수의학석사 학위논문

First Report of Gammaherpesvirus Skin Infection in Narrow-ridged Finless Porpoise (*Neophocaena asiaeorientalis*) in Korea

국내 상괭이 피부 질환을 유발하는 감마허피스바이러스 최초 보고

2022년 2월

서울대학교 대학원

수의학과 수의병인생물학 및 예방수의학 전공

이 성 빈

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이성빈의 석사 학위 논문을 인준함

2022년 1월

위 원 장 윤 화 영 (인)

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위 원 전 진 우 (인)

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By

Sung Bin Lee

Supervisor: Prof. Se Chang Park, D.V.M., Ph.D.

A dissertation submitted to the faculty of the Graduate School of Seoul National University in partial fulfillment of the requirements for the degree of Master in Veterinary Pathobiology and Preventive Medicine

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(Supervised by Professor Se Chang Park)
Department of Veterinary Pathobiology and Preventive Medicine
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Abstract

Narrow-ridged finless porpoise (*Neophocaena asiaeorientalis*) is one of the endemic and dominant cetacean species in Korean seawater. However, the population of the species has been decreased sharply in for the last 10 years. Accordingly, narrow-ridged finless porpoise was included as Endangered in the red list of the International Union for the Conservation of Nature (IUCN) in 2017. Despite the risk of extinction, pathological research of the porpoise has rarely

been reported yet. To prevent population declines of the species, conservation medicine should be studied more for the health of wildlife, ecosystem, and human. In this study, I examined and detected a viral pathogen from infected skin of a narrow-ridged finless porpoise.

A carcass of female narrow-ridged finless porpoise was stranded on the beach located at Jeju Island, South Korea. Twelve dermatitis lesions were spread on various sites of the body. The results of histopathological examination of the skin lesions indicated moderately to markedly thickened epidermis with accentuated rete pegs, amorphous eosinophilic material in the vacuoles, eosinophilic intranuclear inclusion bodies in the epidermal cells, and marked infiltrates of predominantly mononuclear cells within the dermis. Extracted DNA from the skin lesions and mammary gland were amplified respectively with multiple primers of herpesvirus, papillomavirus, *Lacazia loboi*, and poxvirus to do rule out diagnosis. Consequently, gammaherpesvirus was detected in the molecular analysis. The viral sequence contains same parts with the partial DNA polymerase gene of *Balaenoptera acutorostrata* gammaherpesvirus 2, dwarf sperm whale gammaherpesvirus, and Blainville's beaked whale gammaherpesvirus. Gammaherpesvirus has been usually reported as the pathogen of genital and oral mucosae infections in cetaceans, and the skin

infection has been rarely reported. This study is the first report of gammaherpesvirus infection in narrow-ridged finless porpoise in the Republic of Korea. Besides, additional studies should be required on the latent infection status of the herpesvirus in the narrow-ridged finless porpoise and the spread of the herpesvirus in the Korean sea.

Keywords: cetacean herpesvirus, gammaherpesvirus, skin lesion, narrow-ridged finless porpoise, *Neophocaena asiaeorientalis*

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CONTENTS

Abbreviations

IUCN	International Union for Conservation of Nature
DNA	Deoxyribonucleic acid
bp	Base pair
DNApol	DNA polymerase
HV	Herpesvirus
H&E	Haematoxylin and eosin
PAS	Periodic acid schiff
PCR	Polymerase chain reaction
AD	Atopic dermatitis

Literature Review

The herpesviruses are enveloped viruses with diverse double-stranded DNA (Connolly et al., 2011). Nearly 130 viruses are characterized as the Herpesvirus family (Brown et al., 2011). The structure of herpesvirus includes a capsid which covers the DNA, a viral tegument which is a matrix with proteins, an envelope which covers the tegument, and glycoproteins on the envelope of virome (Yu et al., 2011). Herpesviridae can be classified into three subfamily groups; alphaherpesvirus, betaherpesvirus, and gammaherpesvirinae according to genomic features and biological characters (Pellet and Roizman, 2007). The three subfamily groups target different regions of human or animals to infect and the aspect of disease is also different, respectively. Eight genus human herpesviruses cause various diseases from mild and disseminated sore to fatal Burkitt's lymphoma (Diemen et al., 2016). Therefore, human herpesviruses have been researched well. However, the cases of herpesviral infection in cetaceans have been rarely reported (Blanchard et al., 2001).

Narrow-ridged finless porpoise (*Neophocaena asiaeorientalis*) is the one of endemic cetaceans in Korean seawater (Park et al., 2010). Although they live in the seas around Korea, some coastal regions of China and Japan, and Yangtze river in China in the world, the western and southern seas of Korea is the place where most population of the porpoise lives at in the world (Sohn et al., 2012). Narrow-ridged finless porpoise can be classified into two subspecies; East Asian finless porpoise (*N. asiaeorientalis sunameri*) which lives in East Asian seawater and Yangtze finless porpoise (*N. asiaeorientalis asiaeorientalis*) which lives in Yangtze river in China (Deng et al., 2019). Both porpoises have suffered from the rapid population decline, therefore East Asian finless porpoise was included in Endangered (EN) species and Yangtze finless porpoise was included in Critically Endangered (CR) species respectively in the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species (IUCN, 2021). Because most of them live in salt water or fresh water less than 5 m in depth, human activities can influence far more on their habitats and survival than other cetaceans' (Wang D., 2009).

Introduction

The herpesviruses are widespread viruses in the nature with large double-stranded DNA belonging to the family Herpesviridae (McGeoch et al., 2006). More than 130 herpesviruses have been known and some of them were reported from humans and animals (Brown et al., 2012). There are three subfamilies Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae in the family herpesviridae depending on the genomic structure and biological characters (Pellet and Roizman, 2007). Infection of herpesvirus is common in most of vertebrae including human, however, the cases of herpesviral infection in cetaceans have been rarely reported (Blanchard et al., 2001). Usually, the cetacean herpesviruses have been identified using comparative analysis of the DNA polymerase gene (Noguchi et al., 2013). In the cases of cetacean herpesvirus, alpha- and gammaherpesviruses have been discovered, and a few of alphaherpesviruses cause dermatitis such as Delphinid herpesvirus-7 (DeHV-7), -3, and -8 in bottlenose dolphins (*Tursiops truncates*) (Benson et al., 2006; Maness et al., 2011) and

Stenella coeruleoalba herpesvirus in striped dolphin (*Stenella coeruleoalba*) (Sierra et al., 2015). Only phocoenid herpesvirus 1 (PhoHV1) which was identified from harbour porpoises (*Phocoena phocoena*) has been recognized as the skin-infectious gammaherpesvirus before (van Beurden et al., 2015). Mostly, alphaherpesviruses are associated with skin lesions, on the other hand, gammaherpesviruses are associated with genital and oral mucosae infections, respectively.

Narrow-ridged finless porpoise (*Neophocaena asiaeorientalis*) is one of the dominant coastal odontocetes in Korean seawater (Park et al., 2010). As widely known, cetaceans are indicator species of ocean pollution and take a role supplying phosphorus that is one of essential nutrients in marine ecosystem from ocean floor to surface. However, more than 1,000 narrow-ridged finless porpoises have been bycaught by stow net annually in Korean seawater, consequently, the population of them had decreased from 36,000 in 2005 (Park et al., 2007) to 13,000 in 2011 (Park et al., 2015). Because of anticipated population decline in the future, the species was included as Endangered in the International Union for

the Conservation of Nature (IUCN) Red List of Threatened Species in 2017 (IUCN, 2021). Despite the risk of extinction, pathological research of narrow-ridged finless porpoise has rarely been reported yet. More veterinary research should be needed for disease prevention and population control of narrow-ridged finless porpoise in the future.

In this study, the first gammaherpesvirus infection of narrow-ridged finless porpoise is reported and it is worthful as the first skin lesion case of gammaherpesvirus in the porpoise additionally.

Materials & Methods

Necropsy and sample collection

On 15 December 2020, a carcass of female narrow-ridged finless porpoise (*Neophocaena asiaeorientalis*), snout to tail length 148.5 cm, was stranded on the beach located at Hado-ri, Gujwa-eup, Jeju Island, South Korea (Figure 1). The age was estimated with the body length and growth layers in teeth (Hohn A. A., 2009). On the skin, multiple proliferative lesions were observed. The animal was designated NA20-1215 and stored at - 50 °C freezer for further examinations. During necropsy, the calcified mammary gland and the epithelial proliferative lesions were collected from multiple part including oral and genital region with appropriate size and contained in 70% EtOH for molecular analysis, and in 10% buffered formalin for histopathological analysis.

