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A new enzyme-linked immunosorbent assay for serological diagnosis of seal parapoxvirus infection in marine mammals

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Abstract

Introduction: Seal parapoxvirus (SPPV) infection has been reported among pinnipeds in aquaria in Japan; however, its seroprevalence is unknown. Therefore, an enzyme-linked immunosorbent assay (ELISA) was developed for serological diagnosis of SPPV infection. **Material and Methods:** The gene encoding the major envelope protein of SPPV was cloned into the eukaryotic expression vector pAcGFP1-N1, which encodes the green fluorescence protein (GFP), thereby producing a fusion protein (Env-GFP). Parental and cloned vector DNA was independently transfected into cultured seal cells for the expression of GFP and Env-GFP. The wells of an ELISA plate were coated with either GFP- or Env-GFP-transfected cell lysates. The light absorbance of each serum sample was adjusted by subtracting the absorbance of GFP-coated wells from that of Env-GFP-coated wells. Sera from two spotted seals (*Phoca largha*), six beluga whales (*Delphinapterus leucas*), three Pacific white-sided dolphins (*Lagenorhynchus obliquidens*), and ten bottlenose dolphins (*Tursiops truncatus*) from an aquarium in Japan were examined using the ELISA. **Results:** Positive reactions were not observed, except in one preserved sample collected ten years ago from a naturally SPPV-infected spotted seal. **Conclusion:** The established ELISA could be useful in screening marine mammal sera for anti-SPPV antibodies.

Keywords: aquarium, ELISA, parapoxvirus, pinnipeds, Western blotting.

Introduction

Marine mammals such as seals, sea lions, walruses, sea otters, porpoises, dolphins, and whales are considered sentinel species for the ocean ecosystem and human health: they provide early indications of environmental pollution, toxicants, and epizootics of animal and human infectious diseases (5). Pinnipeds are carnivorous, fin-footed marine mammal species inhabiting marine/terrestrial interfaces (9). They are classified into three families: *Otariidae* (fur seals and sea lions), *Phocidae* (true seals), and *Odobenidae* (walruses) (9).

Infection due to viruses belonging to the genus *Parapoxvirus* of the family *Poxviridae* has been confirmed in the following wild and captive pinniped species: grey seals (*Halichoerus grypus*) (12, 31), Mediterranean monk seals (*Monachus monachus*) (37), harbour seals (*Phoca vitulina*) (2, 7, 24), spotted seals (*Phoca largha)* (7), Weddell seals (*Leptonychotes weddellii*) (38), Antarctic fur seals (*Arctocephalus gazella*) (27), California sea lions (*Zalophus californianus*) (26),

Steller sea lions (*Eumetopias jubatus*) (7), and Atlantic walruses (*Odobenus rosmarus rosmarus*) (32). Seal parapoxvirus (SPPV) was confirmed as a new species within the genus *Parapoxvirus*, according to the International Committee on Taxonomy of Viruses 10th Report (current report) on virus taxonomy (16). The new species was designated as *Grey sealpox virus*, as the whole genome sequences of the virus were retrieved from lesions of an infected grey seal (12). Clinical signs of SPPV infection in pinnipeds include firm skin nodules on the head, neck, and thorax, which can extend to the abdomen, flippers, perineum, and oral and nasal mucosa (2, 38). The disease caused by this virus is a zoonosis (8).

In terrestrial animals, parapoxviruses (PPVs) affect domestic ruminants, including cattle (34), sheep and goats (36), and camels (17), as well as wild ruminants, being known in red deer in New Zealand (*Cervus elaphus*) (11) and Japanese serows (*Capricornis crispus*) (35). The latter species is one of the susceptible terrestrial animals in which serological surveys have demonstrated the seroprevalence of PPV infection in Japan (14, 15), and cattle and sheep also have noted seroprevalence in the country (20, 33).

