by PCR. This PCR method was the breakthroughs in identification of novel herpesviruses. In the assay, degenerate primers were targeted to highly conserved sequences of DNA polymerase gene of herpesviruses.

Recently, several new methods for DNA sequencing were developed (Ronaghi *et al*, 1996; Ronaghi *et al*., 2000; Brenner *et al*., 2000; Bentley *et al*., 2008). These massively parallel sequencing methods are called as the next-generation sequencing (NGS) methods. These technologies made it possible to detect and identify novel herpesviruses more easily. Especially, detection and identification of herpesviruses derived from bats were reported (Wu *et al*., 2012; Sano *et al*., 2015; Shabman *et al*., 2016; Geldenhuys *et al*., 2018).

1.2. Virus properties

Herpesviruses are viruses belonging to the order *Herpesvirales*. The virion of herpesviruses is spherical and the diameter of HHV-1 is from 170 to 200 nm (Grünewald *et al*., 2003).

The virion of herpesviruses consists of nucleocapsid, tegument, and envelope. Herpesviral genome is a linear double stranded DNA. Nucleocapsid consists of an icosahedral capsid and a herpesviral genome contained in the capsid. Tegument, which is a virus structure characteristic of herpesvirus, is located in the space between the envelope and capsid. The envelope is derived from the host cell membrane. Herpesviral envelope contains viral proteins, especially glycoproteins (Pellett *et al*., 2011).

Structures of herpesviral genome are classified into six types (type A to F)

based on the location of repeat sequences and the number of isomers (Roizman and Pellet, 2001; Davison 2007). In type A, the genome is composed of a pair of direct repeats (Terminal Repeat; TR) and a unique region sandwiched between them. The size of TR is diverse. For example, the size of direct repeat of equine herpesvirus 5 (EHV-5) is 10 bp, and the size of direct repeat of equine herpesvirus 2 (EHV-2), belonging to the same genus as EHV-5, is more than 15k bp (Wilkie *et al*., 2015). In type B, the genome is composed of a pair of direct repeats and a unique region. The difference from type A is that the direct repeat consists of tandem repeat sequences, and the number of repeats is variable. In type C, the genome structure is basically similar to type B, but the repeat sequences, not related to the terminal repetitive sequence, are inserted in the unique region. In type D, there are two unique regions (U_L and U_S) on the genome. The U_L and U_S are sandwiched between different inverted repeats, TR_L/IR_L and IR_S/TR_S respectively. IR_L and IR_S are adjacent. In type E, the genome structure is basically similar to type D, but the difference with type D is that the direct repeats locate at the both termini of viral genome and an inverted repeat of direct repeats insert between IR_L and IR_S. In type F, the genome doesn't include definite direct or inverted repeats.

Herpesvirus genomes contain a lot of open reading frames (ORFs). The number of ORFs ranges from 70 to more than 200 (Pellett *et al*., 2011). The genomes of several herpesviruses also encode microRNA gene (Piedade and Azevedo-Pereira, 2016). In the mammalian and avian herpesvirus group, about 40 genes are commonly shared, which are termed core genes. These genes are classified into several gene blocks. They encode following proteins concerning for DNA replication machinery, peripheral enzymes, processing and packaging of DNA, egress of capsids from nucleus, capsid assembly, structure of capsid and tegument, envelope glycoproteins, multifunctional regulator of gene expression, and nuclear protein (Davison, 2007; Pellett *et al*., 2011).

1.3. Classification and distribution of herpesvirus

The classification of herpesviruses will be described according to virus taxonomy released by International Committee on Taxonomy of Viruses (ICTV) and information of nucleotide sequences registered in National Center for Biotechnology Information (NCBI) database.

Herpesviruses belong to the order *Hepesvirales*. The order *Herpesvirales* are classified into three families; *Herpesviridae*, *Alloherpesviridae*, and *Malacoherpesviridae* (Davison *et al*., 2009). *Herpesviridae* is further classified into three subfamilies: *Alpha*-, *Beta*-, and *Gammaherpesvirinae*.

The subfamily *Alphaherpesvirinae* includes mammalians, birds, and turtles herpesviruses. The subfamily *Alphaherpesvirinae* consists of five genera: *Iltovirus*, *Mardivirus*, *Scutavirus*, *Simplexvirus* and *Varicellovirus*. The type specie of the genus *Simplexvirus* is HHV-1. The genome organization of several simplexviruses belongs to type E. The genus *Simplexvirus* mainly includes primates herpesviruses. Bovine herpesvirus 2 also belongs to the genus *Simplexvirus*. The type specie of *Varicellovirus* is human herpesvirus 3 (HHV-3), varicella-zoster virus. The genome organization of several varicelloviruses belongs to type D. This genus includes herpesviruses derived from a wide range of animal species in comparison with simplexviruses. The genera *Mardivirus* and *Iltvirus* consist of viruses infecting to birds. All avian herpesviruses belong to the subfamily *Alphaherpesvirinae* or unassigned herpesvirus, not to Beta- or gammaherpesviruses. The type specie of the genus *Mardivirus* is gallid herpesvirus 2, which is the pathogen of Marek's disease. The type specie of the genus *Iltovirus* is gallid herpesvirus 1, which is the pathogen of infectious laryngotracheitis. The type specie of the genus *Scutavirus* is chelonid alphaherpesvirus 5 (ChHV-5), which is associated with

fibropapillomatosis in sea turtles. Chelonid alphaherpesvirus 6 (ChHV-6) is the alphaherpesvirus derived from turtles, but this herpesvirus was not defined as the genus *Scutavirus*. ChHV-6 is associated with lung-eye-trachea disease in sea turtles.

The subfamily *Betaherpesvirinae* consists of four genera: *Cytomegalovirus*, *Muromegalovirus*, *Proboscivirus*, and *Roseolovirus*. The type specie of the genus *Cytomegalovirus* is human herpesvirus 5 (HHV-5). The genus *Cytomegalovirus* includes herpesviruses mainly infecting to primates. The type specie of the genus *Muromegalovirus* is murid herpesvirus 1. The genus *Muromegalovirus* includes rodents herpesvirus. The type specie of the genus *Roseolovirus* is human herpesvirus 6A. The genus *Roseolovirus* includes herpesviruses in human and several non-human primates. The type specie of the genus *Proboscivirus* is elephantid herpesvirus 1 (ElHV-1), which is known as elephant endotheliotropic herpesvirus. The genus *Proboscivirus* contains several elephants herpesviruses.

The subfamily of *Gammaherpesvirinae* consists of four genera: *Lymphocryptovirus*, *Macavirus*, *Percavirus* and *Rhadinovirus*. The type specie of the genus *Lymphocryptovirus* is human herpesvirus 4 (HHV-4), known as Epstein-Barr virus. The genus *Lympocryptovirus* includes herpesviruses derived from human and non-human primates. The type specie of the genus *Rhadinovirus* is human herpesvirus 8 (HHV-8), known as Kaposi's sarcoma-associated herpesvirus. The genus *Rhadinovirus* include herpesviruses derived from various mammalians; human, non-human primates, rodents, ruminants, cetaceans and bats. The type specie of the genus *Macavirus* is alcelaphine herpesvirus 1, known as causal virus of malignant catarrhal fever virus. The genus *Macavirus* includes herpesviruses derived from Artiodactyla. The type specie of the genus *Percavirus* is EHV-2. The genus *Percavirus* contains herpesviruses derived from Carnivora and Perissodactyla. Some herpesviruses derived from mammals, birds

and reptiles, have not been assigned into these three subfamilies yet.

The family *Alloherpesviridae* consists of 4 genera, *Batrachovirus*, *Cyprinivirus*, *Ictalurivirus*, and *Salmonivirus*. Frog herpesviruses belong to the genus *Batrachovirus*. Fish herpesviruses belong to the genus *Cyprinivirus*, *Ictavirus*, and *Salmonivirus*. The members of the family *Alloherpesviridae* contain diverse virus species (McGeoch *et al*., 2006).

The family *Malacoherpesviridae* consists of 2 genera, *Aurivirus* and *Ostreavirus*. They infect to Oyster, Abalone and Chlamys.

1.4. Replication of herpesvirus

Generally, cell-virus adsorption and viral entry to cells are essential for establishment of virus infection of virus. In other words, binding of surface proteins of viruses to receptors on cells, followed by membrane fusion, or endocytosis are essential. Envelope glycoproteins of herpesviruses are known as binding proteins to receptor of host cells. Although the number of envelope glycoproteins varies depending on herpesvirus spiecies, at least three glycoproteins are required for entry; gB, gH, and gL (Spear and Longnecker, 2003) which are encoded in core gen. After membrane fusion, the nucleocapsid and tegument proteins of herpesvirus are released into the cytoplasm of infected cells.

Many DNA viruses, including herpesvirus, need to transfer the viral genome into nuclei of infected cells for viral replication. Because nucleocapsid of herpesvirus is too large to pass thorough nuclear pore, they first bind nucleocupsid to cytoplasmic side of the nuclear pore complex via importin. The capsid has been degraded, then the viral genome is transferred into the nucleus (Cohen *et al*., 2011).

After the entry of viral genome into cell nuclei, transcription of viral genome is

carried out by RNA polymerase II derived from host machinery (Costanzo *et al*., 1977). Gene expressions of herpesviruses are divided into three types, immediate early gene, early gene and late gene (Weir, 2001).

In general, viral genomic DNA in host cell nucleus becomes circular (Boehmer and Lehman, 1997). DNA synthesis of herpesvirus genome starts at one or more origins of replication (Mocarski, 2007). In HHV-1, 7 proteins necessary for DNA synthesis are encoded in their genome (Weller and Coen, 2012), and among them, 6 proteins are encoded by core genes. Although it is thought that DNA replication is performed by a rolling cycle, it is more complicated in HHV-1 (Weller and Coen, 2012).

The capsid is assembled in the host cell nucleus. The viral genomic DNA cleaved from concatemer is encapsulated in the mature capsid and form nucleocapsid (Mettenleiter *et al*., 2009). These proteins important for constituting the capsid, its formation, cleaving the concatemer are encoded by core gen. After nucleocapsid leave from the nucleus to the cytoplasm, the final envelope is acquired in cytoplasmic organelle (Mettenleiter *et al*., 2006) and the virion is released from the cell by exocytosis.

1.5. Latency and persistent

In general, herpesviruses establish latent or persistent infection. However, there are some exceptions. For example, HHV-6 vertically infects by integration into the chromosome (Hall *et al*, 2008). Basically, herpesviruses establish latent infection or persistent infection by evasion from the immune mechanism of the host.

Herpesviruses have the evasion mechanisms from complement (Favoreel *et al*., 2003; Agrawal *et al*., 2017), antibody (Hook *et al*., 2007;Farré *et al*., 2017), MHC class I (Griffin *et al*., 2010), NK cell (De Pelsmaeker *et al*., 2018), and apoptosis (Galluzzi *et*

al., 2008). It was reported that homologs of the host protein encoded on herpesviral genome involved in some of these immune evasion mechanisms.

Sites of latency depend on the virus species. For example, HHV-1, -2, and -3, belonging to the subfamily *Alphaherpesvirinae* are latent in sensory and cranial nerve ganglia. HHV-5, -6, and 7, belonging to the subfamily *Betaherpesvirinae*, are latent in following site: monocytes, lymphocytes; various leukocytes; and T-cells, epithelia. HHV-4 and -8, belonging to the subfamily *Gammaherpesvirinae* are latent in B-cells (Grinde, 2013).

1.6. Herpesvirus infection in animals

Alphaherpesvirus infections in animals often cause respiratory diseases: Infectious bovine rhinotracheitis (Muylkens *et al*., 2009), Aujeszky's disease in pigs (Mettenleiter, 2000), avian infectious bronchitis (Ignjatović and Sapats, 2000), equine rhinopneumonitis (van Maanen, 2002), feline viral rhinotracheitis (Gaskell *et al*., 2007), and canine herpesvirus infection (Buonavoglia and Martella, 2007). These infectious diseases sometimes cause death in the animals. Infection of several alphaherpesviruses causes abortion (Smith, 1997). Marek's disease virus, which causes malignant tumor, is significantly different from other alphaherpesviruses (Venugopal, 2000).

The most well-known betaherpesvirus with high pathogenicity in animals is ElHV-1, belonging to the genus *Proboscivirus*. ElHV-1 infection causes acute haemorrhagic disease in young Asian elephants (*Elephas maximus*), and the majority of juvenile Asian elephants deaths are due to ElHV-1 infection (Long *et al*., 2016). Murid herpesvirus 1 (MuHV-1) is one of the most studied betaherpesvirues, because the virus shares many characteristics with human herpesvirus 5 (Krmpotic *et al*., 2003). Suid herpesvirus 2 is an unassigned betaherpesvirus and causes inclusion body rhinitis in

pigs (Edington *et al*., 1976).

The most well-known severe infectious disease caused by gammaherpesvirus infection is malignant catarrhal fever. Although gammaherpesviruses have been isolated from and detected in many mammalian species, their pathogenicity remains unknown, except for some viruses (Ackermann, 2006).

Generally, natural host range of individual herpesviruses was restricted. Nevertheless, cercopithecine herpesvirus 1 (Elmore and Eberle., 2008), suid herpesvirus 1 (Mettenleiter, 2000), alcelaphine herpesvirus 1 and ovine herpesvirus 2 (Russell *et al*., 2009) cause fatal disease with systemic symptoms, when they infected to the other hosts. It was reported that HHV-1, whose natural host is human, caused fatal disease in New World primate (Epstein and Price, 2009).

1.7. Prevention and treatment

For prevention of herpesvirus infection in animals, some vaccines are available. For examples, a combination vaccine with feline calicivirus and feline parvovirus can be used for prevention from feline herpesvirus 1. Both modified live virus (MLV) vaccines and adjuvanted inactivated virus vaccines can be used (Gaskell *et al*., 2007). For preventing Marek's disease, Marek's disease virus type 2 and Turkey herpesvirus are used as vaccine (Gimeno, 2008). Recombinant vaccines with a deletion of genes related with the pathogenicity are also used for preventing animals. Thymidine kinase or envelope glycoprotein, especially envelope glycoprotein gE, deleted recombinant virus vaccine are well known (Jones and Chowdhury, 2007; Freuling *et al*., 2017).

Several studies on antiviral therapy against herpesvirus infections have been carried out, mainly in feline, caprine and equine herpesviruses (Dal Pozzo and Thiry, 2014). Trifluridine, idoxuridine, ganciclovir and acyclovir are recommended for

treatment of disease caused by feline herpesvirus 1. These antiviral drugs may also be co-administered with interferon. However, valaciclovir is known to be toxic in cats (Nasisse *et al*., 1997). Several cases in which ElHV-1-infected calves of elephants were treated with antiviral drugs were reported (Richman *et al*., 2000; Schmitt *et al*., 2000; Dastjerdi *et al*., 2016).

1.8. Cetacean herpesviruses and bat herpesviruses

More than 250 species of herpesviruses have been found from many animals. However, the characterization of these viruses has been performed in limited herpesviruses; herpesviruses derived from human, experimental animals, companion animals and livestock animals.

Cetacean herpesviruses are considered difficult for isolation. By PCR using consensus primers, the gene of many kinds of herpesviruses have been detected from cetaceans in both sea and river (Blanchard *et al*., 2001; Saliki *et al*., 2006; Smolarek *et al*., 2006; Manire *et al*., 2006; van Elk *et al*., 2009; Arbelo *et al*., 2010; Bellière *et al*., 2010; Maness *et al*., 2011; Miyoshi *et al*., 2011; Soto *et al*., 2012; Hart *et al*., 2012; Rehtanz *et al*., 2012; Lecis *et al*., 2014; Sierra *et al*., 2015; van Beurden *et al*., 2015; Bellehumeur *et al*., 2015; Melero *et al*., 2015; van Elk *et al*., 2016; Seade *et al*., 2017; Davison *et al*., 2017; Grattarola *et al*., 2018; Sacristán *et al*., 2018). Complete sequence of the gammaherpesvirus derived from a bottlenose dolphin was determined (Davison *et al*., 2017). However, only one cetacean-derived gammaherpesvirus has been isolated successfully (van Elk *et al*., 2009). Therefore, pathogenicity has hardly been elucidated. A few herpesviruses derived from cetaceans are likely to be related to skin lesions, genital lesions, neurological disease (van Elk *et al*., 2016), and systemic symptoms

(Arbelo *et al*., 2010). Sero-epidemiology of cetacean herpesvirus has been limited (van Elk *et al*., 2009).