Histopathology

The formalin-fixed skin samples were cut into 1.7 × 0.5 cm, 1.7 × 1.2 cm, and 2.0

× 0.7 cm. The samples were commissioned to Korea Vet Lab (Seongnam, Korea) to perform histopathological examination and analyzed at Antech Diagnostics (Fountain Valley, CA). After embedding in paraffin, the tissues were sectioned into 5 μm and then stained with haematoxylin and eosin (H&E). Methenamine silver and PAS stains were performed for yeast and hyphae. Additionally, acid-fast staining and gram stain were performed to detect bacteria.

PCR amplification for herpesvirus

Total DNA products were extracted from the skin samples and mammary gland in 70% EtOH using the DNeasy® Blood & Tissue Kit (Qiagen, Valencia, CA). To detect universal herpesvirus, two nested PCR protocols were performed. The primers; FP1, FP2, and RP1 (Table 1) were used for the first PCR to detect DNA polymerase (DNApol) gene fragments of 215-235 base pairs (bp) for most herpesviruses and 315 bp for cytomegaloviruses (Benson et al., 2006). Total 20 μl PCR mixture included 1 μl of sample, 1 μl of each primer FP1, FP2, and RP1, 5 μl of Maxime™ PCR PreMix (LiliF Diagnostics, Seongnam, Korea), and 11 μl of

distilled water. PCR was carried out with the following parameters: initial denaturation at 94 °C for 2 min, followed by 55 cycles, each consisted of a denaturation step at 94 °C for 20 sec, an annealing step at 46 °C for 30 sec, and an elongation step at 72 °C for 30s. The last elongation step was performed at 72 °C for 10 min. For the nested PCR, the pair of primers; FP3 and RP2 (Table 1) were used. Total 20 µl PCR mixture included 2 µl of the first PCR product, 1 µl of each primer FP3 and RP2, 5 µl of Maxime™ PCR PreMix (LiliF Diagnostics, Seongnam, Korea), and 11 µl of distilled water. PCR protocol for the second PCR were same as the former parameters. To perform further analysis, Expand High Fidelity enzyme mix (Roche Applied Science, Indianapolis, IN) was used with the previously explained protocol (Benson et al., 2006).

The primers; HVF1, HVF2, and HVR1 (Table 1) were used for the first PCR to detect DNApol gene of 215-315 bp (VanDevanter et al., 1996). Total 20 µl PCR mixture included 1 µl of sample, 1 µl of each primer HVF1, HVF2, and HVR1, 5 µl of Maxime™ PCR PreMix, and 11 µl of distilled water. PCR was carried out with the following parameters: initial denaturation at 94 °C for 10 min,

followed by 35 cycles, each consisted of a denaturation step at 94 °C for 30 sec, an annealing step at 46 °C for 1 min, and an elongation step at 72 °C for 3 min. The last elongation step was performed at 72 °C for 7 min. For the nested PCR, the pair of primers; HVF3 and HVR2 (Table 1) were used. Total 20 µl PCR mixture included 2 µl of the first PCR product, 1 µl of each primer HVF3 and HVR2, 5 µl of Maxime™ PCR PreMix (LiliF Diagnostics, Seongnam, Korea), and 11 µl of distilled water. PCR protocol for the second PCR were same as the former parameters.

According to the result of PCR with the primers FP1, FP2, and RP1 (Figure 4), three primers; DHVF1, DHVF2, and DHVR1 (Table 1) were designed based on the partial DNApol gene of dwarf sperm whale gammaherpesvirus (AY949830.1) (Figure 5a). In addition, three pairs of primers; MHVF1, MHVR1, MHVF2, MHVR2, MHVF3, and MHVR3 (Table 1) were designed based on the partial DNApol gene of *Balaenoptera acutorostrata* gammaherpesvirus 2 (KP995688.1) (Figure 5b). Primer-design was performed with NCBI Primer-BLAST (Ye et al., 2012), and all primers were produced by Cosmogenetech

(Seongdong-gu, Seoul, Korea). PCR protocols with the designed primers were same as the previous parameters for FP1, FP2, and RP1 except the number of cycles was 30. The target size of the PCR products was 350-360 bp for DHVF1, DHVF2, and DHVR1, meanwhile, 90-110 bp for MHVF1, MHVR1, MHVF2, MHVR2, MHVF3, and MHVR3.

PCR amplification for papillomavirus

To detect papillomavirus, two PCR protocols were performed. A pair of primers; FAP59 and FAP64 (Table 1) was used to detect the L1 protein gene of human papillomavirus (HPV) of 480 bp (Forslund et al., 1999). Total 20 µl PCR mixture included 1 µl of sample, 1 µl of each primer FAP59 and FAP64, 5 µl of Maxime™ PCR PreMix, and 12 µl of distilled water. PCR was carried out with the following parameters: initial denaturation at 94 °C for 10 min, followed by 48 cycles, each consisted of a denaturation step at 94 °C for 1.5 min, an annealing step at 51.5 °C for 2 min, and an elongation step at 72 °C for 1.5 min. The last elongation step was performed at 72 °C for 10 min.

In addition, three pairs of primers; SNUPPV-1 F and SNUPPV-1 R, SNUPPV-2 F and SNUPPV-2 R, and SNUPPV-3 F and SNUPPV-3 R (Table 1) were designed with dolphin papillomavirus SNU_DeCa_ PV_1 L1 protein gene, partial cds (serial number: MN536507.1) as a PCR template (Han et al., 2020). Primer-design was performed with NCBI Primer-BLAST. PCR protocols for the three pairs of primers were same as the former parameters for FAP59 and FAP64 except the annealing step at 55 °C for 2 min. The target size of the PCR products was almost 350 bp.

PCR amplification for Lacazia loboi

To detect the fungi pathogen of human and dolphins; *Lacazia loboi*, three PCR protocols were performed. A pair of primers; Kex-1F and Kex-2R (Table 1) was used to detect the conserved region of the *Kex* partial DNA sequence of *Lacazia loboi* of 151 bp (Vilela et al., 2009). Total 20 µl PCR mixture included 1 µl of sample, 1 µl of each primer Kex-1F and Kex-2R, 5 µl of Maxime™ PCR PreMix, and 12 µl of distilled water. PCR was carried out with the following parameters:

initial denaturation at 95 °C for 10 min, followed by 40 cycles, each consisted of a denaturation step at 95 °C for 1 min, an annealing step at 60 °C for 2 min, and an elongation step at 72 °C for 3 min. The last elongation step was performed at 72 °C for 10 min.

A pair of primers; MAE and ATO (Table 1) was used to detect the gp43 of *Paracoccidioides brasiliensis* and *Lacazia loboi* of 550 bp (Sano et al., 2001). Total 20 µl PCR mixture included 1 µl of sample, 1 µl of each primers MAE and ATO, 5 µl of Maxime™ PCR PreMix, and 12 µl of distilled water. PCR was carried out with the following parameters: initial denaturation at 95 °C for 4 min, followed by 40 cycles, each consisted of a denaturation step at 94 °C for 1 min, an annealing step at 50 °C for 1.5 min, and an elongation step at 72 °C for 2 min. The last elongation step was performed at 72 °C for 10 min.

A pair of primers; SUM-F1 and SUM-R2 (Table 1) was used to detect the partial sequence of *gp43* of 382 bp (Ueda et al., 2013). Total 20 µl PCR mixture included 1 µl of sample, 1 µl of each primer SUM-F1 and SUM-R2, 5 µl of Maxime™ PCR PreMix, and 12 µl of distilled water. PCR was carried out with

the following parameters: initial denaturation at 95 °C for 4 min, followed by 40 cycles, each consisted of a denaturation step at 94 °C for 1 min, an annealing step at 50 °C for 1.5 min, and an elongation step at 72 °C for 2 min. The last elongation step was performed at 72 °C for 10 min.

PCR amplification for poxvirus

To detect poxvirus, one PCR protocols was performed. A pair of primers; DNA-pol FP and DNA-pol RP (Table 1) was used to detect the partial DNApol gene of 543 bp (Bracht et al., 2006). Total 20 µl PCR mixture included 1 µl of sample, 1 µl of each primers DNA-pol FP and RP, 5 µl of Maxime™ PCR PreMix, and 12 µl of distilled water. PCR was carried out with the following parameters: initial denaturation at 95 °C for 5 min, followed by 40 cycles, each consisted of a denaturation step at 95 °C for 0.5 min, an annealing step at 50 °C for 0.5 min, and an elongation step at 72 °C for 1 min. The last elongation step was performed at 72 °C for 7 min.