In Japan, SPPV infection has previously been reported among pinnipeds, such as a spotted seal (29) and South American sea lions (*Otaria byronia*) (30) in aquaria. Additionally, PPV infection has been reported in cetacean species, specifically bottlenose dolphins (*Tursiops truncatus*) and Pacific white-sided dolphins (*Lagenorhynchus obliquidens*) (19). However, due to a lack of serological diagnostic assays, no serosurveys have been conducted among marine mammals in Japan. Establishment of validated serological assays will be highly valuable for performing sero-epidemiological studies. Therefore, the aim of this study was to establish a new enzyme-linked immunosorbent assay (ELISA) for the serological diagnosis of SPPV infection.

Material and Methods

Cell culture. Primary aortic smooth muscle cells were isolated from the aorta of a dead spotted seal. Isolated cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (cat. no. 189-02145; Fujifilm Wako, Osaka, Japan) supplemented with 15% cosmic calf serum (HyClone, Provo, UT, USA), 1% non-essential amino acids (Gibco, Grand Island, NY, USA), 1 mM sodium pyruvate (Gibco), and ZellShield (Minerva Biolabs, Berlin, Germany). During cell passages, endothelial cells were eliminated due to slower growth than smooth muscle cells. After four passages, pLNCLT plasmid DNA encoding the large T antigen replication origin-defective simian virus 40 (23) was transfected into the cells. Transfected cells were selected by addition of G418 (cat. no. 04727878001; Roche, Mannheim, Germany) and maintained in RPMI 1640 medium supplemented with 10% foetal bovine serum (cat no. A15-701; PAA Laboratories, Pasching, Austria), 100 mg/mL of streptomycin, and 100 U/mL of penicillin. The established smooth muscle cells were designated as Phocid Smooth Muscle SV40T (PhoMT) cells.

Cloning and transformation. The major envelope protein (B2L protein) of SPPV was chosen as the ELISA antigen because of its high immunogenicity (40). As SPPV has not previously been isolated and the live virus is not currently available in Japan, a DNA fragment encoding the whole nucleotide sequence of the gene encoding SPPV major envelope protein (complete genome SPPV isolate AFK76s1, GenBank accession number KY382358) (12), was synthesised using GeneArt Strings DNA Fragments (Invitrogen, Regensburg, Germany). The DNA fragment was cloned into the *Xho* I and *Eco*R I sites of the eukaryotic expression vector, pAcGFP1-N1 (cat. no. 632469; Takara, Kusatsu, Japan), which encodes the green fluorescence protein (GFP) sequence after the multiple cloning site of the vector. This step was executed using NEBuilder HiFi DNA Assembly Master Mix (cat. no. E2621; New England BioLabs, Ipswich, MA, USA). In the cloned vector, the envelope gene was attached in-frame to the N-terminus of the GFP gene, leading to the expression of a fusion protein (Env-GFP). The cloned and parental (empty) vectors were used for subsequent transfection experiments.

Transfection and confocal microscopy. Transfection of PhoMT cells was with cloned (Env-GFP) or empty (GFP) vectors or was sham transfection using Lipofectamine LTX (cat. no. 15338-100, Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. The transfected cells were observed 48 hours post transfection (hpt) with an LSM700 inverted confocal microscope (Carl Zeiss, Oberkochen, Germany) to note protein expression through green fluorescence. Additionally, the intracellular locations of the GFP and Env-GFP proteins were investigated as follows: cells were grown on cover glasses in six-well plates (cat. no. 140675; Thermo Fisher Scientific, Jiangsu, China), transfected, fixed at 48 hpt using 4% paraformaldehyde, quenched by 50 mM NH_4Cl, mounted using 50% glycerol in distilled water, and examined using a 488 mm LED laser and a 20 × or 63 × oil immersion objective confocal microscope lens.