Genetic information of bat herpesviruses is now increasing by NGS and PCR methods (Wibbelt *et al*., 2007; Molnár *et al*., 2008; Razafindratsimandresy *et al*., 2009; Watanabe *et al*., 2009; Watanabe *et al*., 2010; Jánoska *et al*., 2011; Zhang *et al*., 2012; Wu *et al*., 2012; Sasaki *et al*., 2014; Sano *et al*., 2015; Zheng *et al*., 2016; Shabman *et al*., 2016; Host *et al*., 2016; Pozo *et al*., 2016; Salmier *et al*., 2017; Subdhi *et al*., 2018; Wada *et al*., 2018; Geldenhuys *et al*., 2018; Holz *et al*., 2018). To date, three bat herpesviruses were isolated and determined complete nucleotide sequences (Zhang *et al*., 2012; Sasaki *et al*., 2014; Subudhi *et al*., 2018). And cell tropisms, predicted proteins, and phylogenic relationship were analyzed in the studies. Since Chiroptera contains many species following Rodentia among Mammalia (Wilson and Reeder, 2005), it is expected that there must be an enormous number of herpesviruses in bat.

In this study, I attempted to isolate viruses from various animal specimens and succeeded in isolation of herpesviruses from a dead Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) and a greater horseshoe bat (*Rhinolophus ferrumequinum*) and genetically characterization to them.

2. CHAPTER 1

Isolation of a novel herpesvirus from a Pacific white-sided dolphin

2.1. ABSTRACT

During establishment of primary cell culture from the kidney of a dead Pacific white-sided dolphin (*Lagenorhynchus obliquidens*), a cytopathic effect was observed. Polymerase chain reaction with a set of herpesvirus consensus primers yielded a fragment of the expected size. Nucleotide sequencing of the product indicated that the isolated virus was closely related to an alphaherpesvirus detected in a bottlenose dolphin in the United States, but the identity at the protein level was low (86.6%). Phylogenetic analysis of the encoded sequence confirmed that the new isolate belonged to the subfamily *Alphaherpesvirinae* and clustered together with other cetacean alphaherpesviruses. The complete glycoprotein B-encoding gene (2,757 bp) was amplified from the novel isolate; the encoded protein was compared with the corresponding protein of other herpesviruses, revealing that this virus belongs to the genus *Varicellovirus*. Taken together, these results suggest that this virus corresponds to a novel herpesvirus capable of infecting Pacific white-sided dolphins.

2.2. INTRODUCTION

As described in GENERAL INTRODUCTION, herpesviruses have been detected and isolated from a number of animal species and are classified in the order *Herpesvirales* which comprises three families *Alloherpesviridae*, *Herpesviridae* and *Malacoherpesviridae*. The family *Herpesviridae* comprises three subfamilies, *Alpha*-, *Beta*-, and *Gammaherpesvirinae*. The *Alphaherpesvirinae* subfamily is divided into five genera: Iltovirus, Mardivirus, Scutavirus, Simplexvirus and Varicellovirus. Several of the herpesviruses have been recovered from cetaceans, including animals from the genera *Phocoenidae*, *Monodontidae*, *Delphinidae*, *Ziphiidae*, *Kogiidae*, and *Physeteridae* (van Bressem *et al*., 1999; Smolarek *et al*., 2006; Arbelo *et al*., 2010; Miyoshi *et al*., 2011). These cetacean herpesviruses belonged to the subfamily of *Alpha*- or *Gammaherpesvirinae*, and were detected in areas including Japanese coastal waters. However, to our knowledge, only one cetacean-derived gammaherpesvirus has been isolated successfully, and grown on primary kidney cell culture originating from a *Harbor porpoise* (van Elk *et al*., 2009).

Reports of gene sequences for cetacean herpesviruses have been limited to part of a locus encoding DNA polymerase, as amplified by polymerase chain reaction (PCR) using herpesvirus consensus primers (Van Devanter *et al*., 1996). As a result, phylogenetic relationships among cetacean herpesviruses have been analyzed only using this region. Therefore, further genetic analysis was required to clarify the relationship among cetacean herpesviruses and between cetacean herpesviruses and other herpesviruses.

In this study, a novel herpesvirus was isolated from a primary kidney cell culture originating from a dead Pacific white-sided dolphin (*Lagenorhynchus obliquidens*). The consensus primers yielded a fragment corresponding to part of a DNA

polymerase-encoding gene. Further work provided a complete gene for the major immunogenic protein, glycoprotein B, which is conserved among all herpesviruses. Sequence analysis of the gB-encoding gene permitted a determination of the relationship between this virus and the other herpesviruses.

2.3. MATERIALS AND METHODS

2.3.1. Primary cell culture

To establish a primary cell culture from the kidney of a dead Pacific white-sided dolphin, the kidney cells were dispersed through a mesh and cultured in RPMI1640 (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Hyclone®, Otawa, Canada), 100U/ml of penicillin and 100μg/ml of streptomycin (GIBCO) . The cell culture was incubated at 37 °C in 5% CO2 under humid condition.

2.3.2. Identification of the putative virus by molecular analysis

To identify the putative virus, DNA was extracted from the primary kidney cell culture using a DNeasy tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. To amplify the DNA polymerase-encoding gene from a potential herpesvirus, the resulting DNA was subjected to PCR using the KOD -Plus- Ver.2 kit (TOYOBO, Osaka, Japan) with consensus primers (VanDevanter *et al*., 1996). The reaction was carried out via initial denaturation at 94°C for 2 min; 45 cycles of denaturation at 98°C for 10 sec, annealing at 46°C for 30 sec, and extension at 68°C for 60 sec; and final extension at 68°C for 3 min. The PCR product was electrophoresed on agarose; the resulting fragment (approximately 700 bp) was excised and purified using a QIAEX II gel extraction kit (QIAGEN) according to the manufacturer's protocol. Nucleotide sequence of the fragment was determined using a Big Dye Terminator v3.1 kit (Applied Biosystems, Foster City, CA, U.S.A.).

2.3.3. Phylogenetic analysis

The nucleotide and deduced amino acid sequences of the DNA polymerase and envelope glycoprotein B gene were analyzed using blastX search (Johnson *et. al*. 2008)

and Genetyx 8.0 software (Genetyx, Tokyo, Japan). Amino acid sequences were compared with the previously reported sequences using Clustal W in MEGA 5.05 (Tamura *et al*., 2011), and the phylogenetic trees were constructed using the maximum likelihood (ML) method in PhyML 3.0 (Guindon *et al*., 2003).

2.3.4. PCR and sequencing analysis for the complete glycoprotein B gene

First, PCR was performed with the primer pair Dpol R1 (5'-GTC ACA GCC TCA ACA CTT AAC GCC AAG GTT G-3') and HVgBR (5'-YBM KYT CBY KRT TYT GKA GBK BRC ACC ACG-3'), which are expected to span the gB-encoding locus. Reactions were performed using Ex Taq enzyme (Takara Bio Inc., Shiga, Japan), with initial denaturation at 94°C for 2 min; 40 cycles of denaturation at 98°C for 10 sec, annealing at 51°C for 30 sec and extension at 72°C for 12 min; and final extension at 72°C for 10 min. The amplified fragment (approximately 9 kb) was electrophoresed on agarose, excised, and purified as described in 2.3.2. Sequence obtained from one end of the fragment (using the HVgBR primer) was used to design a virus envelope glycoprotein B gene-specific primer gBR1 (5'-GGA GAC CAT GGC CTG GAA GGC AAC-3').

PCR with primers specific for the virus, DpolR1 and gBR1, was performed using LA Taq (Takara Bio Inc.) with initial denaturation at 94°C for 2 min; 40 cycles of denaturation at 98°C for 10 sec followed by annealing and extension at 68°C for 12 min; and final extension at 68°C for 10 min. The PCR product (approximately 9 kb) was purified as described in 2.3.2.

To determine the complete envelope glycoprotein B gene sequence, inverse PCR was performed, taking advantage of a XhoI restriction site noted in the partial sequence of the gB-encoding gene. Specifically, DNA extracted from infected cells was

digested with XhoI, self-ligated, and used as the template for PCR using LA Taq (Takara Bio Inc) with two primers (gBXhoIup, 5'-TCG AGT GGT CGG CTT GAT GTG TG-3'; gBXhoIdw, 5'-CCA CCT TTG TGG GTG AAC CTA TG-3'). The reaction consisted of initial denaturation at 94°C for 2 min; 40 cycles denaturation at 98°C for 10 sec and annealing at 68°C for 10 min; and final extension at 68°C for 10 min. The resulting PCR product was electrophoresed on agarose, excised, purified, and sequenced as described in 2.3.2. using the amplifying primers and primers designed based on the obtained sequences.

2.4. RESULTS

2.4.1. Isolation of the novel virus

During the culturing of the primary from the kidney of a dead Pacific white-sided dolphin, cytopathic effects (CPE) were observed, specifically, the formation of syncytia and multinucleated giant cells, suggesting the presence of a virus.

2.4.2. Identification of the novel virus

PCR with the herpesviral consensus primers yielded a DNA fragment of approximately 700 bp. The nucleotide sequence determined by sequencing analysis was deposited in the DNA data bank of Japan (DDBJ) as accession numbers AB747558. BlastX search with the resulting sequence revealed that the predicted DNA polymerase protein fragment was closely related to that encoded by a bottlenose dolphin herpesvirus detected in the USA (Maness *et al*., 2011), with identity of 86.6% at the amino acid level, revealing that the newly isolated virus belonged to *Alphaherpesvirinae* and represented a novel herpesvirus. Therefore, the isolated virus was tentatively named lagenorhynchus herpesvirus 1.

2.4.3. Phylogenetic analysis using a partial DNA polymerase gene

The ML tree using the amino acid sequences of the DNA polymerase proteins of alphaherpesviruses indicated that this Lagenorhynchus herpesvirus 1 belonged to the subfamily *Alphaherpesvirinae*. In addition, Lagenorhynchus herpesvirus 1 and the other cetacean alphaherpesviruses clustered together and were further divided into three groups. The Lagenorhynchus herpesvirus 1 grouped with herpesviruses detected from striped dolphins in Spain (Bellière *et al*., 2007), a melon-headed whale in Japan (Miyoshi *et al*., 2011), and bottlenose dolphins in the USA (Maness *et al*., 2011;

Blanchard *et al*., 2001) and Germany (Smolarek *et al*., 2006) (Fig.2-1).

2.4.4. The sequence of the complete envelope glycoprotein B gene

To clarify further the phylogenetic relationship with other herpesviruses, a complete gene encoding the envelope glycoprotein B was recovered and analyzed. The nucleotide sequence determined by sequencing analysis was deposited in the DDBJ as accession number AB747559.

The sequence of the complete envelope glycoprotein B-encoding gene (2,757 bp) of the Lagenorhynchus herpesvirus 1 was compared to that of other herepesviruses, revealing that the predicted gB protein (918 amino acids) was most closely related (61.9% identity at the protein level) to that of Canid herpesvirus 1. Phylogenetic analysis using the amino acids sequence of envelope glycoprotein B gene indicated that the Lagenorhynchus herpesvirus 1 belonged to the genus *Varicellovirus* (Fig. 2-2).

2.5. DISCUSSION

To our knowledge, this is the first report of the isolation of an alphaherpesvirus from a cetacean. As a result of phylogenetic analysis of alphaherpesviral DNA polymerase proteins, this virus clustered with herpesvirus of cetaceans in the previous study (Miyoshi *et al*., 2011). Furthermore, phylogenetic analysis using amino acid sequences of envelope glycoprotein B indicated that the Lagenorhynchus herpesvirus 1 belonged to the genus *Varicellovirus*, suggesting that cetacean alphaherpesviruses should be classified into the genus *Varicellovirus*.

In summary, the Lagenorhynchus herpesvirus 1 described here was similar to herpesviruses detected in striped dolphins in Spain (Bellière *et al*., 2007), a melon-headed whale in Japan (Miyoshi *et al*., 2011), and bottlenose dolphins in the United States (Maness *et al*., 2011; Blanchard *et al*., 2001) and Germany (Smolarek *et al*., 2006) (Fig. 2-1). One of them, a herpesvirus detected from a bottlenose dolphin in USA, was associated with systemic infectious disease, as demonstrated by gross, histologic, ultrastructural, and molecular genetic analyses (Blanchard *et al*., 2001). Although the pathogenesis of the Lagenorhynchus herpesvirus 1 is still unknown, it is possible that this novel virus causes disease in these cetaceans.

Until then, since only partial sequences of DNA polymerase-encoding gene were available for cetacean herpesvirus, phylogenetic analysis of cetacean herpesviruses was limited to the partial DNA polymerase protein. Subsequently we succeeded in isolating the virus using the primary dolphin kidney cell culture, providing DNA in amounts sufficient to permit cloning and sequencing of the complete gB-encoding gene from the new virus. This novel isolate is expected to be useful for further genetic analysis of cetacean herpesviruses. However, further study will be required to identify cell lines capable of supporting the growth of this virus. In addition, sero-epidemiology

of cetacean herpesvirus remains poorly characterized. The predicted envelope glycoprotein B, which represents the major antigen protein of herpesvirus, will be useful for diagnosis of herpesvirus infection in white-sided dolphins and other cetaceans.

2.6. LEGENDS FOR FIGURES

Figure 2-1. Maximum likelihood (ML) tree of alphaherpesviruses using amino acid sequences of a portion of the DNA polymerase protein. The Lagenorhynchus herpesvirus 1 is boxed. Analysis was performed by the ML method in phyML 3.0. The number along each branch indicates the bootstrap value. Designations in parentheses are GenBank accession numbers.

Figure 2-2. Maximum likelihood (ML) tree of alphaherpesviruses using amino acid sequences of glycoprotein B. The Lagenorhynchus herpesvirus 1 is boxed. Analysis was performed by the ML method in phyML 3.0. The number along each branch indicates the bootstrap value. Designations in parentheses are GenBank accession numbers.