Sequence analysis

First, each amplified PCR products were analyzed by QIAxcel ScreenGel Software (Qiagen, Valencia, CA) and resolved by 1.0% gel electrophoresis with 0.5 μg/ml ethidium bromide. Then, the bands of DNA fragments were visualized at UV transillumination. The DNA fragments were extracted with QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and analyzed at Cosmogenetech (Seongdong-gu, Seoul, Korea) for genetic analysis. All sequences were BLAST-searched (Altschul et al., 1990).

Results

Characteristics of the narrow-ridged finless porpoise NA20-1215

According to the dental radiography pulp and the total body length (148.5 cm) of the finless porpoise, it is estimated that about 8 years old and had reached the age of puberty. Twelve dermatitis patches were spread on various sites of the body from snout to fluke (Figure 2a) including both flippers (Figure 2b), especially, it was severe on genital, oral, and the abdominal regions (Figure 2c). The size of the dermatitis lesions was diverse from 2.0 × 3.5 cm to 12.0 × 9.0 cm. Around the genial slit, the largest zone of the infected skin was observed (12.8 × 11.0 cm). All lesions were cracked, sessile, elevated, and had diffused margin. Few points of the severe lesions were peeled off and the blubber was exposed. Besides, on the left-ventral flipper, cartilaginous tissue under the epithelium got damaged. Ocean current might affect it while the body was floating. There was no bleeding sign on all skin lesions.

Result of the necropsy

The autopsy revealed that no notable symptom except skin lesions. There were tiny sporadic nodules (the diameter 0.3 cm) in both lungs and I found few parasites in the parenchyma area. Foamy fluid did not exist in lungs, trachea, and bronchi as reliable evidence of asphyxia. On the one hand, five nodules with pus (1.0 × 1.0 × 2.0 cm) were observed in the liver. Mammary glands were mature and one parasite was found in the lumen of right mammary gland. Additionally, I recognized a firm nodule with calcified material (the diameter 0.8 cm) in the caudal part of left mammary gland. There was an active follicle (2.7 × 2.0 cm) on the right ovary (4.0 × 0.9 cm), and four corpus albicanses were found on the left ovary (4.2 × 1.3 cm).

Histopathological examinations

In histological examinations, each of skin biopsies had moderately to markedly thickened epidermis with accentuated rete pegs (Figure 3a), although morphology was not ideal due to the age of the tissue. Within the epidermis, there was vacuolar

change with pale, somewhat amorphous eosinophilic material in the vacuoles and moderate nuclear debris (Figure 3b). Suspicious eosinophilic intranuclear inclusion bodies (INI) were identified in the cells of epidermis (Figure 3c). Within the dermis at the base of the proliferative epidermis, there were moderate to marked infiltrates of predominantly mononuclear cells (Figure 3d). Macrophages, lymphocytes, melanophages and fewer neutrophils were recognized. Methenamine silver and PAS stains were negative for yeast and fungal organisms. Acid-fast staining did not reveal any acid-fast bacteria. Gram staining revealed aggregates of gram-negative bacteria in the dermis and on the epidermal surface, some of which may represent overgrowth (Figure 3e). Differentials for the lesions include lobomycosis caused by *Lacazia loboi*, papillomavirus, poxvirus, and possibly herpesvirus infection. According to the result of the histological analysis, rule out diagnosis were performed with PCR of each possible pathogens.

PCR and sequencing

Each PCR protocols of papillomavirus, *Lacazia loboi*, and poxvirus was tested

negative. Bands of target size were recognized from the amplified DNA samples with papillomavirus primers in the result of electrophoresis. However, notable DNA sequence was not available to detect (Table 2). In contrast, 134 bp and 130 bp sequences were recognized from the amplified DNA samples with herpesvirus primers; FP1, FP2, and RP1. According to the nucleotide BLAST, the 134 bp sequence was identified as the partial DNApol gene of *Balaenoptera acutorostrata* gammaherpesvirus 2 (KP995688.1) with 21% of query coverage, 0.002 of e-value, and 100% of identification (Figure 4a) and the 130 bp sequence was identified as the partial DNApol gene of dwarf sperm whale gammaherpesvirus (AY949830.1) and Blainville's beaked whale gammaherpesvirus (AY949828.1) with 21% of query coverage, 0.007 of e-value, and 100% of indentification (Figure 4b). The pathogen of the skin infection was supposed to gammaherpesvirus with the identified sequences, however, they were too short to perform phylogenetic analysis. Because of multigenic existence in the DNA samples, nonspecific PCR reaction was frequently occurred with the universal herpesvirus primers.

For an in-depth analysis of the specific cetacean gammaherpesvirus sequence, new primers DHVF1, DHVF2, DHVR1, MHVF1, MHVR1, MHVF2, MHVR2, MHVF3 and MHVR3 were designed and used for further PCR reaction and sequence analysis. Consequently, target-sized bands were detected from skin lesions, oral skin, and mammary gland in the result of QIAxcel ScreenGel Software and gel electrophoresis (Figure 5a and 5b). After the PCR reaction with DHVF1 and DHVR1, all samples from the skin lesion, oral skin, and mammary gland were positive, meanwhile, only skin lesion sample showed positive result with DHVF2 and DHVR1 (Table 2) and the skin lesion sample showed clear 173 bp sequencing result. However, the clear 173 bp sequence was identified with 12% of low query coverage as the partial DNApol gene of dwarf sperm whale gammaherpesvirus (AY949830.1) (Figure 5c). To get more accurate gene of the virus, primer walking is currently in progress. On the one hand, the PCR results of MHVF1, MHVR1, MHVF2, MHVR2, MHVF3, and MHVR3 showed target-sized bands in the gel electrophoresis. However, the sequence results were not available to be analyzed because of nonspecific reactions.

Discussion

Histopathological changes of the skin lesions

Even though the animal tissue had been frozen and thawed, few clinical changes were found in the histological examinations of the skin lesions. Rete pegs were markedly elongated in thickened epidermis. Generally, accentuated rete pegs are commonly recognized in the cases of acanthosis in psoriasis (Kempf et al., 2008) or atopic dermatitis (AD) (Bovenschen et al., 2005). In the case of gammaherpesvirus infection in harbor porpoise (*Phocoena phocoena*), thickened epidermis was reported before (van Elk et al., 2016). Various inflammatory cells and overgrown gram-negative bacteria were observed. Some of dermatitis cases have reported that co-infection of microorganisms such as herpesvirus and *Staphylococcus aureus* causes severe or lethal effect to immunocompromised patients potentially (Saintive et al., 2017). Eosinophilic intranuclear inclusion bodies are identified in the most cases of cetacean herpesvirus infections such as harbor porpoises (van Beurden et al., 2015; van Elk et al., 2016). The one of

considerable changes was the marked infiltrates of predominantly mononuclear cells which is also one of histological symptoms of AD (Leung et al., 1983), carcinoma (Håkansson et al., 1997), or more skin disease. Additionally, a viral cytopathic effect was suspected with the vacuolar change of amorphous eosinophilic material and moderate nuclear debris because some skin-infecting viruses including herpesvirus cause intracytoplasmic vacuolization (Prose et al., 1969; Hedrick et al., 1990; and Prose et al., 1971) and hypereosinophilic material (Origgi et al., 2017). In the case of alphaherpesvirus infection in beluga whale (*Delphinapterus leucas*), the development of microvesicles was reported (Nielsen et al., 2017). In histopathological conclusion, viral infection in the dermis might cause the proliferative dermatitis with bacterial co-infection.

Molecular analysis of the cetacean herpesvirus

According to the result of sequencing, the extracted and amplified virus DNA was close with the partial DNApol gene of *Balaenoptera acutorostrata* gammaherpesvirus 2, dwarf sperm whale gammaherpesvirus, and Blainville's

beaked whale gammaherpesvirus. Considering that I used the primers; FP1, FP2, and RP1 which are universal primers to detect DNApol gene of herpesvirus, the sequenced result is rather meaningful as the cetacean herpesvirus. Because herpesvirus infection in narrow-ridged finless porpoise has not been reported yet, this study proved the existence of the virus significantly. However, the sequence was too short to analyze the viral strain's features and phylogenetic classification. Even though the clear 173 bp sequence was detected after the PCR reaction with newly designed primers DHVF2 and DHVR1, the query coverage was only 12% with the partial DNApol gene of dwarf sperm whale gammaherpesvirus (AY949830.1) (Figure 6). I considered that the drifting time or prolonged freezing storage period affected the quality of genetic material. Primer walking is currently in progress to discover the unknown sequence of the virus. Besides, additional detection of the porpoise gammaherpesvirus should be required for further molecular research of the porpoise herpesvirus.