Polymerase chain reaction (PCR). At 48 hpt, PhoMT cells were collected and DNA was extracted from them using a DNeasy Blood & Tissue Kit (cat. no. 69506; Qiagen, Hilden, Germany). Forward and reverse primers (5'-AGTACATCAATGGGCGTGG-3' and 5'-CTGCTTCATGTGATCGGGG-3', respectively) were designed to confirm cloning with a 535 base pairs (bp) PCR product being generated for empty vectors and a 1,655 bp product for cloned vectors. The reaction was performed using GoTaq Hot Start Green Master Mix (cat. no. M5123; Promega, Madison, WI, USA) in a Veriti thermal cycler (Applied Biosystems, Foster City, CA, USA). The constituent volumes in the PCR reactions were: 2 µL of extracted DNA, 10 µL of

2 × GoTaq Hot Start Green Master Mix, 1 µL of each primer (10 µM), and 6 µL of nuclease free water for a total volume of 20 µL. The thermal cycling conditions were as follows: initial denaturation at 95°C for 2 minutes (min) followed by 30 cycles of denaturation at 95°C for 30 seconds (s), annealing at 58°C for 30 s, extension at 72°C for 1 min and 40 s, and a final extension step at 72°C for 7 min. The PCR products generated were sequenced directly using the same primer set.

The same DNA samples were examined by PCR using forward and reverse primers (5'-GCCAAAAGG GTCATCATCTC-3' and 5'-GGGGCCATCCACAGT CTTCT-3', respectively) capable of amplifying the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene (3, 10), which was detected as an internal control for the extracted DNA samples.

Production of an antibody to the SPPV major envelope protein. An antibody against the SPPV major envelope protein was produced as the primary antibody in the positive control reactions for the ELISA. A synthetic peptide comprising CAMITPTATDFHMNHSGGGV (Fig. 1) as 20 amino acids selected from the sequence of the SPPV envelope protein was used to immunise a rabbit, and the rabbit serum was then collected as the anti-envelope serum. Rabbit pre-serum (collected before immunisation) was used as the negative control serum. Production of the synthetic peptide as well as rabbit sera was performed by Eurofins Genomics (Tokyo, Japan).

Serum samples. Serum samples were collected from marine mammal species inhabiting the Port of Nagoya Public Aquarium in Japan as shown in Table 1. Samples were collected from each animal twice, in 2019 and 2020. A female spotted seal (PL1) had previously been infected with SPPV in 2010 (29), and a plasma sample that was collected from this animal at approximately 75 days after the onset of clinical signs (hereafter referred as PL1-2010 plasma) was used to validate the ELISA.

A

MWPFASIPVGANCRVVETLPPEVASLQQGNMGT LDCFLAIIESAKKFLYIASFCCNLQSTKEGLNVKD RLCALAKSGVNVTILVDHQSKEKDAAELREAGIN YYKVKVSNKEGLGSMLGSFWLSDAGHWYVGSA SLTGGSLATIKNLGVYSTNKHLAVDLMNRYNTF SSMVVDPKQPFTRFC**CAMITPTATDFHMNHSG GGV**FFSDSPERFLGFYRTLDEDLVLHRIDAAENSI DLSLLSMVPVVRSGSEVYYWPLIMDALLRAAIN RSVRVRIIVSQWRNADPLSVAAVRALDNFGVGHI DITARWFAIPGRDDASNNTKLLIVDDCFAHVTVA NLDGTHYKHHAFVSVNAENSDAVKQLAAVFER DWRSEYCTPINLK

B

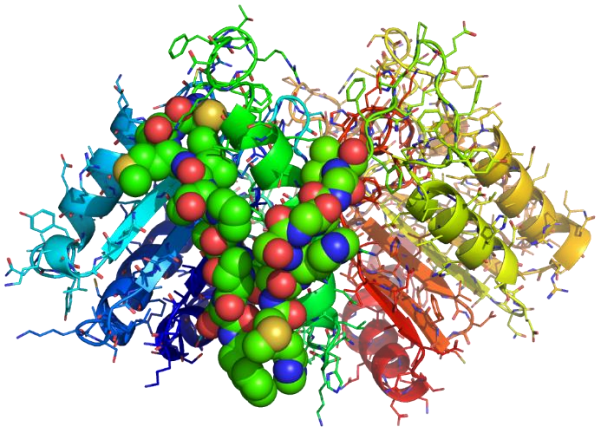

Fig. 1. Selection of the amino acids of the synthetic peptide used for the immunisation of a rabbit (for anti-envelope antibody production). A – Amino acid sequence of the seal parapoxvirus (SPPV) major envelope protein and the selected 20-long sequence (from the 183rd to 202nd amino acid) (bold and underlined); B – The predicted 3D structure of the SPPV major envelope protein was generated by SWISS-MODE (39) and the image was created by using PyMol (www.pymol.org). The coloured balls (four different colours) represent the atoms composing the selected amino acids