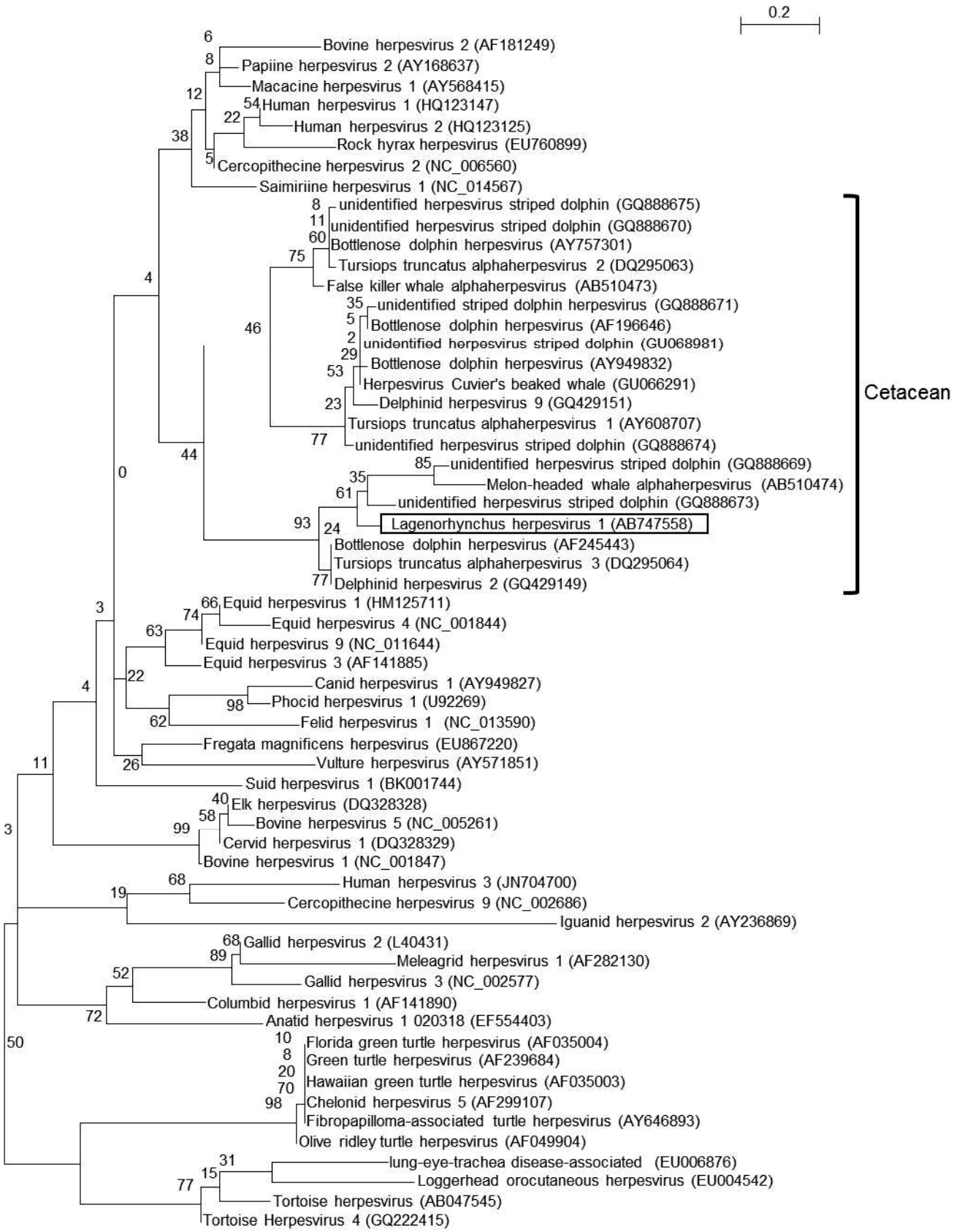

Figure 2-1. Maximum likelihood (ML) tree of alphaherpesviruses using amino acid sequences of a portion of the DNA polymerase protein.

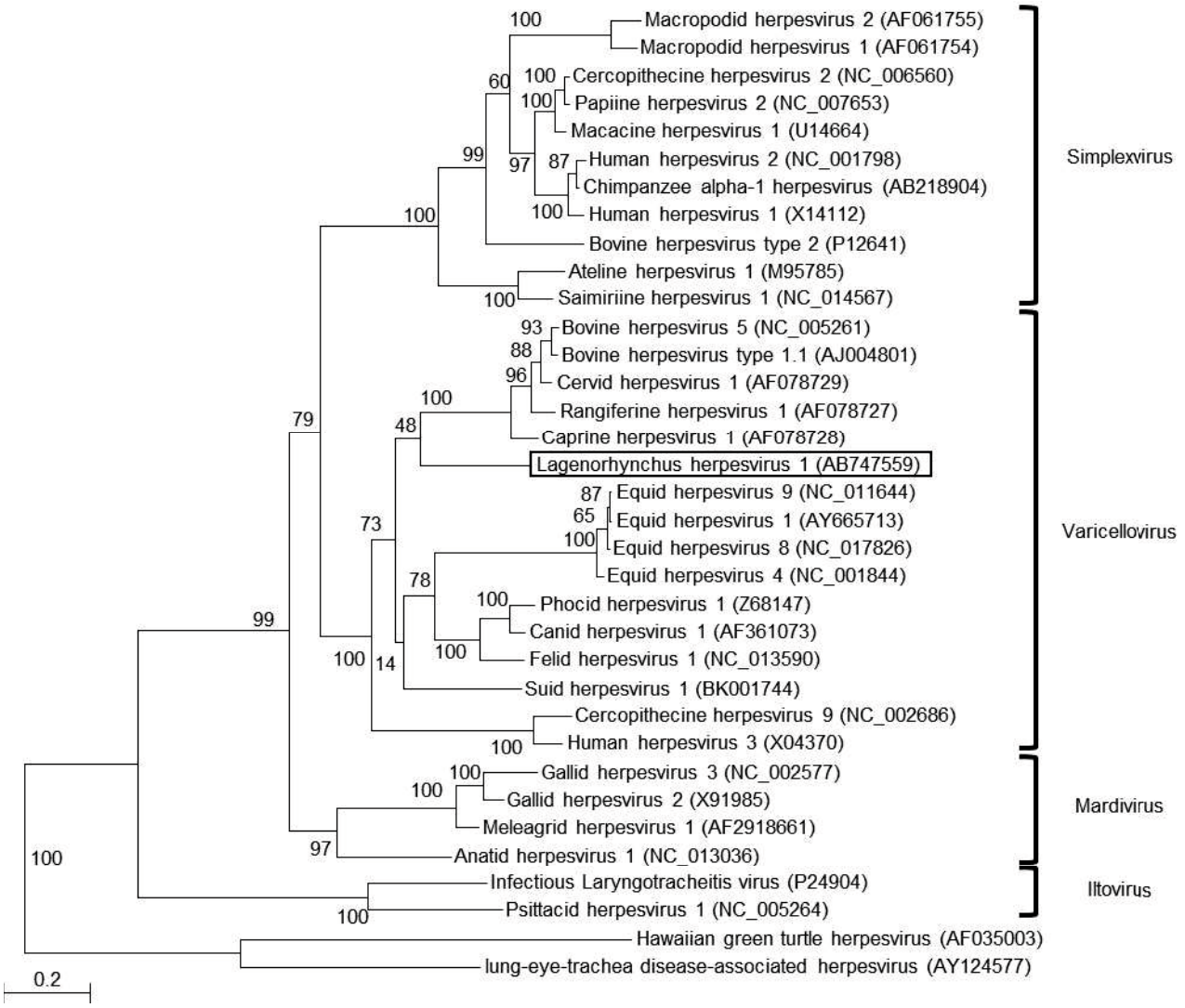

Figure 2-2. Maximum likelihood (ML) tree of alphaherpesviruses using amino acid sequences of the envelope glycoprotein B.

3. CHAPTER 2

The complete genomic sequence of Rhinolophus gammaherpesvirus 1 isolated from a greater horseshoe bat

3.1. ABSTRACT

In a comprehensive research project on bat viruses, we successfully isolated a novel herpesvirus from the spleen of a greater horseshoe bat (*Rhinolophus ferrumequinum*) in Japan using the cell line established from the kidney of the same bat. This herpesvirus was a novel gammaherpesvirus (Rhinolophus gammaherpesvirus 1; RGHV-1), which belonged to the genus Percavirus. Measurement of the growth of RGHV-1 showed that Vero and CRFK cells were more sensitive to RGHV-1 than other cell lines. Using next-generation sequencing and direct sequencing, the whole RGHV-1 genome was determined (147,790 bp). Twelve of the 84 genes predicted to contain open reading frames did not show any homology with those of other herpesviruses. One of the genes was similar to human Bcl-xl. The accumulation of information on bat viruses will be useful for understanding the emergence of bat-originated zoonotic viruses.

3.2. INTRODUCTION

Bats are the natural hosts of many viruses that cause serious diseases, such as Ebola virus, Marburg virus, Hendra virus, Nipah virus, rabies virus, and rabies-associated lyssaviruses (Calisher *et al*., 2006). Bats often become reservoirs of these zoonosis and emerging infectious diseases because there are many species of bats (over 1,000 species), they have a relatively long life span for their body size, they undergo hibernation and torpor, during which both virus replication and immune function are reduced, they exhibit a wide range of movements when they fly, and they live in densely populated conditions, especially cave bats (Luis *et al*., 2013).

Greater horseshoe bats (*Rhinolophus ferrumequinum*), which inhabit Palaearctic areas, including Japan (Piraccini, 2016), usually rest in caves, but sometimes they also rest in houses in Japan (Abe *et al*., 2008). Since severe acute respiratory syndrome (SARS)-like coronaviruses have been detected and isolated from this bat species in China, greater horseshoe bats are considered to be a natural host of SARS coronavirus (Lau *et al*., 2005; Ge *et al*., 2013). It is important to accumulate information on these viruses that infect greater horseshoe bats to better understand the emergence of bat-originated zoonotic viruses.

Herpesviruses are enveloped and double-stranded DNA viruses. As All herpesviruses derived from mammalians are classified into the family *Herpesviridae*, which comprises three subfamilies: *Alpha*-, *Beta*-, and *Gammaherpesvirinae*. The *Gammaherpesvirinae* subfamily is divided into four genera: *Lymphocryptovirus*, *Macavirus*, *Percavirus*, and *Rhadinovirus* (Davison *et al*., 2009). Although gammaherpesviruses have been isolated from and detected in many mammalian species, their pathogenicity remains unknown, with the exception of several kinds of viruses isolated from human, mouse, and sheep (Ackermann, 2006).

In our previous studies, we established several bat-derived cell lines for a comprehensive analysis of bat virus infections (Maeda *et al*., 2008; Maruyama *et al*., 2014; Maruyama *et al*., 2016). In addition, we succeeded in isolating a novel adenovirus derived from bats using our established bat kidney T1 (BKT1) cell line, which was derived from the kidney of a horseshoe bat (*R. ferrumequinum*) (Maeda *et al*., 2008). In the current study, a novel gammaherpesvirus was isolated from the spleen of a greater horseshoe bat using BKT1 cells, and the complete genome sequence of the virus was determined.

3.3. MATERIALS AND METHODS

3.3.1. Cell line

In this study, our established five cell lines derived from bats, BKT1, FBKT1, YubFKT1, YubFKT2 and DemKT1, and five cultured cell lines, Vero, CRFK, fcwf-4, MDCK and BHK, derived from the other mammals were used. BKT1 were established as described in 3.3.2. The other bat cell lines, FBKT1, YubFKT1, YubFKT2 and DemKT1 were derived from the kidney of a *Pteropus dasymallus yayeyamae*, the kidney of a *Miniopterus fuliginosus*, the kidney of a *Miniopterus fuliginosus* and the kidney of *Rousettus leschenaultia*, respectively. All of them were transformed with the expression plasmid DNA encoding the large T antigen of replication origin-defective simian virus 40.

All cell lines were maintained with Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10 % fetal calf serum and incubated at 37 °C in 5% CO2 under humid condition.

3.3.2. Primary cell culture and virus cultivation

One horseshoe bat, *Rhinolophus ferrumquinum*, was caught in a cave in Yamaguchi Prefecture in 2006 under the permission of the governor. The primary cell culture was established from the kidney and spleen. During cultivation, CPE) were appeared in the primary spleen cell culture. On the other hand, the primary cells from the kidney were established and transfected with the expression plasmid DNA encoding the large T antigen of replication origin-defective simian virus 40 (pLNCLT), kindly provided by Dr. Yasumoto. Finally, we established BKT1 cell line over 100 passages. The supernatant from the primary spleen cell culture were inoculated on BKT1 cells, the cells were passaged a few times until CPE was observed and the supernatant was

collected and used for further experiments.

3.3.3. Identification of the putative virus

DNA was extracted from the BKT1 infected with the virus using a DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's protocol and was used as a template in the following PCR. PCR with the herpesvirus consensus primers (VanDevanter *et al*., 1996) and sequencing analysis was carried out as described in 2.3.2.

3.3.4. Titration

The titer of the virus suspensions of RGHV-1 was determined by the 50% tissue culture infectious dose (TCID50) assay using 96-wells flat bottom microplate. 100 μl of serial 10-fold dilution of virus suspension was applied to each well. Then, 100 μl of Vero cells (5 x 10^4/ml) were applied to each well and incubated at 37 °C with 5 % CO2 for 8 days. Each well was observed for checking CPE, and the titer of RGHV-1 was calculated using Reed-Muench calculation (Reed et al., 1938).

3.3.5. Growth curve

Five mammalian cell lines, Vero, BHK, CRFK, fcwf-4, and MDCK, and five bat cell lines, BKT1, FBKT1, YubFKT1, YubFKT2 and DemKT1, were passaged to 6 well plates. After cells were 80% confluent, cells were inoculated with RGHV-1 at a multiplicity of infection (MOI) of 0.001. After 7 days post-infection, supernatants of cells were harvested. After centrifugation at 3,500 rpm for 5 minutes at 4 °C, the supernatants were collected and stored at -80 °C. The titer of the virus suspensions was calculated by the 50% tissue culture infectious dose (TCID50) assay using Vero cells.

For growth curve, Vero and CRFK cells were inoculated with RGHV-1 at MOI of 0.001 and the supernatant was collected every 24 hours. The viral titer was determined by TCID50 using Vero cells.

3.3.6. Extraction of genomic DNA

RGHV-1 was inoculated into monolayer of Vero and the supernatant was collected after CPE spread. After centrifugation at 3,000 rpm for 10 minutes at 4 °C, the supernatant was collected. The same operation was carried out at 8,000 rpm for 30 minutes at 4 °C, and the supernatant was recovered. For precipitating of viruses, polyethylene glycol 6,000 (PEG) was added to the virus suspension and mixed by at 4 °C overnight. After centrifugation at 8,000 rpm for 30 minutes at 4 °C, the supernatant was discarded and the pellet was suspended in PBS. This suspension was passed through a layer of 20% sucrose by ultracentrifugation at 25,000 x g for 2 hours and the pellet was suspended in PBS. DNA was extracted from the suspension using DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's protocol.

3.3.7. Next generation sequencing

For Preparation of fragment libraries from genomic DNA and PCR products, Ion Xpress™ Plus gDNA Fragment Library Preparation was used according to the manufacturer's protocol. These libraries were enriched using the Ion OneTouch™ System according to the manufacturer's protocol. Next Generation Sequencing analysis was performed on Ion torrent PGM™ with the Ion 318™ Chip kit. The data obtained in the next generation sequence was assembled with SPAdes (Bankevich *et al*., 2012). Sequences of contigs were confirmed using BWA (Li *et al*., 2009) and CLC workbench (QIAGEN). The positions of the contigs on the genome were predicted by referring to

the BLASTn (Altschul *et al*., 1990) results and the genome sequences of gammaherpesvirus.

3.3.8. PCR and sequence analysis

15 pairs of primer sets for PCR were designed based on sequences determined in the next generation sequence analysis. PCR was performed using TaKaRa LA kit (Takara Bio Inc.) and TaKaRa Gflex kit (Takara Bio Inc.). PCR products were electrophoretically analyzed and purified using QIAEXII kit. Nucleotide sequences of the fragments were determined using Sanger sequence, same as described in 2.3.2., or next generation sequence as described in 3.3.7.

3.3.9. Analysis and annotation

To identify repeat regions and inverted repeat regions, we analyzed the RGHV-1 sequence using Tandem Repeats Finder Version 4.07b (Benson *et al*., 1999) and Inverted Repeats Finder Version 3.07 (Warburton *et al*., 2004) .

The open reading frames (ORFs) and predicted proteins were analyzed using Genetyx software (version 10; Genetyx, Tokyo, Japan) and the BLASTp program (Gish *et al*., 1993), respectively.

We searched domains by using the Conserved Domain Database (Marchler-Bauer *et al*., 2017), and predicted the transmembrane domain by using the SOSUI engine (Hirokawa *et al*., 1998).

3.3.10. Phylogenetic analysis

All sequences of herpesviruses were collected from DDBJ/GenBank/EMBL database. Amino acid sequences were compared with the previously reported sequences

using Clustal W in MEGA 7 (Kumar *et al*., 2016), and the phylogenetic trees were constructed using the maximum likelihood (ML) method using MEGA 7 (Kumar *et al*., 2016).

3.4. RESULTS

3.4.1. Virus isolation

We attempted to establish cell lines from the spleen and kidney of a greater horseshoe bat (*R. ferrumequinum*). From the kidney, we succeeded in establishing cell lines of BKT1 (Maeda *et al*., 2008). However, CPE appeared in the primary spleen cell culture. To propagate the virus, we inoculated the supernatant to BKT1 cells. After several passages, CPE were observed in the BKT1 cells.