Autopsy report of NA20-1215

Narrow-ridged finless porpoise is the most bycaught cetacean species in Korean seawater (Kim et al., 2013). Therefore, most of the porpoises suffocate in nets and the stranded bodies usually have clinical signs of asphyxia such as net injuries on the skin (Tregenza et al., 1994), disseminated gas bubbles in many organs, froth in trachea (Quirós et al., 2018), and reddish eyes (Puig-Lozano et al., 2020). However, the clinical signs of suffocation were not recognized in NA20-1215 and the most noticeable symptom was the skin lesions. Despite of the severe proliferative dermatitis, all lymph nodes had normal size. Several parasites were found from the lungs and mammary glands. In the liver and mammary gland, few nodules which are assumed previous parasite infection were recognized. As a veterinarian and the first author of this study, I diagnosed that gammaherpesvirus skin infection and immunocompromised state had the possibility to affect the death of NA20-1215.

Comparative analysis between the gammaherpesvirus infection of animals

Gammaherpesvirus infection has been reported from the skin lesion of various

vertebrae. For example, gammaherpesviruses were identified from the ulcerative skin lesions of South American fur seal (*Arctocephalus australis*), fisher (*Martes pennanti*), sheep (*Ovis aries*), and so on (Sacristán et al., 2018; Gagnon et al., 2011; Ackermann, 2005). Meanwhile, few gammaherpesviruses were identified from cetaceans and the viruses mostly infect to genital lesions, lymph node, or central nervous system (Smolarek-Benson et al., 2006; Belliére et al., 2010; Vargas-Castro et al., 2020). Skin infection of gammaherpesvirus has been rarely reported in cetaceans. Only *Phocoenid* HV1 was identified from the cutaneous lesions of harbour porpoises (*Phocoena phocoena*) (van Beurden et al., 2015). Therefore, this study can be significant as another skin infection case of gammaherpesvirus in cetaceans.

Latent infection of herpesvirus and further research

One of the representative characters of the family Herpesviridae is latent infection which makes the molecular diagnosis difficult from the lesions. Among the Herpesviridae, the target organs for latency period are diverse depending on the

subfamilies. Gammaherpesvirus is highly lymphotropic, while alphaherpesvirus infects latently in neurons and betaherpesvirus has variable tropism (Speck and Ganem, 2010). Due to the latent infection, herpesvirus has also been detected from several cetacean cases without clear symptoms or manifestations (Bellière et al., 2010). This study focuses on gammaherpesvirus infection from the only one individual; NA20-1215 with the severe and clear skin dermatitis. At the same time, this study raises more needs to detect latent existence of herpesvirus from the healthy narrow-ridged finless porpoises. There is a possibility that numerous narrow-ridged finless porpoises are latent-infected with herpesvirus in the Korean seawater. Cetacean herpesviruses are mostly associated with genitally transmitted disease, which should affect the animals' sexual behavior and their distribution in a negative way. The population of narrow-ridged finless porpoise has declined dramatically for the last decade. For the conservation of the porpoises and, by extension, marine ecosystem, the role of herpesvirus in the Korean sea should be investigated more.

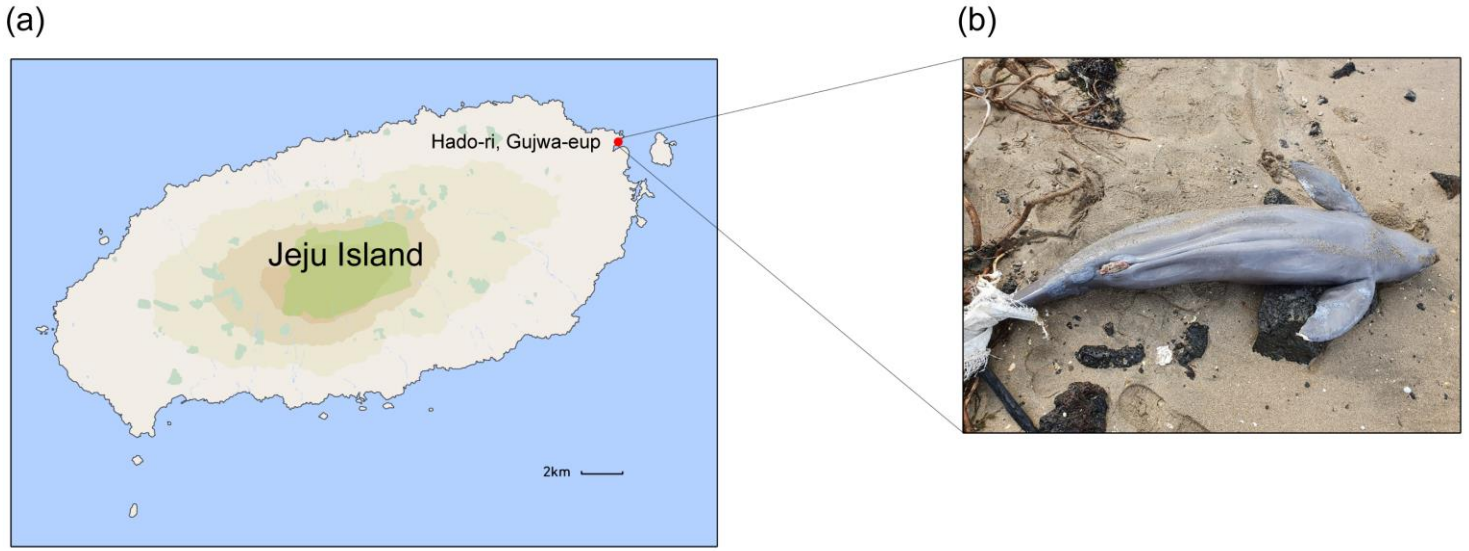

Fig 1. Location of the sample in Jeju Island. A stranded body of narrow-ridged finless porpoise was found at Hado-ri, Gujwa-eup, Jeju-si, Jeju-do, Republic of Korea (33°31.140′ N 126°54.206′ E).