Table 1. Marine mammal species used in this study

Species	Animal identification	Sex	Age in years (as of October, 2020)
Spotted seal (*Phoca largha*)	PL1	F	11
	PL2	M	10
Beluga whale (*Delphinapterus leucas*)	DL1	M	42
	DL5	F	21
	DL6	F	25
	DL9	F	13
	DL11	M	8
	DL12	M	12
Pacific white-sided dolphin (*Lagenorhynchus obliquidens*)	LO2	M	20
	LO4	F	21
	LO5	F	11
Bottlenose dolphin (*Tursiops truncatus*)	TTC	F	20
	TTH	M	4
	TTL	F	23
	TTP	M	23
	TTQ	M	22
	TTR	F	3
	TTS	M	2
	TTT	M	25
	TTW	F	20
	TTX	F	21

PL – *Phoca largha*; DL – *Delphinapterus leucas*; LO – *Lagenorhynchus obliquidens*; TT – *Tursiops truncatus*; F – female; M – male

Table 2. Optimisation of the enzyme-linked immunosorbent assay (ELISA) conditions

Factor	Optimal condition	Other evaluated but not chosen conditions
Transfection reagent	Lipofectamine LTX	FuGENE HD (E2311, Promega, Madison, WI, USA), and Polyethylenimine HCL MAX (24765, Polysciences, Taiwan)
Cell lysis buffer	Using both RIPA and denaturing lysis buffers	1% NP40, RIPA buffer alone, and denaturing lysis buffer alone
Antigen preparation	Pellets of lysed cells	Supernatants of lysed cells, and whole cells lysed in the dish without pelleting
Time of collection of transfected cells (hours post transfection)	48	60
Antigen concentration	25 µg/well (100 µL/well at 0.25 µg/µL)	6.25, 12.5, 50, 100, and 200 µg/well
Blocking reagent	PBS containing 1% Block ACE	c-block-e, h-block-e, k-block-e, and b-block-e (BCL-BKSE-01, Beacle, Kyoto, Japan)
Primary sera/plasma dilution	1:50 (100 µL/well) diluted in PBS-T containing 0.4% Block ACE	1:100
HRP-conjugated protein A/G dilution	1:10,000 (100 µL/well) diluted in PBS-T containing 0.4% Block ACE	1:5,000

RIPA – radioimmunoprecipitation; NP40 – nonyl phenoxypolyethoxylethanol 40; PBS – phosphate-buffered saline; PBS-T – phosphate-buffered saline with Tween 20; HRP – horseradish peroxidase. The RIPA lysis buffer was 1% sodium deoxycholate, 1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.5 mM ethylenediaminetetraacetic acid. The denaturing lysis buffer was 2% sodium dodecyl sulphate, 770 mg dithiothreitol, and 4.5 mL of 500 mM Tris-HCl, pH 6.8, in 10 mL of PBS