3.4.2. Identification of novel herpesvirus

To identify the isolate, RNA was extracted from the virus-infected cells and determination of viral RNA sequence (RDV) method was used to determine the sequences (Mizutani *et al*., 2007). The results indicated that one fragment possessed 51.4% homology with EHV-2 tegument protein at the amino acid sequence level. For the detection of herpesvirus genes, polymerase chain reaction (PCR) was performed using herpesvirus consensus primers (VanDevanter *et al*., 1996), and the nucleotide sequence was determined. The results showed that 682 bp of the amplified gene was homologous to those of gammaherpesviruses. The nucleotide sequence was deposited into the DNA Data Bank of Japan (DDBJ) under accession number AB298558. The isolated virus was tentatively designated as Rhinolophus gammaherpesvirus 1 (RGHV-1).

3.4.3. Propagation

RGHV-1 was propagated in BKT1 cells, but it was difficult to measure the virus titers using BKT1 cells, because CPE appeared in the BKT1 cells after a few passages following inoculation with RGHV-1. For the propagation of RGHV-1, we

inoculated RGHV-1 to Vero cells and CPE were observed 7 days after infection without passaging of the cells. In further experiments, we used Vero cells for titrating RGHV-1. To compare the sensitivity of the cell lines, 10 mammalian cell lines, including Vero (monkey), BHK (hamster), CRFK (cat), fcwf-4 (cat), MDCK (dog), and five of our established bat cell lines, BKT1, FBKT1 (Ryukyu flying fox), YubFKT1, YubFKT2 (Eastern bent-winged bat), and DemKT1 (Leschenault's rousette), were inoculated with RGHV-1 at a multiplicity of infection of 0.001. During 7 days of observation, CPE were observed only in the Vero and CRFK cells, with Vero cells showing more CPE than the CRFK cells. Seven days after infection, supernatants were collected and the viral titers were determined using the 50% tissue culture infectious dose (TCID50) method in Vero cells. The virus titers in Vero, CRFK, fcwf-4, and DemKT1 cells were all over 100 TCID50/ml. The levels were particularly high in Vero and CRFK cells, with virus titers exceeding 1,000 TCID50/ml. In contrast, the virus titers in the other six cell lines were less than 100 TCID50/ml (Fig. 3-1).

Next, virus growth was sequentially compared between the Vero and CRFK cells. Results showed that RGHV-1 grew better in CRFK cells than in Vero cells, and the virus kept growing for 9 days (Fig. 3-2).

3.4.4. Determination of the complete genome

To determine the complete genome of RGHV-1, we extracted DNA from the viral supernatant of Vero cells infected with RGHV-1. Next-generation sequencing was performed, and 314,997 reads were obtained. Next, we performed PCR for the sequences between the contigs, and the PCR products were analyzed by direct sequencing and next-generation sequencing using Ion torrent PGM™. All obtained sequences were assembled. Finally, the complete genome sequence of RGHV-1 was

confirmed by mapping.

To predict the termini of the RGHV-1 genome, we performed a search for the pac-1 signal sequence (Deiss *et al*., 1986). It is known that the pac-1 site is present in the vicinity of the 5'-end in equine herpesviruses 2 and 5 (Agius *et al*., 1992). In the RGHV-1 genome, a pac-1-like sequence (5'-GGGGGGTCAACTAAGGGGGGGG-3') was found. Next, concatemeric DNA was confirmed by PCR using primers flanking the pac-1 site and nucleotide sequence analysis. The complete genome of RGHV-1 was deposited into the DDBJ under accession number LC333428.

The complete genome of RGHV-1 was 147,790 bp, and it had a G+C content of 44.7%. The genome structure included a unique region flanked by tandem terminal repeats (4,721 bp) of identical sequences at each end (Fig. 3-3). The terminal repeats had a G+C content of 55.3%. We found several repeat regions, but did not find any of the telomeric repeats that were observed in several herpesviruses (Kishi *et al*., 1998).

3.4.5. ORFs in the genome

Eighty-four ORFs were identified (Fig. 3-3 and Table 3-1), and a homology search indicated that 44 ORFs were herpesvirus core genes that have been described in previous studies (Arvin *et al*., 2002). Of the other 40 ORFs, 28 ORFs encoded proteins that were homologues of other herpesvirus proteins, while the remaining 12 ORFs did not.

Among the 12 ORFs that did not show homology with proteins in other herpesviruses, the functions of two ORFs were predicted. The first was a protein encoded by ORF 4 that had 26% homology with the RING finger membrane protein of *Cordyceps brongniartii* by BLASTp analysis. The results of domain search showed that the RING-variant domain was located in the first third of the protein, and two

transmembrane helices were also in the middle of the protein. This structure was similar to that of a modulator of immune recognition (MIR) in herpesviruses (Lehner *et al*., 2005) despite the lack of homology, so it was speculated that this protein might function as a MIR. In addition, RGHV-1 possessed another ORF 3 encoding MIR.

The other predicted protein was encoded by ORF 13, which had 30.3% homology with human Bcl-xl, but no homology with viral Bcl-xl, and the BH-1, -2, and -4 domains were conserved.

3.4.6. The results of phylogenetic analysis

Phylogenic analysis of glycoprotein B revealed that RGHV-1 belongs to genus *Percavirus* (Fig. 3-5), and phylogenic analysis of the other core gene also suggested a similar classification.

3.5. DISCUSSION

To our knowledge, this is the first report of the complete sequence of a virus of the genus Percavirus derived from bats. Recently, the nucleotide sequence of Myotis herpesvirus 8 (MyHV-8) was reported using next-generation sequencing, but the terminal region could not be determined (Host *et al*., 2016). Within the genus Percavirus, the complete genomes of EHV-2, -5, and Felis catus gammaherpesvirus 1 have been reported, but the sizes of the terminal repeat regions and the inverted repeat sequence regions differed from those of RGHV-1 (Fig. 3-4). Thus, this RGHV-1 might have unique characteristics. A repeat region similar to the telomere-like repeats of EHV-2 was found in the terminal repeats, but telomeric sequences were not included. In this unique region, a pair of inverted repeat sequences was found, but the position appeared to differ from those of EHV-2 and -5.

Since RGHV-1 possesses several proteins related to immune evasion, it was hypothesized that these viral proteins might efficiently function in bat cell lines and persistent infection might have been established. Host *et al*. also reported that Myotis gammaherpesvirus 8 (MyHV-8) isolated from tumor-derived bat cultured cells did not cause any observable CPE on bat cell lines during infection (Host *et al*., 2016).

Susceptibility of cell lines indicated that cats might be susceptible to RGHV-1. Results showed that RGHV-1 grew better in CRFK cells than in Vero cells (Fig. 3-2). In addition, the other cat cell line, fcwf-4 cells, also supported the growth of RGHV-1 (Fig. 3-1). Further studies will be required to determine the pathogenicity of RGHV-1 in mammals, including bats and cats.

Among the herpesviruses, only human herpesvirus 8 (Lehner *et al*., 2005) and bovine herpesvirus 4 (Goto *et al*., 2003) have multiple types of MIR. These MIR homologues might suppress major histocompatibility complex expression and

contribute to the immune evasion of RGHV-1. The ORF 13 protein might have a function similar to that of Bcl-xl as an apoptosis inhibitor. In addition, RGHV-1 possessed another Bcl-2 homolog, which was encoded by ORF 8 and was similar to the BALF1 of Epstein-Barr virus that protects cells from apoptosis (Marshall *et al*., 1999). These two Bcl-2 homologues might have anti-apoptotic activity during RGHV-1 infection.

RGHV-1 was very close to Myotis ricketti herpesvirus 2, with 97.6% homology in the amino acid sequences, indicating that these two viruses must be the same species. The natural host of RGHV-1 needs to be clarified in further analyses.

In conclusion, we isolated a novel gammmaherpesvirus, RGHV-1, and determined its complete genome. The accumulation of information on bat viruses will be useful for understanding the emergence of bat-originated zoonotic viruses.

3.6. LEGENDS FOR FIGURES

Figure 3-1. Comparison of the sensitivity to RGHV-1 among 10 mammalian cell lines. Vero (monkey), BHK (hamster), CRFK (cat), fcwf-4 (cat), MDCK (dog), BKT1, FBKT1 (Ryukyu flying fox), YubFKT1, YubFKT2 (Eastern Bent-winged Bat), and DemKT1 (Leschenault's rousette) cells were infected with RGHV-1 at a multiplicity of infection of 0.001. The supernatants were collected 7 days after infection, and the titers were measured by the TCID50 method.

Figure 3-2. Growth kinetics of RGHV-1 in Vero and CRFK cells. Cells were infected with RGHV-1 at a multiplicity of infection of 0.001, and the supernatants were collected every day. The mean values from two independent experiments are shown. Filled boxes and open circles indicate the virus titers in CRFK and Vero cells, respectively.

Figure 3-3. Genome structure of RGHV-1. Arrows with diagonal stripes indicate ORFs encoding homologues of herpesvirus proteins. Arrows with black dots on a white background indicate ORFs encoding proteins homologous to proteins other than herpesvirus proteins. White arrows indicate ORFs encoding unknown proteins.

Figure 3-4. Comparison of the gammaherpesvirus genome structures. Genome structures of Equine herpesvirus 2 (EHV-2), Equine herpesvirus 5 (EHV-5), Human herpesvirus 4 (HHV-4), and Human herpesvirus 8 (HHV-8) were obtained from accession numbers indicated.

Figure 3-5. Maximum-likelihood (ML) tree of gammaherpesviruses constructed using the amino acid sequences of glycoprotein B. All sequences of herpesviruses were

collected from the DDBJ/GenBank/EMBL databases.

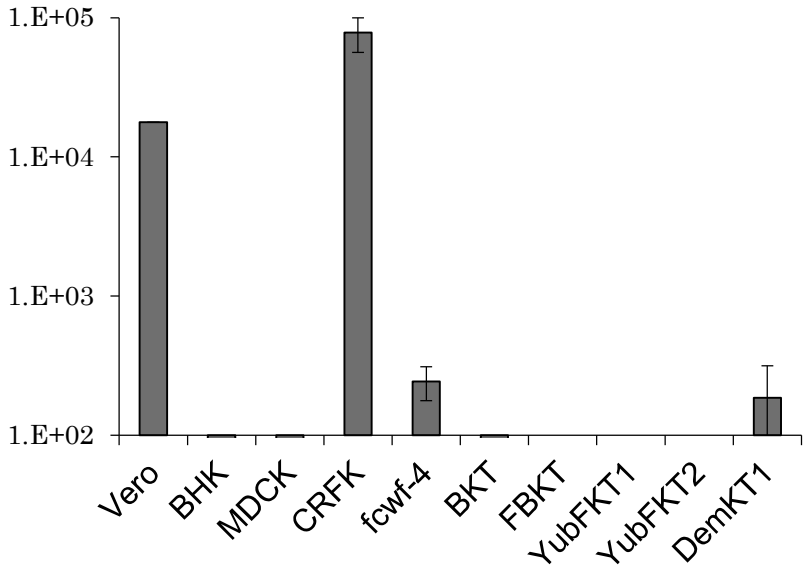

Figure 3-1. Comparison of the sensitivity to RGHV-1 among 10 mammalian cell lines.

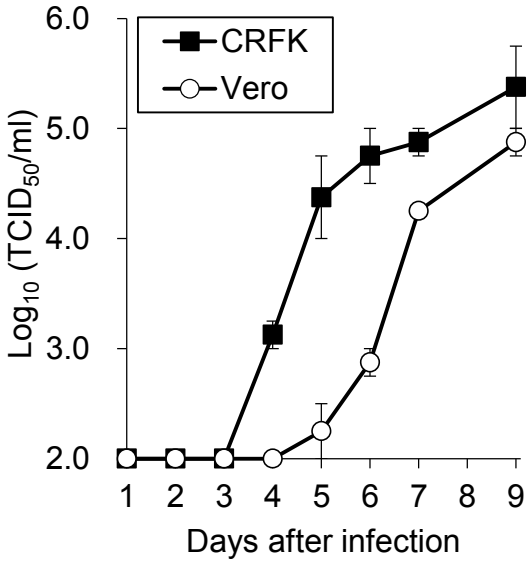

Figure 3-2. Growth kinetics of RGHV-1 in Vero and CRFK cells.

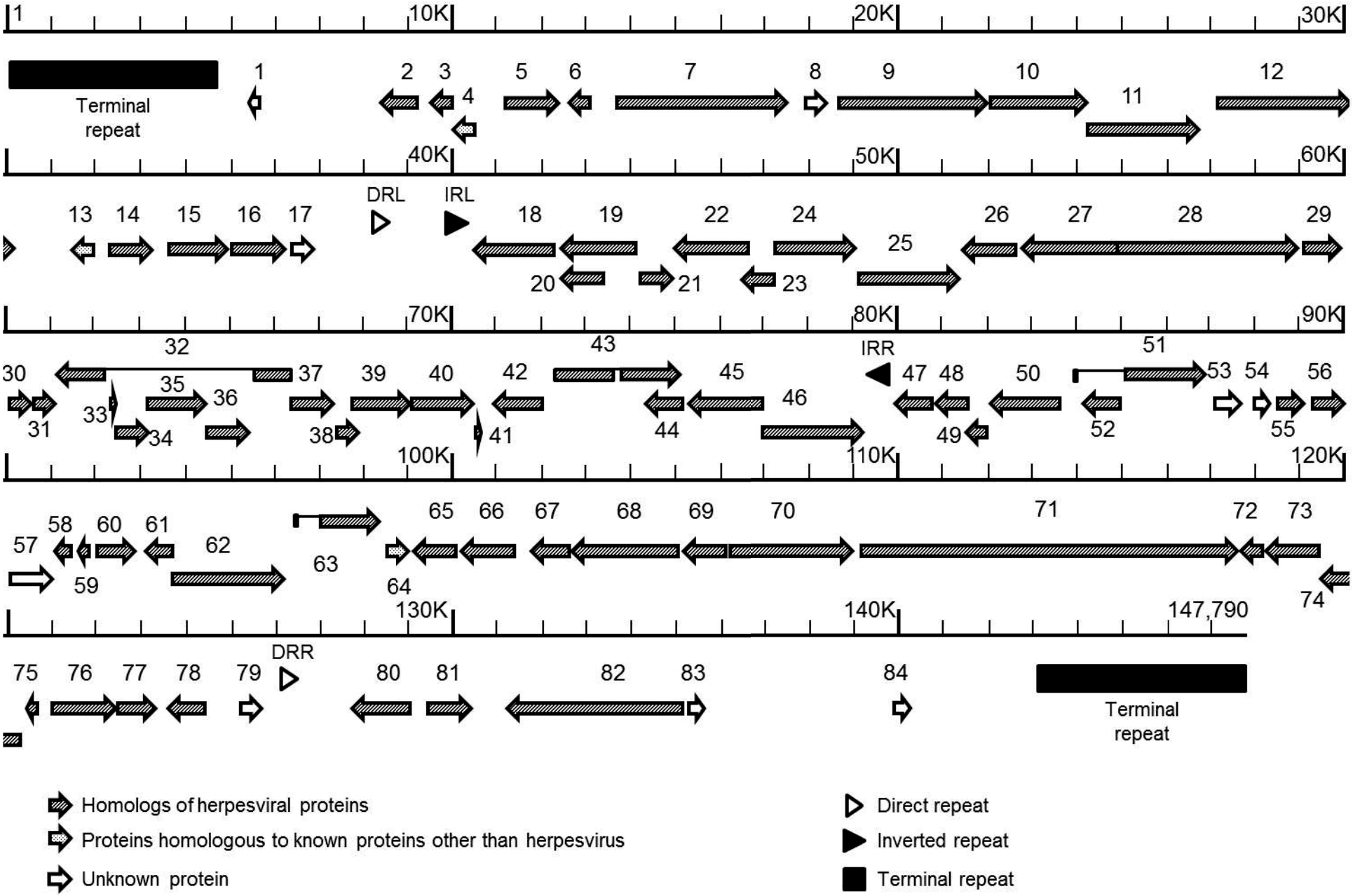

Figure 3-3. Genome structure of RGHV-1.