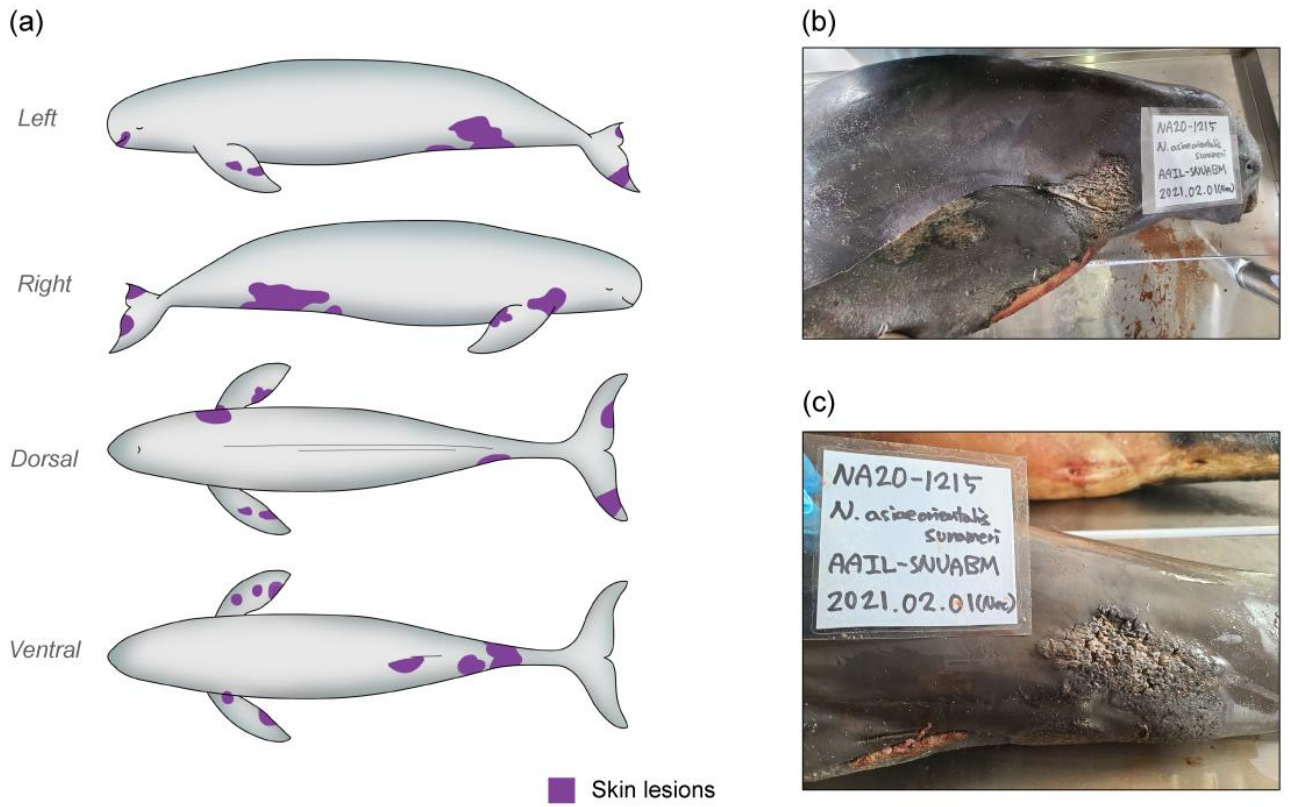

Fig 2. Clinical lesions of herpesvirus infection on the skin. (a) Starting from the top, left, right, dorsal, and ventral view of NA20-1215. Observed skin lesions were marked with the purple color. Total twelve skin lesions were observed on the whole body. (b) Dermatitis lesions were found on both flippers and fluke as well. (c) The largest skin lesion was 12.8 × 11.0 cm around the genital slit.

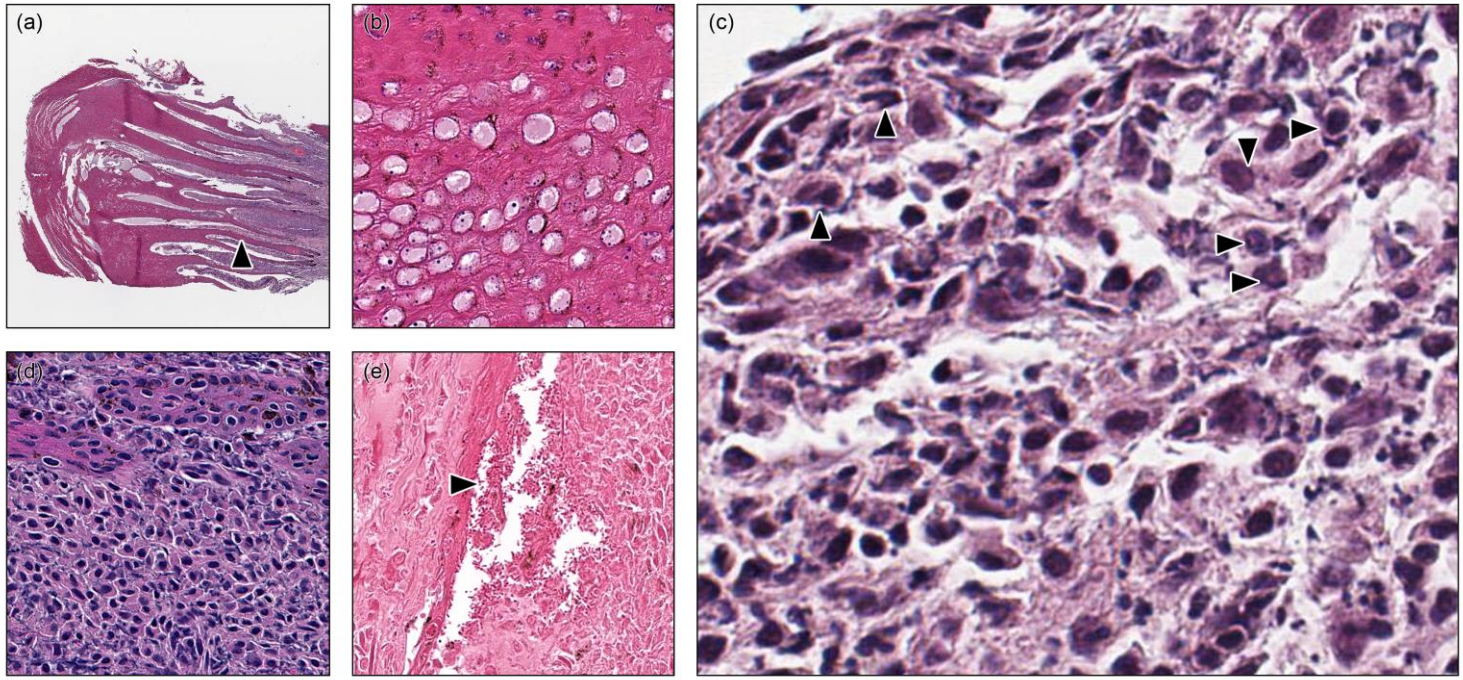

Fig 3. Histopathological examination of skin lesions. (a) Epidermis was moderately to markedly thickened. The black arrow (▲) indicates accentuated rete pegs into the underlying connective tissue. Hematoxylin and eosin (HE). (b) There was vacuolar change with amorphous eosinophilic material in the vacuoles and moderate nuclear debris within the epidermis (HE). (c) Intranuclear eosinophilic inclusion body (INI) was observed in the cells of epidermis (▲, ▶, ▼). (d) Mononuclear cells infiltrated predominantly within the dermis at the base of the proliferative epidermis (HE). (e) Overgrown gram-negative bacteria was observed in the dermis (▶)(HE).

PCR primers	Primer sequence	Target gene
Herpesvirus		
FP1	5’- GAY TTY GCI AGY YTI TAY CC- 3’	DNA polymerase gene
FP2	5’- TCC TGG ACA AGC AGC ARI YSG CIM TIA A -3’	DNA polymerase gene
RP1	5’- GTC TTG CTC ACC AGI TCI ACI CCY TT -3’	DNA polymerase gene
FP3	5’- TGT AAC TCG GTG TAY GGI TTY ACI GGI GT -3’	DNA polymerase gene
RP2	5’- CAC AGA GTC CGT RTC ICC RTA IAT -3’	DNA polymerase gene
HVF1	5’- GAY TTY GCN AGY YTN TAY CC -3’	DNA polymerase gene
HVF2	5’- TCC TGG ACA AGC AGA RNY SGC NMT NAA -3’	DNA polymerase gene
HVR1	5’- GTC TTG CTC ACC AGN TCN ACN CCY TT -3’	DNA polymerase gene
HVF3	5’- TGT AAC TCG GTG TAY GGN TTY ACN GGN GT -3’	DNA polymerase gene
HVR2	5’- CAC AGA GTC CGT RTC NCC RTA NAT -3’	DNA polymerase gene
DHVF1	5’- ATC GGC ATC CCA ATC TGA CC -3’	DNA polymerase gene
DHVF2	5’- CTG ACC CCC GAA GAC TTT GA -3’	DNA polymerase gene
DHVR1	5’- CAT TCT CGC ACG TAT CGG GT -3’	DNA polymerase gene
MHVF1	5’- GTC TCC AAA CCA AGT GCA GC -3’	DNA polymerase gene
MHVR1	5’- TTT GCT GCT AGG CTG TCC AT -3’	DNA polymerase gene
MHVF2	5’- AGG TGC CTT TTC AGA GAG CC -3’	DNA polymerase gene
MHVR2	5’- ACC CTT TTG TCT TGC TCA CCA -3’	DNA polymerase gene
MHVF3	5’- TCA GGG CTT CTC CCG TGT A -3’	DNA polymerase gene
MHVR3	5’- TGT GTG ATG AGT TGC TGC AC-3’	DNA polymerase gene
Papillomavirus		
FAP59	5’- TAA CWG TIG GIC AYC CWT ATT -3’	L1 protein gene
FAP64	5’- CCW ATA TCW VHC ATI TCI CCA TC -3’	L1 protein gene
SNUPPV-1 F	5’- ATC AAT ATA GGG CTA TGC GA -3’	L1 protein gene
SNUPPV-1 R	5’- TTG CTC CTT TTC CTT TTC AC -3’	L1 protein gene
SNUPPV-2 F	5’- AAT CAA TAT AGG GCT ATG CGA -3’	L1 protein gene