Western blotting (WB). Assays were also performed to confirm expression of the proteins. Transfected PhoMT cells cultured in six-well plates were collected at 48 hpt using 25 µL/well of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 5 min. To examine marine mammals' sera for SPPV antibodies, the ELISA antigen was prepared using pellets obtained from cells that were transfected in 100 mm dishes (cat. no. 430167; Corning, Oneonta, NY, USA), lysed with radioimmunoprecipitation (RIPA) buffer (1% sodium deoxycholate, 1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.5 mM ethylenediaminetetraacetic acid (EDTA)), and pelleted by centrifugation. The pellets were suspended in 100 µL of SDS-PAGE sample buffer, boiled for 15 min with periodic vortex mixing, and electrophoresed in 10% SDS-PAGE. Separated proteins were blotted onto an Immobilon-P polyvinylidene difluoride membrane (Merck Millipore, Cork, Ireland). Blots were blocked with 5% skimmed milk, washed using Tris-buffered saline (TBS) (100 mM Tris-HCl, pH 8.0 and 30 mM NaCl) containing 0.1% Tween-20 (TBS-T) and incubated. This operation was with either rabbit sera or marine mammal sera/plasma as primary antibodies diluted at 1:4,000 in TBS-T containing 1% skim milk or with anti-GFP-Tag rabbit polyclonal antibody (cat. no. 29779; AnaSpec, Fremont, CA, USA) diluted at 1:1,000. Next, the blots were washed and incubated with horseradish peroxidase (HRP)-conjugated protein A/G (cat. no. 32490; Thermo Fisher Scientific, Rockford, IL, USA) diluted at 1:7,500 or peroxidase-linked ECL anti-rabbit IgG (cat. no. NA9340; GE Healthcare, Buckinghamshire, UK) diluted at 1:2,000. The chimeric protein A/G conjugate was used as a probe replacing the secondary antibody in the WB and ELISA assays because of its non-species-specific binding with the Fc region of antibodies, which commends its use in detection of antibodies from a wide variety of wild animals, including marine mammal species (28). The bound antibodies were detected using a Pierce ECL Plus Western blotting substrate (Thermo Fisher Scientific, Waltham, MA, USA) and visualised using a ChemiDoc XRS+ imaging system (Bio-Rad, Hercules, CA, USA).

Optimisation and establishment of ELISA. For establishment of the ELISA, several experimental conditions were compared to find the optimal components and parameters (Table 2). The conditions showing the largest difference in optical density (OD) values between Env-GFP- and GFP-coated wells incubated with rabbit anti-envelope serum or PL1-2010 plasma and the lowest OD values in both types of cell lysate-coated wells incubated with rabbit pre-serum were chosen. The assay as finally developed is as follows: PhoMT cells in 100 mm dishes were transfected, collected at 48 hpt using 500 µL of RIPA buffer containing protease inhibitor cocktail set I (cat. no. 165-26021; Wako), incubated on ice for 30 min, sonicated, and then incubated on ice again for 15 min. Cells were centrifuged at 13,000 x *g* for 5 min at 4°C, the supernatant was removed, 100 µL of the denaturing lysis buffer (1) was added to the cell pellet, and the buffer and pellet were boiled for 15 min with periodical vortex mixing. Protein concentrations were measured by a Lowry assay (21) using the *DC* Protein Assay (Bio-Rad). Each well pair of a 96-well plate (U96 MAXISORP, NUNC-Immuno plate, Thermo Fisher Scientific, Roskilde, Denmark) was coated in parallel with cell lysates diluted in 50 mM carbonate-bicarbonate buffer (pH 9.6): one of the wells was coated with Env-GFP cell lysates and the other with GFP cell lysates. The plates were incubated for 2 hours (h) at 37°C and then overnight at 4°C. They were then washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-T), blocked with 200 µL/well of PBS containing 1% Block ACE (UK-B40, DS Pharma Biomedica, Sapporo, Japan)

for 1 h at 37°C, washed, incubated with serum/plasma samples for 1 h at 37°C, washed again, incubated with HRP-conjugated protein A/G for 1 h at 37°C, and washed a final time. The reaction was colourised using 100 µL/well of KPL 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) peroxidase substrate system (cat. no. 5120-0032; SeraCare Life Sciences, Milford, MA, USA) and incubated for 30 min at 37°C. Finally, the reaction was stopped by adding 100 µL/well of 5% SDS, and the OD values were measured at 405 nm using a Multiscan FC plate reader (Thermo Fisher Scientific, Shanghai, China). The adjusted OD value of each sample was calculated by subtracting the OD value of GFP-coated wells from that of the corresponding Env-GFP-coated wells. Rabbit anti-envelope serum and pre-serum were included as positive and negative control reactions, respectively. Additionally, the PL1-2010 plasma was included as another positive control.