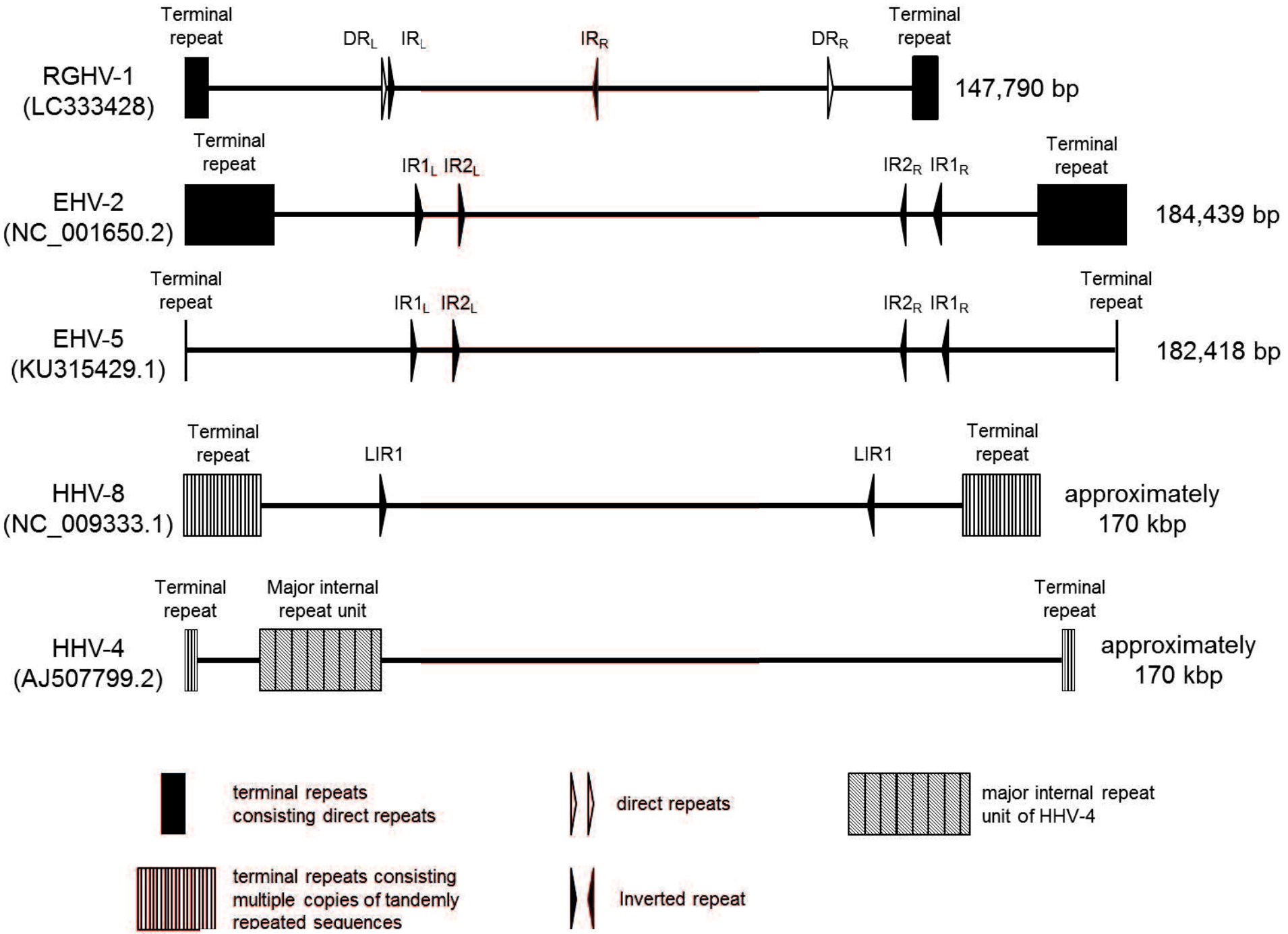

Figure 3-4. Comparison of the gammaherpesvirus genome structures.

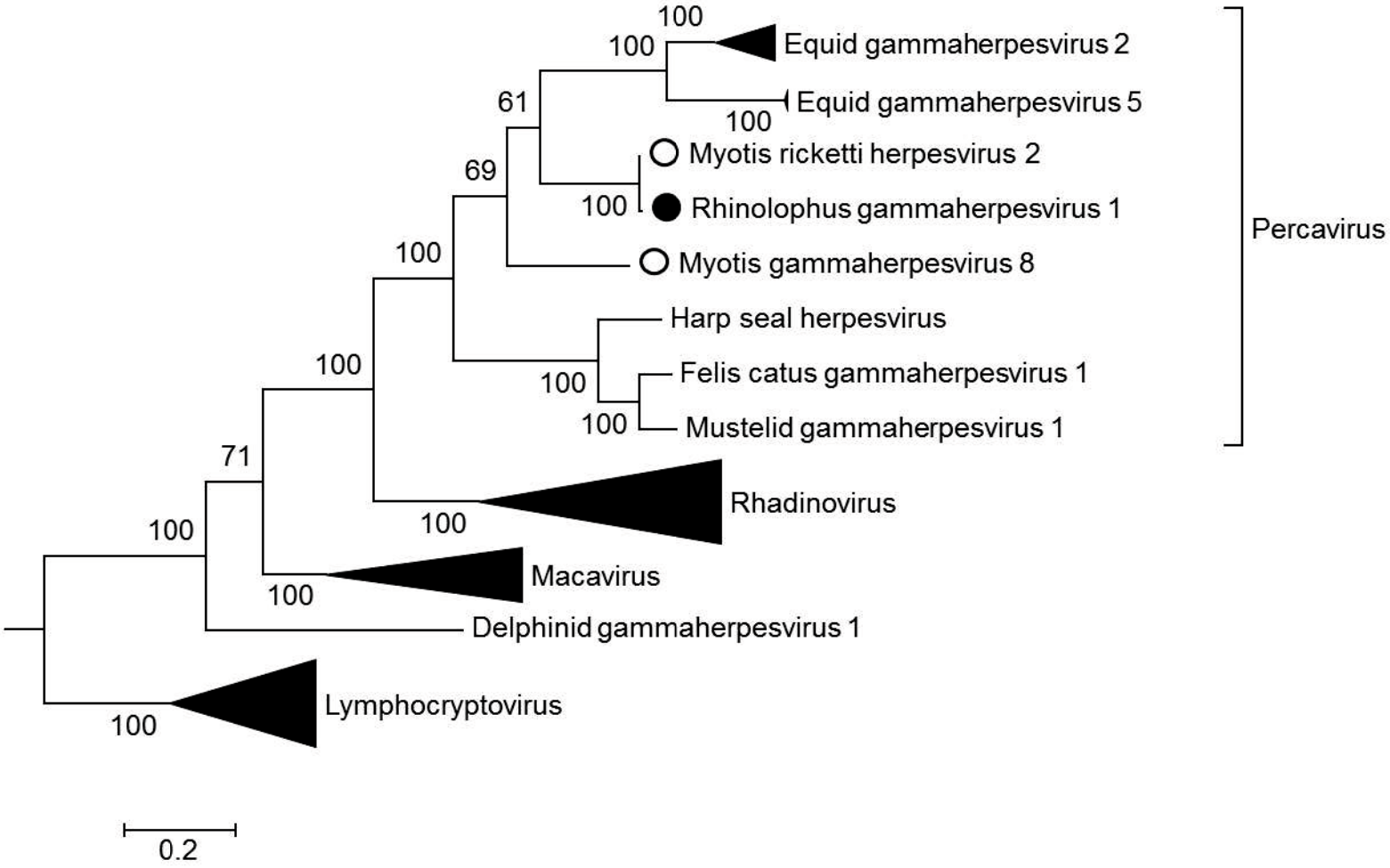

Figure 3-5. Maximum-likelihood (ML) tree of gammaherpesviruses constructed using the amino acid sequences of glycoprotein B.

Table 3-1. Features of predicted protein-coding regions in the RGHV-1 genome.

Location	Name of gene	Strand	Size of protein	Note
5420-5734	ORF1	+	104	Hypothetical protein
8356-9228	ORF2	+	290	Hypothetical protein, homolog of equid gammaherpesvirus 2 E2
9508-10062	ORF3	-	184	MIR-like membrane protein
9969-10505	ORF4	-	178	MIR-like membrane protein
11155-12354	ORF5	+	399	Hypothetical protein, homolog of equid gammaherpesvirus 2 E3
12537-13016	ORF6	-	159	Hypothetical protein
13703-17575	ORF7	+	1290	Hypothetical protein, homolog of saimiriine gammaherpesvirus 2 ORF3
17889-18440	ORF8	+	183	Bcl-2
18667-22065	ORF9	+	1132	Single-stranded DNA-binding protein
22094-24223	ORF10	+	709	DNA packaging terminase subunit 2
24214-26820	ORF11	+	868	Glycoprotein B
27170-30196	ORF12	+	1008	DNA polymerase catalytic subunit
31447-31947	ORF13	-	166	Bcl-2
32275-33285	ORF14	+	336	Hypothetical protein, homolog of equid gammaherpesvirus 2 E6
33553-34935	ORF15	+	460	Hypothetical protein, homolog of human herpesvirus 8 ORF10
35004-36320	ORF16	+	438	Hypothetical protein, homolog of human herpesvirus 8 ORF11
36390-36860	ORF17	-	156	Hypothetical protein
40400-42274	ORF18	-	624	Glycoprotein
42428-44182	ORF19	-	584	Capsid maturation protease
9508-10062	ORF20	-	184	Capsid scaffold protein
44181-44993	ORF21	+	270	Hypothetical protein, homolog of human herpesvirus 8 ORF18
44990-46645	ORF22	-	551	Hypothetical protein, homolog of human herpesvirus 8 ORF19
46494-47219	ORF23	-	241	Hypothetical protein, homolog of human herpesvirus 8 ORF20
47218-49071	ORF24	+	617	Thymidine kinase
49071-51419	ORF25	+	782	Glycoprotein H
51416-52618	ORF26	-	400	Hypothetical protein, homolog of human herpesvirus 8 ORF23
52676-54898	ORF27	-	740	Hypothetical protein, homolog of human herpesvirus 8 ORF24
54900-59036	ORF28	+	1378	Major capsid protein
59093-59995	ORF29	+	300	Capsid triplex subunit 2
60022-60516	ORF30	+	164	Hypothetical protein, homolog of human herpesvirus 8 ORF27
60652-60993	ORF31	+	113	Hypothetical protein, homolog of human herpesvirus 8 ORF28
61091-62233, 65497-66411	ORF32	-	685	DNA packaging terminase subunit 1
62323-62577	ORF33	+	84	Hypothetical protein, homolog of human herpesvirus 8 ORF30

62460-63173	ORF34	+	237	Hypothetical protein, homolog of human herpesvirus 8 ORF31
63119-64501	ORF35	+	460	Hypothetical protein, homolog of human herpesvirus 8 ORF32
64494-65507	ORF36	+	337	Hypothetical protein, homolog of human herpesvirus 8 ORF33
66428-67396	ORF37	+	322	Hypothetical protein, homolog of human herpesvirus 8 ORF34
67383-67856	ORF38	+	157	Hypothetical protein, homolog of human herpesvirus 8 ORF35
67741-69075	ORF39	+	444	Tegument serine/threonine protein kinase
69102-70544	ORF40	+	480	Deoxyribonuclease
70499-70723	ORF41	+	74	Myristylated tegument protein
70884-72023	ORF42	-	379	Glycoprotein M
72282-73707, 73799-74361	ORF43	+	662	Helicase-primase subunit
74356-75180	ORF44	-	274	Hypothetical protein, homolog of human herpesvirus 8 ORF42
75217-76923	ORF45	-	568	Capsid portal protein
76898-79267	ORF46	+	789	Helicase-primase helicase subunit
79999-80787	ORF47	-	262	Hypothetical protein
80797-81552	ORF48	-	251	Uracil-DNA glycosylase
81509-81967	ORF49	-	152	Glycoprotein L
82060-83592	ORF50	-	510	Hypothetical protein, homolog of human herpesvirus 8 ORF48
83928-83945, 85019-86923	ORF51	+	640	Protein Rta
84089-84991	ORF52	-	300	Hypothetical protein, homolog of human herpesvirus 8 ORF49
87054-87758	ORF53	+	234	Hypothetical protein
87942-88427	ORF54	+	161	Hypothetical protein
88481-89056	ORF55	+	191	Dihydrofolate reductase
89275-90000	ORF56	+	241	Hypothetical protein, homolog of equid gammaherpesvirus 2 E7A
89997-90995	ORF57	+	332	Hypothetical protein
91035-91463	ORF58	-	142	Hypothetical protein
91523-91858	ORF59	-	111	Glycoprotein N
91972-92844	ORF60	+	290	Deoxyuridine triphosphatase
93042-93707	ORF61	-	221	Hypothetical protein, homolog of human herpesvirus 8 ORF55
93680-96292	ORF62	+	870	Helicase-primase primase subunit
96415-96466, 96986-98361	ORF63	+	475	Multifunctional expression regulator
98551-99039	ORF64	+	162	Hypothetical protein
99043-100107	ORF65	-	354	Hypothetical protein, homolog of human herpesvirus 8 ORF58
100118-101416	ORF66	-	432	DNA polymerase processivity subunit
101688-102605	ORF67	-	305	Ribonucleotide reductase subunit 2
102654-105047	ORF68	-	797	Ribonucleotide reductase subunit 1
105106-106122	ORF69	-	338	Capsid triplex subunit 1
106239-109055	ORF70	+	938	Hypothetical protein, homolog of human herpesvirus 8

			ORF63
109128-117659	ORF71	+	2843	Large tegument protein
117696-118154	ORF72	-	152	Small capsid protein
118181-119413	ORF73	-	410	Hypothetical protein, homolog of human herpesvirus 8 ORF66
119413-120261	ORF74	-	282	Nuclear egress membrane protein
120358-120633	ORF75	-	91	Hypothetical protein, homolog of human herpesvirus 8 ORF67A
120923-122374	ORF76	+	483	Hypothetical protein, homolog of human herpesvirus 8 ORF68
122395-123315	ORF77	+	306	Nuclear egress lamina protein
123525-124394	ORF78	-	289	Thymidylate synthase
125197-125700	ORF79	+	167	Hypothetical protein
127721-129001	ORF80	-	426	LANA-1
129385-130365	ORF81	+	326	Hypothetical protein, homolog of human herpesvirus 8 ORF74
131132-135121	ORF82	-	1329	Hypothetical protein, homolog of human herpesvirus 8 ORF75
135246-135659	ORF83	+	137	Hypothetical protein
139879-140217	ORF84	+	112	Hypothetical protein

Table 3-2. Features of repeat regions in the RGHV-1 genome.

Location	Name of repeat	Feature of repeat
1-4721	Terminal repeat	End of RGHV-1 genome
38259-38637	DR_L	Direct repeat of nt 126086 to 126455
39890-40372	IR_L	Inverted repeat of nt 79289 to 79762
79289-79762	IR_R	Inverted repeat of nt 39890 to 40372
126086-126455	DR_R	Direct repeat of nt 38259 to 38637
143070-147790	Terminal repeat	End of RGHV-1 genome

4. GENERAL CONCLUSION

Herpesviruses infect from mammals to molluscs. The site of latent infection, the pathogenicity and the proliferative property are different among subfamilies. Alphaherpesviruses, which cause various diseases in humans, companion animals, and livestock animals, have been well studied. On the other hand, betaherpesviruses and gammaherpesviruses in animals have not been unclear.

In this study, I isolated two novel herpesviruses from wild animals and analyzed.

In CHAPTER 1, I analyzed the herpesvirus isolated from the kidneys of dead domestic dolphins.

In primary cell culture of the kidney of the dolphin died in the aquarium in Japan, cytopathic effect (CPE) mainly composed of syncytia was observed. As the result of PCR using herpesvirus consensus primers and sequencing analysis, the virus was a novel herpesvirus belonging to the subfamily *Alphaherpesvirinae* (Lagenorhynchus herpesvirus 1). Phylogenetic analysis of the amino acid sequences of DNA polymerase region showed that the novel herpesvirus belongs to the same clade with the other alphaherpesviruses derived from cetaceans.

In order to clarify the phylogenetic relationship with the other alphaherpesviruses, the complete nucleotide sequence (2,757 bp) of the envelope glycoprotein gB gene was determined by PCR using newly designed primers and inverse PCR, suggesting that this herpesvirus belongs to the genus *Varicellovirus* and is closely related to herpesvirus derived from ruminants. This is the first report on the isolation of alphaherpesvirus derived from cetaceans. In addition, our results suggested

that herpesviruses evolved with their host.