SNUPPV-2 R	5’- TTC ACA ATG CTT TTC CAC CA -3’	L1 protein gene
SNUPPV-3 F	5’- ACA TGA AAG GTT GGT TTG GT -3’	L1 protein gene
SNUPPV-3 R	5’- GCC ATC CTG TAT ATG CGT AG -3’	L1 protein gene
Lacazia loboi		
Kex-1F	5’- TGC TTY GGT TTG GGG TTG -3’	*Kex* gene
Kex-2R	5’- CAC TGG ARC CGT CAG CTA -3’	*Kex* gene
MAE	5’- TGC TGC GGC GGG GTT AAA CCA TGT C -3’	*gp43* gene
ATO	5’- GTT GTG GTA TGT GTC GAT GTA GAC G -3’	*gp43* gene
SUM-F1	5’- GTC ATC GAT CTC CAT GGT GTT AAG -3’	*gp43* gene
SUM-R2	5’- GGC AGA RAA GCA TCC GAA A -3’	*gp43* gene
Poxvirus		
DNApol-FP	5’- ATA CAG AGC TAG TAC ITT AAT AAA AG -3’	DNA polymerase gene
DNApol-RP	5’- CTA TTT TTA AAT CCC ATT AAA CC -3’	DNA polymerase gene

Table 1. Primer list for rule out diagnosis of the cetacean skin lesions. 19 primers were used to detect partial DNA polymerase gene of herpesvirus. 8 primers were used to detect partial L1 gene of papillomavirus. 6 primers were used to detect partial *Kex* gene or *gp43* gene of *Lacazia loboi*. A pair of primers were used to detect partial DNA polymerase gene of poxvirus.

PCR primers	Target size (bp)	Result of electrophoresis			Identified sequence
		Skin lesions	Skin (Oral area)	Mammary gland	
Herpesvirus					
FP1, FP2, RP1 (1st)	215-315	+	+	+	Gammaherpesvirus (Low query coverage)
FP3, RP2 (2nd)		-	-	-	
HVF1, HVF2, HVR1 (1st)	215-315	+	+	+	
HVF3, HVR2 (2nd)		-	-	-	
DHVF1, DHVR1	360	+	+	+	
DHVF2, DHVR1	350	+	-	-	Gammaherpesvirus (Low query coverage)
MHVF1, MHVR1	110	+	+	+	
MHVF2, MHVR2	110	-	-	+	
MHVF3, MHVR3	90	+	+	+	
Papillomavirus					
FAP59, FAP64	480	+/-	+/-	-	
SNUPPV-1 F, SNUPPV-1 R	350	+/-	+/-	-	
SNUPPV-2 F, SNUPPV-2 R	350	+/-	+/-	-	
SNUPPV-3 F, SNUPPV-3 R	350	+/-	+/-	-	
Lacazia loboi					
Kex-1F, Kex-2R	151	-	-	-	
MAE, ATO	550	-	-	-	
SUM-F1, SUM-R2	382	-	-	-	
Poxvirus					
DNApol-FP, DNApol-RP	543	-	-	-	

Table 2. The result of electrophoresis, and the identified sequence according to the respective PCR primers. + and - represents positive and negative result, respectively. Although all samples including skin lesions of genital slit and oral area and mammary gland had positive PCR result with the herpesvirus primers, most of the bands were weak and failed to be sequenced because nonspecific reactions frequently occured. Only the PCR product with the primers FP1, FP2, and RP1 was sequenced successfully and gammaherpesvirus was identified as the result with low query coverage. In addition, newly designed primers; DHVF1, DHVF2, DHVR1, MHVF1, MHVR1, MHVF2, MHVR2, MHVF3, and MHVR3 were used to detect DNApol gene of cetacean herpesvirus, then, most of the results was positive. Amplified DNA with DHVF2 and DHVR1 was identified as gammaherpesvirus with low query coverage. On the one hand, the PCR results of MHVF1, MHVR1, MHVF2, MHVR2, MHVF3, and MHVR3 were not available to be analyzed because of nonspecific reactions. PCR with the papillomavirus primers had shown weak bands in the gel, however, the PCR products were also failed to be sequenced. *Lacazia loboi* and poxvirus were negative.

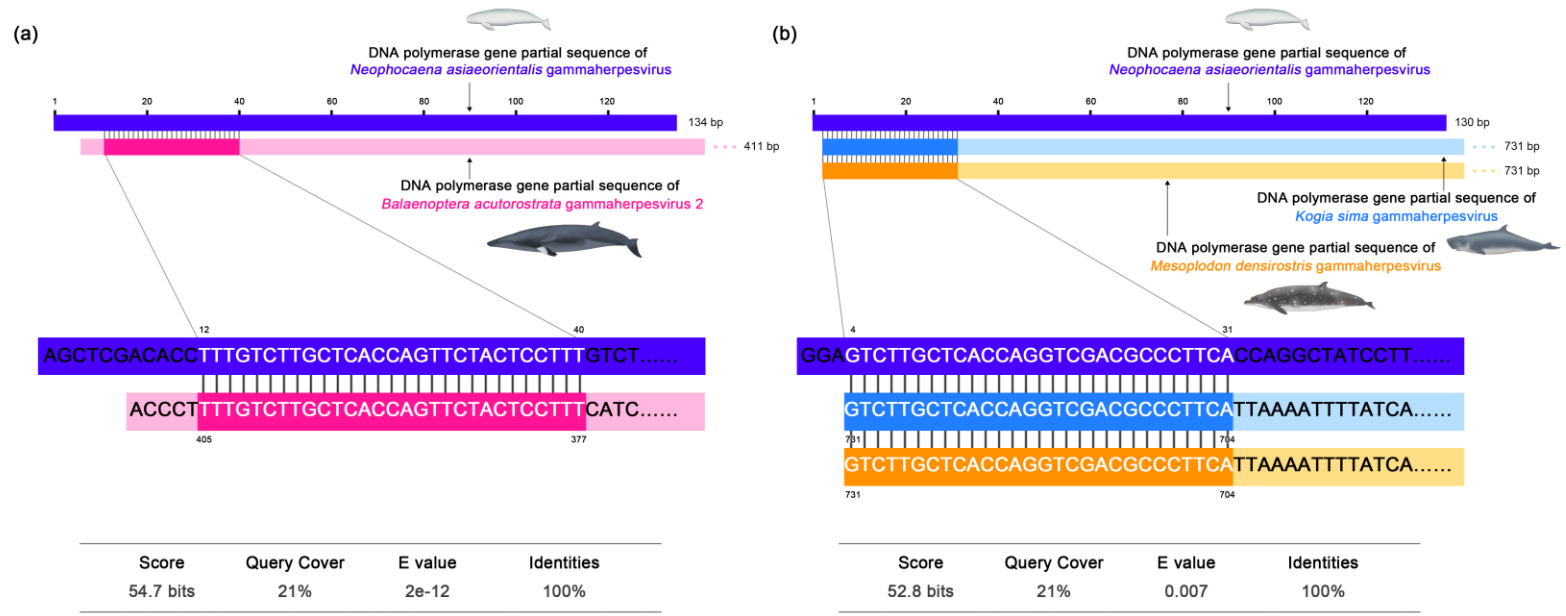

Fig 4. The comparative analysis between DNA polymerase gene partial sequences of *Neophocaena asiaeorientalis* gammaherpesvirus, *Balaenoptera acutorostrata* gammaherpesvirus 2 (KP995688.1), dwarf sperm whale (*Kogia sima*) gammaherpesvirus (AY949830.1), and Blainville's beaked whale (*Mesoplodon densirostris*) gammaherpesvirus (AY949828.1). (a) Purple line indicates the 134 bp sequence of the partial DNA polymerase gene of *Neophocaena asiaeorientalis* gammaherpesvirus and pink line indicates the 411 bp sequence of the partial DNA polymerase gene of *Balaenoptera acutorostrata* gammaherpesvirus 2. In the sequence of *Neophocaena asiaeorientalis* gammaherpesvirus, 29 bp sequence was identified as the DNA polymerase gene partial sequence of *Balaenoptera*

acutorostrata gammaherpesvirus 2 with 21% of query coverage, 0.002 of e-value, and 100% of identification. (b) Purple line indicates the 130 bp sequence of the partial DNA polymerase gene of *Neophocaena asiaeorientalis* gammaherpesvirus. Blue and yellow lines indicate the 731 bp sequence of the partial DNA polymerase gene of dwarf sperm whale gammaherpesvirus and Blainville's beaked whale gammaherpesvirus, respectively.