In CHAPTER 2, the novel herpesvirus, RGHV-1 was isolated from the spleen of a great horseshoe bat. RGHV-1 was characterized and the complete nucleotide sequence of RGHV-1 was determined.

I successfully isolated a novel herpesvirus from the spleen of a greater horseshoe bat (*Rhinolophus ferrumequinum*) in Japan using the cell line established from the kidney of the same bat. The result of RDV methods indicated that this virus is the novel gammaherpesvirus (Rhinolophus gammaherpesvirus 1; RGHV-1). Vero cells and CRFK cells supported viral growth more than the other cell lines.

Using next-generation sequencing and direct sequencing, the whole RGHV-1 genome was determined (147,790 bp). The unique region of RGHV-1 was flanked by terminal direct sequences (4,721bp). In the genome, there are 84 predicted ORFs. 12 ORFs did not show any homology with those of the other herpesviruses. One of the 12 genes was similar to human Bcl-xl. The structure of the other predicted protein was similar to the structure of MIR of HHV-8. Phylogenetic analysis showed that RGHV-1 belongs to the genus *Percavirus* in the subfamily *Gammaherpesvirinae*. RGHV-1 was very close to Myotis ricketti herpesvirus 2, with 97.6% homology in the amino acid sequences, indicating that these two viruses must be the same species. The natural host of RGHV-1 needs to be clarified in further analyses.

In this study, I succeeded in isolation of one alphaherpesvirus from a cetacean and one gammaherpesvirus from a bat. Both herpesviruses are novel herpesviruses. Usage of cell cultures and cell lines derived from natural host of virus was effective for

isolation of viruses from animals. Isolation of these viruses made it possible to determine the complete nucleotide sequence of glycoprotein gB of the dolphin herpesvirus and the whole nucleotide sequence of RGHV-1.

Herpesviruses derived from cetaceans are thought to be associated with skin lesions, genital legions, and systemic symptoms. For the virus isolated in CHAPTER 1, further study including more effective viral growth is thought to lead to the diagnosis, prevention and treatment of cetaceans in the zoo.

Some proteins involved in immune evasion were identified in the genome of the RGHV-1 isolated in CHAPTER 2. Analysis of these proteins will be useful for understand of the immune system of the bat, known as the host of multiple zoonotic infections.

5. ACKNOWLEDGMENTS

These studies were carried at the Laboratory of Veterinary Microbiology, Joint Faculty of Veterinary Medicine, Yamaguchi University, Japan, from 2011 to 2018.

First of all, the author would like to show my greatest appreciation to my supervisor, **Dr. Ken Maeda** (Laboratory of Veterinary Microbiology, Yamaguchi University). Despite the author had been absent from the graduate school for more than two years, he provided the author the opportunity to complete these studies with support and advice. The author cannot thank him enough.

The author is deeply grateful to all of his co-supervisors, **Dr. Hiroyuki Iwata** (Laboratory of Veterinary Hygiene, Yamaguchi University), **Dr. Tsuyoshi Yamaguchi** (Laboratory of Veterinary Hygiene, Tottori University), **Dr. Kazuo Nishigaki** (Laboratory of Molecular Immunology and Infectious Disease, Yamaguchi University), and **Dr. Takashi Yamanaka** (Japan Racing Association) for giving comments in details, useful suggestion and discussion on his study.

The author is deeply grateful to **Dr. Masayuki Shimojima** (National Institute of Infectious Diseases), **Dr. Ryusei Kuwata**, **Dr. Hiroshi Shimoda** (Laboratory of Veterinary Microbiology, Yamaguchi University) and **Dr. Yutaka Terada** (Research Institute for Microbial Diseases, Osaka University) for their support and advice on his experiments.

The author sincerely thanks to his laboratory members for their support in his experiments and sharing his joy.

Finally, the author would like to thank his father, mother, older sister and brothers for assisting the author until he received a Ph.D.

6. REFERENCES

Abe H, Ishi N, Ito T, Kaneko Y, Maeda K, Miura S, Yoneda M (2008) A guide of the mammals of Japan. Tokai Univ. Press. pp.30 (in Japanese)

Ackermann M (2006) Pathogenesis of gammaherpesvirus infections. *Vet Microbiol*, 113(3–4): 211–222.

Agius CT, Nagesha HS, Studdert MJ (1992) Equine herpesvirus 5: comparisons with EHV2 (equine cytomegalovirus), cloning, and mapping of a new equine herpesvirus with a novel genome structure. *Virology*, 191(1): 176–186.

Agrawal P, Nawadkar R, Ojha H, Kumar J, Sahu A (2017) Complement Evasion Strategies of Viruses: An Overview. *Front Microbiol*, 8: 1117.

Albrecht P, Blaskovuc D, Jakubik J, Lesso J (1963) Demonstration of pseudorabies virus in chick embryo cell cultures and infected animals by the fluorescent antibody technique. *Acta Virol*, 7: 289–296.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol*, 215(3): 403–410.

Antinone SE and Smith GA (2010) Retrograde axon transport of herpes simplex virus and pseudorabies virus: a live-cell comparative analysis. *J Virol*, 84(3): 1504–1512.

Arbelo M, Sierra E, Esperón F, Watanabe TT, Bellière EN, Espinosa de los Monteros A, Fernández A (2010) Herpesvirus infection with severe lymphoid necrosis affecting a beaked whale stranded in the Canary Islands. *Dis Aquat Org*, 89(3): 261–264.

Aujeszky A (1902). Über eine neue Infektionskrankheit bei Haustieren. *Zbl Bakt Orig*, 32: 353–357.

Baer R, Bankier AT, Biggin MD, Deininger PL, Farrell PJ, Gibson TJ, Hatfull G, Hudson GS, Satchwell SC, Séguin C, Tuffnell PS, Barrell BG. (1984) DNA

sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature*. 310(5974): 207–211.

Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler, Alekseyev MA, Pevzner PA (2012) SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol*, 19(5): 455–477.

Bellehumeur C, Lair S, Romero CH, Provost C, Nielsen O, Gagnon CA (2015) Identification of a novel herpesvirus associated with a penile proliferative lesion in a beluga (*Delphinapterus leucas*). *J Wildl Dis*, 51(1): 244–249.

Bellière EN, Esperón F, Arbelo M, Muñoz MJ, Fernández A, Sánchez-Vizcaíno JM (2010) Presence of herpesvirus in striped dolphins stranded during the cetacean morbillivirus epizootic along the Mediterranean Spanish coast in 2007. *Arch Virol*, 155(8): 1307–1311.

Benson G (1999) Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acids Res*, 27(2): 573–580.

Bentley DR, Balasubramanian S, Swerdlow HP, Smith GP, Milton J, Brown CG, Hall KP, Evers DJ, Barnes CL, Bignell HR, Boutell JM, Bryant J, Carter RJ, Keira Cheetham R, Cox AJ, Ellis DJ, Flatbush MR, Gormley NA, Humphray SJ, Irving LJ, Karbelashvili MS, Kirk SM, Li H, Liu X, Maisinger KS, Murray LJ, Obradovic B, Ost T, Parkinson ML, Pratt MR, Rasolonjatovo IM, Reed MT, Rigatti R, Rodighiero C, Ross MT, Sabot A, Sankar SV, Scally A, Schroth GP, Smith ME, Smith VP, Spiridou A, Torrance PE, Tzonev SS, Vermaas EH, Walter K, Wu X, Zhang L, Alam MD, Anastasi C, Aniebo IC, Bailey DM, Bancarz IR, Banerjee S, Barbour SG, Baybayan PA, Benoit VA, Benson KF, Bevis C, Black PJ, Boodhun A,

Brennan JS, Bridgham JA, Brown RC, Brown AA, Buermann DH, Bundu AA, Burrows JC, Carter NP, Castillo N, Chiara E Catenazzi M, Chang S, Neil Cooley R, Crake NR, Dada OO, Diakoumakos KD, Dominguez-Fernandez B, Earnshaw DJ, Egbujor UC, Elmore DW, Etchin SS, Ewan MR, Fedurco M, Fraser LJ, Fuentes Fajardo KV, Scott Furey W, George D, Gietzen KJ, Goddard CP, Golda GS, Granieri PA, Green DE, Gustafson DL, Hansen NF, Harnish K, Haudenschild CD, Heyer NI, Hims MM, Ho JT, Horgan AM, Hoschler K, Hurwitz S, Ivanov DV, Johnson MQ, James T, Huw Jones TA, Kang GD, Kerelska TH, Kersey AD, Khrebtukova I, Kindwall AP, Kingsbury Z, Kokko-Gonzales PI, Kumar A, Laurent MA, Lawley CT, Lee SE, Lee X, Liao AK, Loch JA, Lok M, Luo S, Mammen RM, Martin JW, McCauley PG, McNitt P, Mehta P, Moon KW, Mullens JW, Newington T, Ning Z, Ling Ng B, Novo SM, O'Neill MJ, Osborne MA, Osnowski A, Ostadan O, Paraschos LL, Pickering L, Pike AC, Pike AC, Chris Pinkard D, Pliskin DP, Podhasky J, Quijano VJ, Raczy C, Rae VH, Rawlings SR, Chiva Rodriguez A, Roe PM, Rogers J, Rogert Bacigalupo MC, Romanov N, Romieu A, Roth RK, Rourke NJ, Ruediger ST, Rusman E, Sanches-Kuiper RM, Schenker MR, Seoane JM, Shaw RJ, Shiver MK, Short SW, Sizto NL, Sluis JP, Smith MA, Ernest Sohna Sohna J, Spence EJ, Stevens K, Sutton N, Szajkowski L, Tregidgo CL, Turcatti G, Vandevondele S, Verhovsky Y, Virk SM, Wakelin S, Walcott GC, Wang J, Worsley GJ, Yan J, Yau L, Zuerlein M, Rogers J, Mullikin JC, Hurles ME, McCooke NJ, West JS, Oaks FL, Lundberg PL, Klenerman D, Durbin R, Smith AJ (2008) Accurate whole human genome sequencing using reversible terminator chemistry. *Nature*, 456(7218): 53–59.

Biegeleisen JZ Jr, Scott LV, Lewis V Jr (1959) Rapid diagnosis of herpes simplex virus

infections with fluorescent antibody. *Science*, 129(3349): 640–641.

Blanchard TW, Santiago NT, Lipscomb TP, Garber RL, McFee WE, Knowles S (2001) Two novel alphaherpesviruses associated with fatal disseminated infections in Atlantic bottlenose dolphins. *J Wildl Dis*, 37(2): 297–305.

Boehmer PE and Lehman IR (1997) Herpes simplex virus DNA replication. *Annu Rev Biochem*, 66: 347–384.

Brenner S, Johnson M, Bridgham J, Golda G, Lloyd DH, Johnson D, Luo S, McCurdy S, Foy M, Ewan M, Roth R, George D, Eletr S, Albrecht G, Vermaas E, Williams SR, Moon K, Burcham T, Pallas M, DuBridge RB, Kirchner J, Fearon K, Mao J, Corcoran K (2000) Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays. *Nat Biotechnol*, 18(6): 630–634.

Buonavoglia C and Martella V (2007) Canine respiratory viruses. *Vet Res*, 38(2): 355–373.

Calisher CH, Childs JE, Field HE, Holmes KV, Schountz T (2006) Bats: important reservoir hosts of emerging viruses. *Clin Microbiol Rev*, 19(3): 531–545.

Cohen S, Au S, Panté N (2011) How viruses access the nucleus. *Biochim Biophys Acta*, 1813(9): 1634–1645.

Costanzo F, Campadelli-Fiume G, Foa-Tomasi L, Cassai E (1977). Evidence that herpes simplex virus DNA is transcribed by cellular RNA polymerase B. *J. Virol*, 21(3): 996–1001.

Dal Pozzo F and Thiry E (2014) Antiviral chemotherapy in veterinary medicine: current applications and perspectives. *Rev Sci Tech*, 33(3): 791–801.

Dastjerdi, A., Seilern-Moy, K., Darpel, K., Steinbach, F. & Molenaar, F. (2016) Surviving and fatal Elephant Endotheliotropic Herpesvirus-1A infections in

juvenile Asian elephants - lessons learned and recommendations on anti-herpesviral therapy. *BMC Vet. Res*, 12(1): 178.

Davison AJ (2002) Evolution of the herpesviruses. *Vet Microbiol*, 86(1–2): 69–88.

Davison AJ (2007) Comparative analysis of the genomes. In: Arvin, A, Campidelli-Fiume G, Mocarski, E., Moore, PS, Roizman, B, Whitley, R, Yamanishi K (eds.). Human Herpesviruses: Biology, Therapy and Immunoprophylaxis. Cambridge University Press, Cambridge. Chapter 2. Downloaded on 01 November 2018 from https://www.ncbi.nlm.nih.gov/books/NBK47439/

Davison AJ, Eberle R, Ehlers B, Hayward GS, McGeoch DJ, Minson AC, Pellett PE, Roizman B, Studdert MJ, Thiry E (2009) The Order *Herpesvirales Arch Virol*, 154(1): 171–177.

Davison AJ, Subramaniam K, Kerr K, Jacob JM, Landrau-Giovannetti N, Walsh MT, Wells RS, Waltzek TB (2017) Genome sequence of a gammaherpesvirus from a common bottlenose dolphin (*Tursiops truncatus*). *Genome Announc*, 5(31). pii: e00777-17.

De Pelsmaeker S, Romero N, Vitale M, Favoreel HW (2018) Herpesvirus evasion of natural killer cells. *J Virol*, 92(11). pii: e02105-17.

Deiss LP, Chou J, Frenkel N (1986) Functional domains within the sequence involved in the cleavage-packaging of herpes simplex virus DNA. *J Virol*, 59(3): 605–618.

Edington N, Plowright W, Watt RG (1976) Generalized porcine cytomegalic inclusion disease: distribution of cytomegalic cells and virus. *J Comp Pathol*, 86(2): 191–202.

Elmore D and Eberle R (2008) Monkey B virus (Cercopithecine herpesvirus 1). *Comp Med*, 58(1): 11–21.

Enders JF, Weller TH, Robbins FC (1949) Cultivation of the lansing strain of poliomyelitis virus in cultures of various human embryonic tissues. *Science*, 109(2822): 85–87.

Epstein JH and Price JT (2009) The significant but understudied impact of pathogen transmission from humans to animals. *Mt Sinai J Med*, 76(5): 448–455.

Farré D, Martínez-Vicente P, Engel P, Angulo A (2017) Immunoglobulin superfamily members encoded by viruses and their multiple roles in immune evasion. *Eur J Immunol*, 47(5): 780–796.

Favoreel HW, Van de Walle GR, Nauwynck HJ, Pensaert MB (2003) Virus complement evasion strategies. *J Gen Virol*, 84(1): 1–15.

Freuling CM, Müller TF, Mettenleiter TC (2017) Vaccines against pseudorabies virus (PrV). *Vet Microbiol*, 206: 3–9.

Galluzzi L, Brenner C, Morselli E, Touat Z, Kroemer G (2008) Viral control of mitochondrial apoptosis. *PLoS Pathog*, 4(5):e1000018.

Gaskell R, Dawson S, Radford A, Thiry E. Feline herpesvirus (2007) *Vet Res*, 38(2): 337–354.

Ge XY, Li JL, Yang XL, Chmura AA, Zhu G, Epstein JH, Mazet JK, Hu B, Zhang W, Peng C, Zhang YJ, Luo CM, Tan B, Wang N, Zhu Y, Crameri G, Zhang SY, Wang LF, Daszak P, Shi ZL (2013) Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. *Nature*, 503(7477): 535–538.

Geldenhuys M, Mortlock M, Weyer J, Bezuidt O, Seamark ECJ, Kearney T, Gleasner C, Erkkila TH, Cui H, Markotter W (2018) A metagenomic viral discovery approach identifies potential zoonotic and novel mammalian viruses in Neoromicia bats within South Africa. *PLoS One*, 13(3): e0194527.

Gimeno IM (2008) Marek's disease vaccines: a solution for today but a worry for tomorrow? *Vaccine*, 26 Suppl 3: C31–C41.

Gish W and States DJ (1993) Identification of protein coding regions by database similarity search. *Nat Genet*, 3(3): 266–272.

Goto E, Ishido S, Sato Y, Ohgimoto S, Ohgimoto K, Nagano-Fujii M, Hotta H (2003) c-MIR, a human E3 ubiquitin ligase, is a functional homolog of herpesvirus proteins MIR1 and MIR2 and has similar activity. *J Biol Chem*, 278(17): 14657–14668.

Grattarola C, Giorda F, Iulini B, Pautasso A, Ballardini M, Zoppi S, Marsili L, Peletto S, Masoero L, Varello K, Garibaldi F, Scaglione FE, Di Guardo G, Dondo A, Goria M, Serracca L, Mignone W, Casalone C (2018) Occlusive mycotic tracheobronchitis and systemic Alphaherpesvirus coinfection in a free-living striped dolphin Stenella coeruleoalba in Italy. *Dis Aquat Organ*, 127(2): 137–144.

Griffin BD, Verweij MC, Wiertz EJ (2010) Herpesviruses and immunity: the art of evasion. *Vet Microbiol*, 143(1): 89–100.

Grinde B (2013) Herpesviruses: latency and reactivation - viral strategies and host response. *J Oral Microbiol*, 5. 22766; http://dx.doi.org/ 10.3402/jom.v5i0.22766.

Green M (1965) Chemistry and structure of animal virus particles. *Am J Med*, 38(5): 651–668.

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol*, 52(5): 696–704.

Grünewald, Desai P, Winkler DC, Heymann JB, Belnap DM, Baumeister W, Steven AC (2003) Three-dimensional structure of herpes simplex virus from cryo-electron tomography. *Science*, 302(5649): 1396–1398.

Hall CB, Caserta MT, Schnabel K, Shelley LM, Marino AS, Carnahan JA, Yoo C, Lofthus GK, McDermott MP (2008) Chromosomal integration of human herpesvirus 6 is the major mode of congenital human herpesvirus 6 infection. *Pediatrics*, 122(3): 513–520.

Hart LB, Rotstein DS, Wells RS, Allen J, Barleycorn A, Balmer BC, Lane SM, Speakman T, Zolman ES, Stolen M, McFee W, Goldstein T, Rowles TK, Schwacke LH (2012) Skin lesions on common bottlenose dolphins (Tursiops truncatus) from three sites in the Northwest Atlantic, USA. *PLoS One*, 7(3): e33081.

Hirokawa T, Boon-Chieng S, Mitaku S (1998) SOSUI: classification and secondary structure prediction system for membrane proteins. *Bioinformatics*, 14(4): 378–379.

Hogue IB, Bosse JB, Hu JR, Thiberge SY, Enquist LW (2014) Cellular mechanisms of alpha herpesvirus egress: live cell fluorescence microscopy of pseudorabies virus exocytosis. *PLoS Pathog*, 10(12): e1004535.

Holz PH, Lumsden LF, Druce J, Legione AR, Vaz P, Devlin JM, Hufschmid J (2018) Virus survey in populations of two subspecies of bent-winged bats (Miniopterus orianae bassanii and oceanensis) in south-eastern Australia reveals a high prevalence of diverse herpesviruses. *PLoS One*, 13(5): e0197625.

Hook LM and Friedman HM (2007) Subversion of innate and adaptive immunity: immune evasion from antibody and complement. In: Arvin A, Campadelli-Fiume G, Mocarski E, Moore P, Roizman B, Whitley R, Yamanishi K (eds.). Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis. Cambridge: Cambridge University Press. Chapter 63. Downloaded on 01 November 2018 from https://www.ncbi.nlm.nih.gov/books/NBK47404/

Host KM and Damania B (2016) Discovery of a novel bat gammaherpesvirus. *mSphere*,

1(1): e00016-16.

Roizman B and Pellett PE (2001) The family *Herpesviridae*: a brief introduction. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (eds.). Fields Virology, 4th edn., Philadelphia: Lippincott, Williams and Wilkins. pp. 2381–2398

Ignjatović J and Sapats S (2000) Avian infectious bronchitis virus. *Rev Sci Tech*, 19(2): 493–508.

Jánoska M, Vidovszky M, Molnár V, Liptovszky M, Harrach B, Benko M (2011) Novel adenoviruses and herpesviruses detected in bats. *Vet J*, 189(1): 118–121.

Jones C and Chowdhury (2007) A review of the biology of bovine herpesvirus type 1 (BHV-1), its role as a cofactor in the bovine respiratory disease complex and development of improved vaccines. *Anim Health Res Rev*, 8(2): 187–205.

Kishi M, Harada H, Takahashi M, Tanaka A, Hayashi M, Nonoyama M, Josephs SF, Buchbinder A, Schachter F, Ablashi DV (1988) A repeat sequence, GGGTTA, is shared by DNA of human herpesvirus 6 and Marek's disease virus. *J Virol*, 62(12): 4824–4827.

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol Biol Evol*, 33(7): 1870–1874.

Lau SK, Woo PC, Li KS, Huang Y, Tsoi HW, Wong BH, Wong SS, Leung SY, Chan KH, Yuen KY (2005) Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. *Proc Natl Acad Sci U S A*, 102(39): 14040–14045.

Lecis R, Tocchetti M, Rotta A, Naitana S, Ganges L, Pittau M, Alberti A (2014) First gammaherpesvirus detection in a free-living Mediterranean bottlenose dolphin. *J Zoo Wildl Med*, 45(4): 922–925.

Lehner PJ, Hoer S, Dodd R, Duncan LM. (2005) Downregulation of cell surfacereceptors by the K3 family of viraland cellular ubiquitin E3 ligases *Immunol Rev*. 207(1): 112–125.

Li H and Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler Transform. *Bioinformatics*. 25(14): 1754–1760.

Loewenstein A (1919) Experimental transmission of HSV-1 from human lesions to rabbit corneas. *Munch Med Wochenschr*. 66: 769–770.

Long SY, Latimer EM, Hayward GS (2016) Review of Elephant Endotheliotropic Herpesviruses and Acute Hemorrhagic Disease. *ILAR J*, 56(3): 283–296.

Luis AD, Hayman DT, O'Shea TJ, Cryan PM, Gilbert AT, Pulliam JR, Mills JN, Timonin ME, Willis CK, Cunningham AA, Fooks AR, Rupprecht CE, Wood JL, Webb CT (2013) A comparison of bats and rodents as reservoirs of zoonotic viruses: are bats special? *Proc Biol Sci*, 280(1756): 2753–2757.

Maeda K, Hondo E, Terakawa J, Kiso Y, Nakaichi N, Endoh D, Sakai K, Morikawa S, Mizutani T (2008) Isolation of novel adenovirus from fruit bat (*Pteropus dasymallus yayeyamae*). *Emerg Infect Dis*. 14(2): 347–349.

Maness HT, Nollens HH, Jensen ED, Goldstein T, LaMere S, Childress A, Sykes J, St Leger J, Lacave G, Latson FE, Wellehan JF Jr (2011) Phylogenetic analysis of marine mammal herpesviruses. *Vet Microbiol*, 149(1–2): 23–29.

Manire CA, Smolarek KA, Romero CH, Kinsel MJ, Clauss TM, Byrd L (2006) Proliferative dermatitis associated with a novel alphaherpesvirus in an Atlantic bottlenose dolphin (*Tursiops truncatus*). *J Zoo Wildl Med*, 37(2): 174–181.

Marshall WL, Yim C, Gustafson E, Graf T, Sage DR, Hanify K, Williams L, Fingeroth J, Finberg RW (1999) Epstein-Barr virus encodes a novel homolog of the bcl-2

oncogene that inhibits apoptosis and associates with Bax and Bak. *J Virol*, 73(6): 5181–5185.

Marchler-Bauer A, Bo Y, Han L, He J, Lanczycki CJ, Lu S, Chitsaz F, Derbyshire MK, Geer RC, Gonzales NR, Gwadz M, Hurwitz DI, Lu F, Marchler GH, Song JS, Thanki N, Wang Z, Yamashita RA, Zhang D, Zheng C, Geer LY, Bryant SH. (2017) CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. *Nucleic Acids Res*, 45(D1): D200–D203.

Marek J (1907) Multiple nervenentzündung (polyneuritis) bei hühnern. *Deutsche Tierärztliche Wochenschrift*, 15, 417–421.

Maruyama J, Miyamoto H, Kajihara M, Ogawa H, Maeda K, Sakoda Y, Yoshida R, Takada A (2014) Characterization of the envelope glycoprotein of a novel filovirus, lloviu virus. *J Virol*, 88(1): 99–109.

Maruyama J, Nao N, Miyamoto H, Maeda K, Ogawa H, Yoshida R, Igarashi M, Takada A (2016) Characterization of the glycoproteins of bat-derived influenza viruses. *Virology*. 488: 43–50.

Maxam AM and Gilbert W (1977) A new method for sequencing DNA. *Proc Natl Acad Sci U S A*, 74(2): 560–564.

Maness HT, Nollens HH, Jensen ED, Goldstein T, LaMere S, Childress A, Sykes J, St Leger J, Lacave G, Latson FE, Wellehan JF Jr (2011) Phylogenetic analysis of marine mammal herpesviruses. *Vet Microbiol*, 149(1–2): 23–29.

McGeoch DJ and Cook S (1994) Molecular phylogeny of the *Alphaherpesvirinae* subfamily and a proposed evolutionary timescale. *J Mol Biol*, 238(1): 9–22.

McGeoch DJ, Cook S, Dolan A, Jamieson FE, Telford EA (1995) Molecular phylogeny and evolutionary timescale for the family of mammalian herpesviruses. *J Mol Biol*,

247(3): 443–458.

McGeoch DJ, Rixon FJ, Davison AJ (2006) Topics in herpesvirus genomics and evolution. *Virus Res*, 117(1): 90–104.

McKnight SL (1980) The nucleotide sequence and transcript map of the herpes simplex virus thymidine kinase gene. *Nucleic Acids Res*. 8(24): 5949–5964.

Melero M, Crespo-Picazo JL, Rubio-Guerri C, Garcia-Parraga D, Sanchez-Vizcaino JM (2015) First molecular determination of herpesvirus from two mysticete species stranded in the Mediterranean Sea. *BMC Vet Res*, 11:283.

Mettler CC and Mettler FA (1947) History of Medicine. Philadelphia: Blakiston Co. p.356

Mettenleiter TC (2000) Aujeszky's disease (pseudorabies) virus: the virus and molecular pathogenesis--state of the art, June 1999. *Vet Res*, 31(1): 99–115.

Mettenleiter TC (2002) Herpesvirus assembly and egress. *J Virol*. 76(4): 1537–1547.

Mettenleiter TC, Klupp BG, Granzow H (2006) Herpesvirus assembly: a tale of two membranes. *Curr Opin Microbiol*, 9(4): 423–429.

Mettenleiter TC, Klupp BG, Granzow H (2009) Herpesvirus assembly: an update. *Virus Res*, 143(2): 222–234.

Miyoshi K, Nishida S, Sone E, Tajima Y, Makara M, Yoshioka M, Nakamura M, Yamada TK, Koike H (2011) Molecular identification of novel alpha- and gammaherpesviruses from cetaceans stranded on Japanese coasts. *Zool Sci*, 28(2): 126–133.

Mizutani T, Endoh D, Okamoto M, Shirato K, Shimizu H, Arita M, Fukushi S, Saijo M, Sakai K, Lim CK, Ito M, Nerome R, Takasaki T, Ishii K, Suzuki T, Kurane I, Morikawa S, Nishimura H (2007) Rapid Genome Sequencing of RNA Viruses.

Emerg Infect Dis. 13(2): 322–324.

Mocarski J (2007) Comparative analysis of herpesvirus-common proteins. In: Arvin AM, Campadelli-Fiume G, Mocarski ES, Moore PS, Roizman B, Whitley R, Yamanishi K (eds.). Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis. Cambridge: Cambridge University Press. Chapter 4. Downloaded on 01 November 2018 from https://www.ncbi.nlm.nih.gov/books/NBK47403/

Molnár V, Jánoska M, Harrach B, Glávits R, Pálmai N, Rigó D, Sós E, Liptovszky M (2008) Detection of a novel bat gammaherpesvirus in Hungary. *Acta Vet Hung*, 56(4): 529–538.

Muylkens B, Thiry J, Kirten P, Schynts F, Thiry E (2007) Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis. *Vet Res*, 38(2): 181–209.

Nandi S, Kumar M, Manohar M, Chauhan RS (2009) Bovine herpes virus infections in cattle. *Anim Health Res Rev*, 10(1): 85–98.

Nasisse MP, Dorman DC, Jamison KC, Weigler BJ, Hawkins EC, Stevens JB (1997) Effects of valacyclovir in cats infected with feline herpesvirus 1. *Am J Vet Res*, 58(10): 1141–1144.

Nicola AV and Straus SE (2004) Cellular and viral requirements for rapid endocytic entry of herpes simplex virus. *J Virol*, 78(14): 7508–7517.

Pellett PE, Davison AJ, Eberle R, Ehlers B, Hayward GS, Lacoste V, Minson AC, Nicholas J, Roizman B, Studdert MJ, Wang F (2011) Order Herpesvirales, In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ (eds.). Virus taxonomy. Ninth report of the International Committee on Taxonomy of Viruses. San Diego: Elsevier Academic Press. p 99–107.

Piedade D and Azevedo-Pereira JM (2016) The role of microRNAs in the pathogenesis

of herpesvirus infection. *Viruses*, 8(6): pii:E156.

Piraccini, R (2016) Rhinolophus ferrumequinum. The IUCN Red List of Threatened Species 2016: e.T19517A21973253. Downloaded on 01 January 2018.

Pozo F, Juste J, Vázquez-Morón S, Aznar-López C, Ibáñez C, Garin I, Aihartza J, Casas I, Tenorio A, Echevarría JE (2016) Identification of Novel Betaherpesviruses in Iberian Bats Reveals Parallel Evolution. *PLoS One*, 11(12): e0169153.

Razafindratsimandresy R, Jeanmaire EM, Counor D, Vasconcelos PF, Sall AA, Reynes JM (2009) Partial molecular characterization of alphaherpesviruses isolated from tropical bats. *J Gen Virol*, 90(1): 44–47.

Reed LJ and Muench H (1938) A simple method of estimating fifty per cent endpoints. *Am J Hyg*, 27(3): 493–497.

Rehtanz M, Bossart GD, Fair PA, Reif JS, Ghim SJ, Jenson AB (2012) Papillomaviruses and herpesviruses: who is who in genital tumor development of free-ranging Atlantic bottlenose dolphins (*Tursiops truncatus*)? *Vet Microbiol*, 160(3–4): 297–304.

Richman LK, Montali RJ, Cambre RC, Schmitt D, Hardy D, Hildbrandt T, Bengis RG, Hamzeh FM, Shahkolahi A, Hayward GS (2000) Clinical and pathological findings of a newly recognized disease of elephants caused by endotheliotropic herpesviruses. *J Wildl Dis*, 36(1): 1–12.

Ronaghi M, Karamohamed S, Pettersson B, Uhlén M, Nyrén P (1996) Real-time DNA sequencing using detection of pyrophosphate release. *Anal Biochem*, 1996 242(1): 84–89.

Ronaghi M (2001) Pyrosequencing sheds light on DNA sequencing. *Genome Res*, 11(1): 3–11.

Russell GC, Stewart JP, Haig DM (2009) Malignant catarrhal fever: a review. *Vet J*, 179(3): 324–335.

Sacristan C, Esperon F, Ewbank AC, Diaz-Delgado J, Ferreira-Machado E, Costa-Silva S, Sanchez-Sarmiento AM, Groch KR, Neves E, Pereira Dutra GH, Gravena W, Da Silva VMF, Marcondes MCC, Castaldo Colosio A, Cremer MJ, Carvalho VL, Meirelles ACO, Marigo J, Catao-Dias JL (2018) Novel Herpesviruses in riverine and marine cetaceans from south america. *Acta Trop*, 190: 220–227

Saddington RS (1932) Cultivation of herpesvirus, and use of the mouse in its titration. *Proc Soc Exper Biol & Med*, 29(8): 1012–1014

Sanders M., Kiem I, Lagunoff D (1953) Cultivation of viruses. A critical review. *AMA Arch Pathol.* 56(2): 148–225.