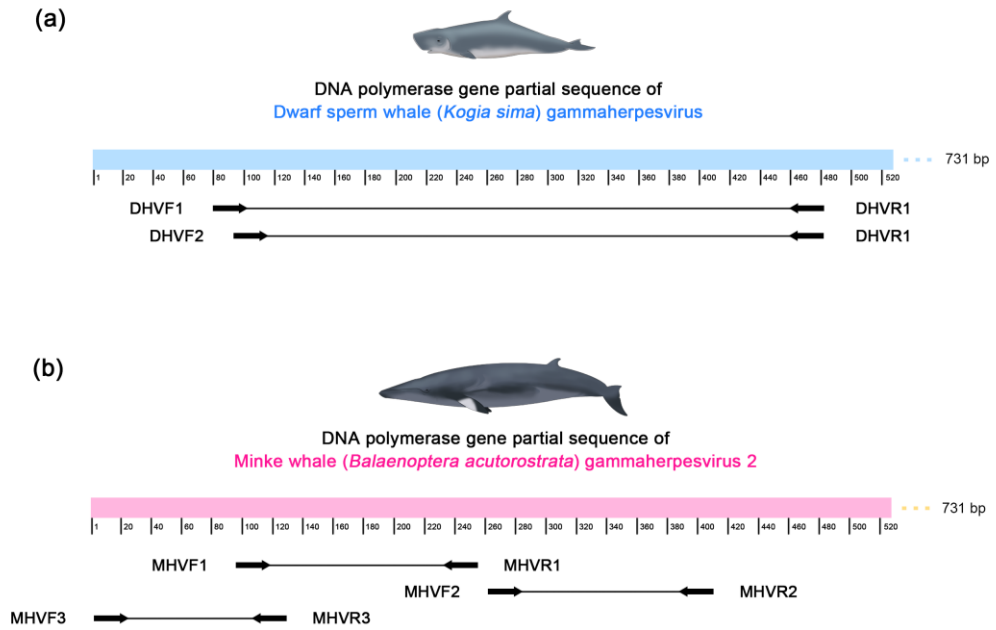

Fig 5. Designing primers based on DNApol genes of cetacean gammaherpesviruses. (a) Three primers DHVF1, DHVF2, and DHVR1 were designed with NCBI Primer-BLAST. The partial DNApol gene of Dwarf sperm whale gammaherpesvirus (AY949830.1) was used as a template. Each pair of primers was expected to amplify approximately 350 bp of the gene. (b) Primers MHVF1, MHVR1, MHVF2, MHVR2, MHVF3, and MHVR3 were designed using the partial DNApol gene of *Balaenoptera acutorostrata* gammaherpesvirus (KP995688.1) as a template. Each pair of primers was expected to amplify approximately 90-110 bp of the gene.

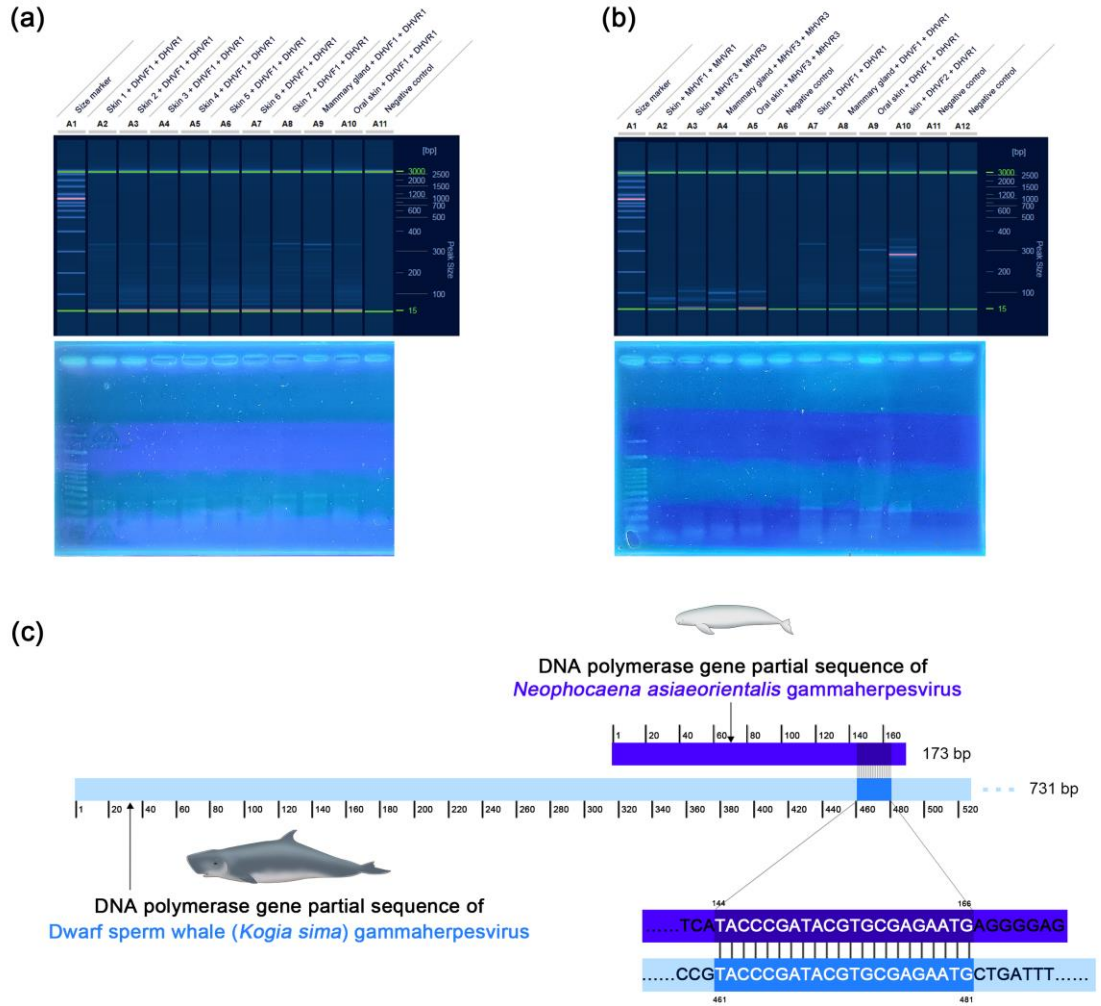
(a)
Size marker
Skin 1 + DHVF1 + DHVR1
Skin 2 + DHVF1 + DHVR1
Skin 3 + DHVF1 + DHVR1
Skin 4 + DHVF1 + DHVR1
Skin 5 + DHVF1 + DHVR1
Skin 6 + DHVF1 + DHVR1
Skin 7 + DHVF1 + DHVR1
Mammary gland + DHVF1 + DHVR1
Oral skin + DHVF1 + DHVR1
Negative control
A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11
[bp]
3000 2500 2000 1500 1200 1000 700 600 500 400 300 200 100 15
Pixel Size
(b)
Size marker
Skin + MHVF1 + MHVR1
Skin + MHVF3 + MHVR3
Mammary gland + MHVF3 + MHVR3
Oral skin + MHVF3 + MHVR3
Negative control
Skin + DHVF1 + DHVR1
Mammary gland + DHVF1 + DHVR1
Oral skin + DHVF1 + DHVR1
skin + DHVF2 + DHVR1
Negative control
Negative control
A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 A12
[bp]
3000 2500 2000 1500 1200 1000 700 600 500 400 300 200 100 15
Pixel Size
(c)
DNA polymerase gene partial sequence of
Neophocaena asiaeorientalis gammaherpesvirus
1 20 40 60 80 100 120 140 160
173 bp
731 bp
1 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 460 480 500 520
DNA polymerase gene partial sequence of
Dwarf sperm whale (Kogia sima) gammaherpesvirus
144
166
.....TCATACCCGATACGTGCGAGAATGAGGGGAG
.....CCGTACCCGATACGTGCGAGAATGCTGATTT.....
461
481

Fig 6. The results of QIAxcel ScreenGel software, gel electrophoresis, and sequencing after PCR reactions. (a) PCR products with the primers DHVF1, DHVF2, and DHVR1 were analyzed by QIAxcel ScreenGel software and gel electrophoresis. Nearly all of samples showed target-sized bands which are estimated 350 bp. (b) PCR products with the primers MHVF1, MHVR1, MHVF3, MHVF3, DHVF1, DHVF2, and DHVR1 were analyzed by QIAxcel ScreenGel software and gel electrophoresis. Nearly all of samples showed target-sized bands which are estimated 90-110 bp with MHVF1, MHVR1, MHVF3, and MHVF3 and 350 bp with DHVF1, DHVF2, and DHVR1, respectively. (c) Skin lesion sample showed positive result with DHVF2 and DHVR1 and the PCR product showed clear 173 bp sequencing result. However, the clear sequence was identified with 12% of low query coverage as the partial DNApol gene of dwarf sperm whale gammaherpesvirus (AY949830.1).