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A*, 74(12): 5463–5467.

Saliki JT, Cooper EJ, Rotstein DS, Caseltine SL, Pabst DA, McLellan WA, Govett P, Harms C, Smolarek KA, Romero CH (2006) A novel gammaherpesvirus associated with genital lesions in a Blainville's beaked whale (*Mesoplodon densirostris*). *J Wildl Dis*, 42(1): 142–148.

Salmier A, Tirera S, de Thoisy B, Franc A, Darcissac E, Donato D, Bouchier C, Lacoste V, Lavergne A (2017) Virome analysis of two sympatric bat species (*Desmodus rotundus* and *Molossus molossus*) in French Guiana. *PLoS One*, 12(11): e0186943.

Sano K, Okazaki S, Taniguchi S, Masangkay JS, Puentespina R Jr, Eres E, Cosico E, Quibod N, Kondo T, Shimoda H, Hatta Y, Mitomo S, Oba M, Katayama Y, Sassa Y, Furuya T, Nagai M, Une Y, Maeda K, Kyuwa S, Yoshikawa Y, Akashi H, Omatsu T, Mizutani T (2015) Detection of a novel herpesvirus from bats in the Philippines.

Virus Genes, 51(1): 136–139.

Sasaki M, Setiyono A, Handharyani E, Kobayashi S, Rahmadani I, Taha S, Adiani S, Subangkit M, Nakamura I, Sawa H, Kimura T (2014) Isolation and characterization of a novel alphaherpesvirus in fruit bats. *J Virol*, 88(17): 9819–9829.

Schipper IA and Chow TL (1968) Detection of infectious bovine rhinotracheitis (IBR) by immunofluorescence. *Can J Comp Med*, 32(2):412–415.

Schmitt DL, Hardy DA, Montali RJ, Richman LK, Lindsay WA, Isaza R, West G (2000) Use of famciclovir for the treatment of endotheliotrophic herpesvirus infections in Asian elephants (Elephas maximus). *J Zoo Wildl Med*, 31(4): 518–522.

Seade GCC, Cerqueira VD, Sierra E, Chaves JF, Moura MAO, Montao DP, Riet-Correa G, Oliveira CA, Siciliano S, Emin-Lima R, Costa AF, Fernandez A, Bezerra Junior PS (2017) Herpesviral infection in a Guiana dolphin (Sotalia guianensis) from the northern coast of Brazil. *J Vet Diagn Invest*, 29(6): 877–879.

Shabman RS, Shrivastava S, Tsibane T, Attie O, Jayaprakash A, Mire CE, Dilley KE, Puri V, Stockwell TB, Geisbert TW, Sachidanandam R, Basler CF (2016) Isolation and Characterization of a Novel Gammaherpesvirus from a Microbat Cell Line. *mSphere*, 1(1). pii:e00070–15.

Sierra E, Diaz-Delgado J, Arbelo M, Andrada M, Sacchini S, Fernandez A (2015) Herpesvirus-associated genital lesions in a stranded striped dolphin (Stenella coeruleoalba) in the canary islands, Spain. *J Wildl Dis*, 51(3): 696–702.

Smith KC (1997) Herpesviral abortion in domestic animals. *Vet J*, 153(3): 253–268.

Smolarek Benson KA, Manire CA, Ewing RY, Saliki JT, Townsend FI, Ehlers B, Romero CH (2006) Identification of novel alpha- and gammaherpesviruses from cutaneous and mucosal lesions of dolphins and whales. *J Virol Methods*, 136(1–2):

261–266.

Solari S and Baker RJ (2007) Mammal Species of the World: A Taxonomic and Geographic Reference by D. E. Wilson; D. M. Reeder. *J Mammal*, 88(3): 824–830

Soto S, Gonzalez B, Willoughby K, Maley M, Olvera A, Kennedy S, Marco A, Domingo M (2012) Systemic herpesvirus and morbillivirus co-infection in a striped dolphin (*Stenella coeruleoalba*). *J Comp Pathol*, 2146(2–3): 269–273.

Spear PG and Longnecker R (2003) Herpesvirus entry: an update. *J Virol*, 77(19): 10179–10185.

Stevens TD and Watkins HM (1969) Rapid identification of viruses by indirect immunofluorescence: standardization and use of antiserum pool to nine respiratory viruses. *Appl Microbiol*, 17(3): 384–393.

Subudhi S, Rapin N, Dorville N, Hill JE, Town J3, Willis CKR, Bollinger TK, Misra V (2018) Isolation, characterization and prevalence of a novel Gammaherpesvirus in Eptesicus fuscus, the North American big brown bat. *Virology*, 516: 227–238.

Szpara ML, Gatherer D, Ochoa A, Greenbaum B, Dolan A, Bowden RJ, Enquist LW, Legendre M, Davison AJ (2014) Evolution and diversity in human herpes simplex virus genomes. *J Virol*, 88(2): 1209–1227.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol*, 28(10): 2731–2739.

van Beurden SJ, IJsseldijk LL, Ordonez SR, Forster C, de Vrieze G, Grone A, Verheije MH, Kik M (2015) Identification of a novel gammaherpesvirus associated with (muco)cutaneous lesions in harbour porpoises (*Phocoena phocoena*). *Arch Virol*, 160(12): 3115–3120.

van Bressem MF, van Waerebeek K, Raga JA (1999) A review of virus infections of cetaceans and the potential impact of morbilliviruses, poxviruses and papillomaviruses on host population dynamics. *Dis Aquat Org*, 38(1): 53–65.

van Maanen C (2002) Equine herpesvirus 1 and 4 infections: an update. *Vet Q*, 24(2): 58–78.

VanDevanter DR, Warrener P, Bennett L, Schultz ER, Coulter S, Garber RL, Rose TM (1996) Detection and analysis of diverse herpesviral species by consensus primer PCR. *J Clin Microbiol*, 34(7): 1666–1671.

van Elk CE, van de Bildt MW, de Jong AA, Osterhaus AD, Kuiken T (2009) Genital herpesvirus in bottlenose dolphins (*Tursiops truncatus*): cultivation, epidemiology, and associated pathology. *J Wildl Dis*, 45(4): 895–906.

van Elk C, van de Bildt M, van Run P, de Jong A, Getu S, Verjans G, Osterhaus A, Kuiken T (2016) Central nervous system disease and genital disease in harbor porpoises (Phocoena phocoena) are associated with different herpesviruses. *Vet Res*, 47: 28.

Venugopal K (2000) Marek's disease: an update on oncogenic mechanisms and control. *Res Vet Sci*, 69(1): 17–23.

Wada Y, Sasaki M, Setiyono A, Handharyani E, Rahmadani I, Taha S, Adiani S, Latief M, Kholilullah ZA, Subangkit M, Kobayashi S, Nakamura I, Kimura T, Orba Y, Sawa H (2018) Detection of novel gammaherpesviruses from fruit bats in Indonesia. *J Med Microbiol*, 67(3): 415–422.

Warburton PE, Giordano J, Cheung F, Gelfand Y, Benson G (2004) Inverted repeat structure of the human genome: the X-chromosome contains a preponderance of large, highly homologous inverted repeats that contain testes genes. *Genome Res*,

14(10A): 1861–1869.

Watanabe S, Ueda N, Iha K, Masangkay JS, Fujii H, Alviola P, Mizutani T, Maeda K, Yamane D, Walid A, Kato K, Kyuwa S, Tohya Y, Yoshikawa Y, Akashi H (2009) Detection of a new bat gammaherpesvirus in the Philippines. *Virus Genes*, 39(1): 90–93.

Watanabe S, Maeda K, Suzuki K, Ueda N, Iha K, Taniguchi S, Shimoda H, Kato K, Yoshikawa Y, Morikawa S, Kurane I, Akashi H, Mizutani T (2010) Novel betaherpesvirus in bats. *Emerg Infect Dis*, 16(6): 986–988.

Weller SK and Coen DM (2012) Herpes simplex viruses: mechanisms of DNA replication. *Cold Spring Harb Perspect Biol*, 4(9): a013011.

Weir JP (2001) Regulation of herpes simplex virus gene expression. *Gene*. 271(2): 117–130.

Wibbelt G, Kurth A, Yasmum N, Bannert M, Nagel S, Nitsche A, Ehlers B (2007) Discovery of herpesviruses in bats. *J Gen Virol*, 88(10): 2651–2655.

Wilkie GS, Kerr K, Stewart JP, Studdert MJ, Davison AJ (2015) Genome sequences of equid herpesviruses 2 and 5. *Genome Announc*, 3(2): pii: e00119-15.

Wu Z, Ren X, Yang L, Hu Y, Yang J, He G, Zhang J, Dong J, Sun L, Du J, Liu L, Xue Y, Wang J, Yang F, Zhang S, Jin Q (2012) Virome analysis for identification of novel mammalian viruses in bat species from Chinese provinces. *J Virol*, 86(20): 10999–11012.

Zhang H, Todd S, Tachedjian M, Barr JA, Luo M, Yu M, Marsh GA, Crameri G, Wang LF (2012) A novel bat herpesvirus encodes homologues of major histocompatibility complex classes I and II, C-type lectin, and a unique family of immune-related genes. *J Virol*, 86(15): 8014–8030.

Zheng XY, Qiu M, Chen SW, Xiao JP, Ma LZ, Liu S, Zhou JH, Zhang QH, Li X, Chen Z, Wu Y, Chen HF, Jiang LN, Xiong YQ, Ma SJ, Zhong XS, Huo ST, Ge J, Cen SW, Chen Q (2016) High prevalence and diversity of viruses of the subfamily *Gammaherpesvirinae*, family *Herpesviridae*, in fecal specimens from bats of different species in southern China. *Arch Virol*, 161(1): 135–140.

学位論文要旨

山口大学大学院連合獣医学研究科

氏名　　石嶋　慧多

指導教官　　前田 健

Isolation and analysis of herpesviruses from wild animals
(野生動物由来ヘルペスウイルスの分離及び解析)

ヘルペスウイルスはヘルペスウイルス目に分類されるウイルスの総称である。その宿主域は哺乳類から軟体動物まで多岐に渡る。特に哺乳類を宿主とするヘルペスウイルスはヘルペスウイルス科に属し、アルファ、ベータ、ガンマヘルペスウイルスの 3 亜科に分類される。生体内において潜伏感染する箇所、あるいは病原性・増殖性などは特に亜科ごとで大きく異なる。また、ヒト、伴侶動物、生産動物において多くの疾病が報告されているアルファヘルペスウイルスは比較的病原性について明らかになっている部分もあるが、ガンマヘルペスウイルスについては、その病原性について不明な部分が多い。動物園動物を含む、野生動物由来のヘルペスウイルスについては分離の報告も少ない。未だ、各種動物が保有するヘルペスウイルスに関して不明な点が多いのが現状である。

本研究において、新規に分離された野生動物由来の 2 種のヘルペスウイルスの同定並びに解析を行った。

【第 1 章】　カマイルカから分離された新規のヘルペスウイルス

本章では、国内の死亡したカマイルカの腎臓から分離されたヘルペスウイルスについて、その解析を行った。

国内の水族館で死亡したカマイルカの腎臓から培養細胞の作出を試みた結果、合胞体を主体とする細胞変性効果（CPE）が確認された。ヘルペスウイルスコンセンサスプライマーによる PCR、および塩基配列を決定した結果、分離ウイルスはアルファヘルペスウ

イルス亜科に属する新規のヘルペスウイルスであることが明らかになった(Lagenorhynchus herpesvirus 1)。決定したDNA ポリメラーゼ領域で系統解析を行った結果、他の鯨類由来アルファヘルペスウイルスと同じクレードを形成した。鯨類由来アルファヘルペスウイルスの系統学的位置をより詳細に明らかにするため、新規に設計したプライマーとインバース PCR によって、エンベロープ糖蛋白 gB 遺伝子の全長(2,757 bp)の決定に成功した。系統解析の結果、本ウイルスはバリセロウイルス属に属し、反芻類由来のヘルペスウイルスと近縁であることが明らかになった。

鯨類由来のアルファヘルペスウイルスの分離については、本報告が初めてである。反芻類由来のアルファヘルペスウイルスと本ウイルスが近縁だったことは、ヘルペスウイルスと宿主が共進化しているという仮説と一致していた。

【第2章】キクガシラコウモリガンマヘルペスウイルスの分離及び全塩基配列の決定

本章では、国内のキクガシラコウモリから分離されたガンマヘルペスウイルスについて、その性状解析と全塩基配列の決定を行った。

国内で捕獲したキクガシラコウモリの脾臓から培養細胞の作出を試みた結果、継代の途中で CPE が認められた。上清を同一のキクガシラコウモリから作出した腎由来培養細胞株である Bat kidney T (BKT)-1 細胞に接種、継代したところ、CPE が認められた。Rapid determination of viral RNA sequence 法によって、新規のガンマヘルペスウイルスであることが明らかになった (Rhinolophus gammaherpesvirus 1; RGHV-1)。RGHV-1 の増殖性について、10 種類の細胞を用いて評価した。5 種類の翼手目由来の細胞 (BKT1、FBKT1、YubFKT1、YubFKT2、DemKT1)及び 5 種類の翼手目以外の哺乳類由来の細胞 (Vero、BHK、CRFK、fcwf-4、MDCK)において RGHV-1 の増殖性を比較した結果、Vero 細胞と CRFK 細胞では他の細胞に比較してよく増殖した。

RGHV-1 感染 Vero 細胞から抽出した DNA から全塩基配列の決定を試み、PCR、ダイレクトシークエンス、並びに次世代シークエンス法でその全塩基配列 (147,790 bp) を決定した。4,721 bp の末端反復配列が両端に存在するゲノム構造をとり、そのゲノム上には

84 個の蛋白をコードしている open reading frame (ORF)が推定された。12 個の ORF は、既知のヘルペスウイルス由来の蛋白と相同性のない配列であったが、そのうち 2 個の ORF については機能が推測された。一つはアポトーシス抑制因子であるヒト bcl-xl と相同性のある蛋白で、もう一つは構造的にヒトヘルペスウイルス 8 型の modulator of immune recognition (MIR)に類似していた。糖蛋白 gB 遺伝子の全長による系統解析の結果、ペルカウイルス属に属することが確認された。また、過去に報告のある、ホオヒゲコウモリの糞便から検出されたガンマヘルペスウイルスと 97.6 %の相同性が認められ、同一の種と考えられた。いずれのコウモリ種が RGHV-1 の自然宿主かについてはより詳細な検討が必要である。

本章では、新規に分離されたキクガシラコウモリ由来のヘルペスウイルスについて、その全塩基配列を決定した。ゲノム構造の決定ならびに遺伝子解析によって、既知のガンマヘルペスウイルスとは異なる特徴を有するヘルペスウイルスであることが明らかになった。

本研究において、野生動物由来のヘルペスウイルスとして、鯨類由来のアルファヘルペスウイルスと翼手目由来のガンマヘルペスウイルスの分離に成功した。いずれも過去に分離報告がないヘルペスウイルスであり、宿主の臓器由来の初代培養細胞、並びに宿主に由来する培養細胞株がウイルス分離で有用であることが改めて示された。また、分離に成功したことで、カマイルカヘルペスウイルスでは糖蛋白 gB の全長が、キクガシラコウモリ由来ヘルペスウイルスでは塩基配列の全長が決定された。

鯨類由来のヘルペスウイルスについては、皮膚病変や、あるいは全身症状との関連が疑われるにも関わらず、分離に成功した例は本報告を除けば現時点では 1 例のみである。第一章で分離されたウイルスについて、今後、培養細胞による増殖系の確立を含めてより詳細な研究をすることは、動物園における鯨類の予防・治療につながると考えられる。

第 2 章において分離されたウイルスのゲノム中には、既知のヘルペスウイルスとは大

きく異なる蛋白を含めて、免疫回避に関与する蛋白が複数同定されている。これらの蛋白の解析を進めることは、複数の人獣共通感染症の宿主として知られているコウモリの免疫系を知る手がかりになると考えられる。