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Summary

A carcass of female narrow-ridged finless porpoise was stranded on the beach located at Jeju Island, South Korea. Twelve dermatitis lesions were spread on various sites of the body. In histopathological examination of the skin lesions, few manifestations were identified such as moderately to markedly thickened epidermis, accentuated rete pegs, vacuolization and amorphous eosinophilic material in the vacuoles, eosinophilic intranuclear inclusion bodies in the epidermal cells, and marked infiltrates of predominantly mononuclear cells within the dermis. Extracted DNA from the skin lesions and mammary gland were amplified respectively with multiple primers of few possible pathogens; herpesvirus, papillomavirus, *Lacazia loboi*, and poxvirus to do rule out diagnosis. Consequently, gammaherpesvirus was detected in the molecular analysis. The viral sequence contains same parts with the partial DNA polymerase gene of *Balaenoptera acutorostrata* gammaherpesvirus 2, dwarf sperm whale gammaherpesvirus, and Blainville's beaked whale gammaherpesvirus. The viral sequence should be analyzed more precisely for further phylogenetic analysis. Besides, for the conservation of the porpoises and, by extension, marine ecosystem, the role of herpesvirus in the Korean sea should be investigated more. Gammaherpesvirus has been usually reported as the pathogen of genital and oral mucosae infections in cetaceans, and the skin infection has been rarely reported.

This study is the first report of gammaherpesvirus infection in narrow-ridged finless porpoise in the Republic of Korea.

국문 초록

국내 상괭이 피부 질환을 유발하는 감마허피스바이러스 최초 보고

이 성 빈

서울대학교 대학원 수의학과 수의병인생물학 및 예방수의학 전공

(지도교수: 박 세 창)

상괭이는 한국의 토종 돌고래이자 한국 바다의 우점종으로 잘 알려진 고래이다. 그러나 최근 10년 동안 상괭이의 개체수는 크게 감소하였으며, 그 결과 상괭이는 2017년 국제자연보전연맹(IUCN)의 적색 목록에서 절멸 위기(Endangered)에 처한 동물로 등록되었다. 이러한 멸종 위기에도 불구하고, 상괭이의 병리학적 연구는 아직까지 보고된 바가 많지 않다. 상괭이의 개체수 감소를 넘어 야생동물, 인간, 생태계의 건강을 위해 상괭이 보존 의학이 앞으로 더 연구되어야 할 것으로 보인다. 필자는 상괭이의 피부병에서 바이러스성 질병체를

검출하고, 연구한 내용을 이번 논문을 통해 보고하고자 한다.

암컷 상괭이 사체 하나가 한국 제주도 구좌읍에 위치한 해안가에서 발견되었다. 이 상괭이의 전신에는 약 20여개의 피부병변 부위가 산재해 있었다. 해당 병변의 조직학적 검사 결과 상으로 현저하게 두꺼워진 상피와 길어진 망융선을 관찰할 수 있었고, 그 외에 다수의 액포 형성 및 액포 내 무정형 호산성 물질, 진피 세포 내 호산성 핵내 봉입체, 진피 내 무수히 많은 단핵세포의 침투 등이 특이적이었다. 조직 검사 결과 허피스바이러스, 파필로마바이러스, 라카지아 로보이, 폭스바이러스 등이 해당 피부병의 병인체로 의심되어 피부 병변부에서 DNA를 추출하고, 각 병인체의 프라이머들을 이용하여 DNA를 증폭하였으며, 배제진단 방식으로 진단하였다. 최종적으로 DNA 시퀀스 분석 결과 이 피부병변에서는 감마허피스바이러스의 DNA 중합효소 유전자가 검출되었다. 해당 바이러스 DNA 시퀀스에는 상괭이와 같은 고래목에 속하는 밍크고래, 꼬마향고래, 혹부리고래에서 검출된 감마허피스바이러스와 동일한 시퀀스를 일부 갖고 있음을 PCR 및 유전자 분석을 통해 확인할 수 있었다. 감마허피스바이러스는 고래에서 주로 생식기 점막 또는 구강 점막에 질병을 일으키는 원인체로 알려져 있고, 피부 병변을 일으킨다는 보고는 거의 없었다. 이번 연구는 한국 상괭이에서 피부 질환을 유발하는 감마허피스바이러스를 최초로 보고하는 것에 그 의의가 있다. 또한, 상괭이의 허피스바이러스 잠복감염 실태, 한국 바다의 허피스바이러스 전파 양상 등이 추가적으로 연구되어야 할

것으로 보인다.

핵심어: 고래허피스바이러스, 감마허피스바이러스, 피부병, 상괭이, *Neophocaena asiaeorientalis*

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1. Sib Sankar Giri, Sang Guen Kim, Jeong Woo Kang, Sang Wha Kim, Jun Kwon, **Sung Bin Lee**, Won Joon Jung, Se Chang Park. Applications of carbon nanotubes and polymeric micro-/nano-particles in fish 2 vaccine delivery: progress and future perspectives. Reviews in Aquaculture. https://doi.org/10.1111/raq.12547.

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1. Jun Kwon, Sang Guen Kim, Sib Sankar Giri, Hyoun Joong Kim, Sang Wha Kim, Jung Woo Kang, **Sung Bin Lee**, Won Jun Jeong, Se Chang Park*. Genomic characterization of bacteriophage pSal-SNUABM-01, a novel elongated head phage infecting Salmonella sp. The Korean Society of Veterinary Science Autumn Conference 2021. GSCO Gunsan, North Jeolla Province, Republic of Korea, 28th October - 30th October, 2021.

2. Jun Kwon, Sang Guen Kim, Sang Wha Kim, **Sung Bin Lee**, Won Joon Jung, Jeong Woo Kang, Sib Sankar Giri, Se Chang Park*. Topical bacteriophage application, a promising alternative against dog otitis externa. Oxford Bacteriophage Conference – Phages 2021. Virtual conference, 7th September - 8th September, 2021.

2020

1. Sang Wha Kim, Won Hee Hong, Hyoun Joong Kim, Sang Guen Kim, Jun Kwon, **Sung Bin Lee**, Won Joon Jung, Jeong Woo Kang, Byung Yeop Kim, Se Chang Park*. Assisted reproductive technologies in shark: First report of hormone-induced artificial insemination and sperm cryopreservation in *Chondrichthyes*. The Korean Society of Veterinary Science Autumn Conference 2020. Hongcheon, Gangwon-do, Republic of Korea, 19th November - 21st November, 2020.

2. Sang Wha Kim, Jun Kwon, Hyoun Joong Kim, Sang Guen Kim, **Sung Bin Lee**, Won Joon Jung, Jeong Woo Kang, Se Chang Park*. Bacteriophage phage

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또한 2년이 넘는 시간 동안 저의 연구에 힘이 되어 주신 제주대학교 김병엽 교수님께도 특별히 감사의 인사를 드리고자 합니다. 해양 포유류 연구에 더욱 매진할 수 있도록 항상 도움의 손길을 마다하지 않으신 덕분에 제가 이번 연구를 시작할 수 있었습니다.

저에게 아낌없는 지원을 해주시는 저희 부모님께도 깊은 감사를 전합니다. 6년의 학사 과정과 3년의 공중방역수의사 과정을 거쳐, 늦은 나이에 대학원 생활까지 하게 되었지만, 언제나 응원해주시는 부모님 덕분에 저도 더 힘을 내 석사 과정 마무리 단계까지 오게 되었습니다.

마지막으로, 제 석사학위 논문 심사원으로 참석해주신 윤화영 교수님과 전진우 교수님, 귀한 시간 내주셔서 정말 감사드립니다. 교수님들의 꼼꼼하고 세심한 조언들 덕분에 제 석사 과정이 성공적으로 마무리된 것 같습니다. 많은 분들의 도움을 받으며 대학원 생활을 보내왔기에, 앞으로의 연구에도 더 열중하여 훌륭한 수의사이자 연구자가 되도록 노력하겠습니다.

이번 연구는 제주도청 및 제주대학교의 “2021년 해양보호생물 상괭이 부검 시범연구 [제주권역]” 과제의 지원을 받아 진행되었습니다.