Results

Cloning and expression of the SPPV envelope protein. Cloning was confirmed by the PCR assay using DNA from transfected cells (Fig. 2A) and sequencing (data not shown). The two proteins in expression were confirmed by the detection of green fluorescence in transfected cells (Fig. 2B, upper panels). Expression of Env-GFP was in the cellular cytoplasm and was concentrated in localised sites, especially near the nucleus, whereas expression of GFP was in the whole cell (Fig. 2B, lower panels). The Env-GFP fusion protein (632 amino acids, with an expected size of 70.2 kDa) was detected in the Env-GFP-transfected cell lysates through WB using the rabbit anti-envelope serum, the anti-GFP antibody, or the PL1-2010 plasma (Fig. 2C). Additionally, GFP (239 amino acids, with an expected size of 26.9 kDa) was detected in GFP-transfected cell lysates using anti-GFP antibody (Fig. 2C). These results confirmed the expression of GFP and Env-GFP in the respective transfected cells.

Development of ELISA. In the ELISA plates, the Env-GFP-coated wells incubated with the rabbit anti-envelope serum showed a colour reaction (Fig. 3A), whereas the GFP- and mock-coated wells did not, with higher OD values being observed in the Env-GFP-coated wells than in the GFP-coated wells (Fig. 3B). By comparing different concentrations of the cell lysates, it was found that 25 µg/well was the optimal concentration (Fig. 3A and B). These results indicated the specificity of the prepared rabbit sera. Different combinations of cell lysate concentrations and dilutions of PL1-2010 plasma were compared (Fig. 3C). As observed using rabbit anti-envelope serum, 25 µg/well cell lysate concentration using 1:50 PL1-2010 plasma dilution showed the greatest difference between the Env-GFP- and GFP-coated wells (Fig. 3C). However, the difference using PL1-2010 plasma (Fig. 3C) was much lower than that observed using rabbit anti-envelope serum (Fig. 3B). To examine the hypothesis that plasma might increase non-specific reactions compared to serum, the OD values obtained using serum and plasma samples from the same seals were compared. The OD values in both types of samples showed non-remarkable differences (data not shown), indicating that using plasma samples for ELISA is straightforward.

In repeated experiments (four independent iterations) using 25 µg/well of cell lysates and 1:50 dilution of the PL1-2010 plasma, the adjusted OD values were in the range of $0.1 \leq OD < 0.15$. The lower limit, *i.e.* 0.1, was used as the cut-off value. Therefore, the cut-off value of the assay was set to an adjusted $OD \geq 0.1$ (an adjusted $OD \geq 0.1$ indicated that the animal is considered positive). The lower limit (low conservative cut-off value) was used due to the fact that this would eventually increase the sensitivity of the assay.

Serosurvey in marine mammals inhabiting the Port of Nagoya Public Aquarium. This established ELISA was used to examine serum samples collected from marine mammals inhabiting an aquarium. The OD values for Env-GFP-coated wells were the same as or very similar to those of the GFP-coated wells for the examined samples. None of the examined serum samples passed the pre-determined cut-off value, and these samples were therefore judged to be negative sera. Each serum sample was assayed at least twice.

Confirming ELISA results by WB. To confirm the ELISA results, all seal serum samples were examined again using the WB assay, as were selected serum samples from cetacean species. The positive controls, *i.e.* rabbit anti-envelope serum and PL1-2010 plasma, showed bands as expected using Env-GFP cell lysates but not when using GFP cell lysates (Fig. 4A and B). These bands were not detected with the pre-serum (Fig. 4A and B). The examined seals' sera (collected either in 2019 or 2020) did not show evidence of positive reactions (Fig. 4A), and the examined cetaceans' sera were also negative (Fig. 4B). These results confirmed those obtained using the ELISA established with this research.

Discussion

In this study, a new ELISA was developed to detect anti-SPPV antibodies. In this ELISA, each serum sample is assayed using antigen-containing and antigen-free cell lysates, and the OD value of each sample is adjusted by subtracting the OD of antigen-free cell lysates from that of antigen-containing lysates. This strategy greatly enhances the specificity of the assay due to background (non-specific) absorbance removal. Additionally, antigen preparation is relatively simple. Furthermore, this methodology avoids the use of live viruses and can be used in laboratories with low biosecurity levels, decreasing the risks of contamination as well as public health concerns.

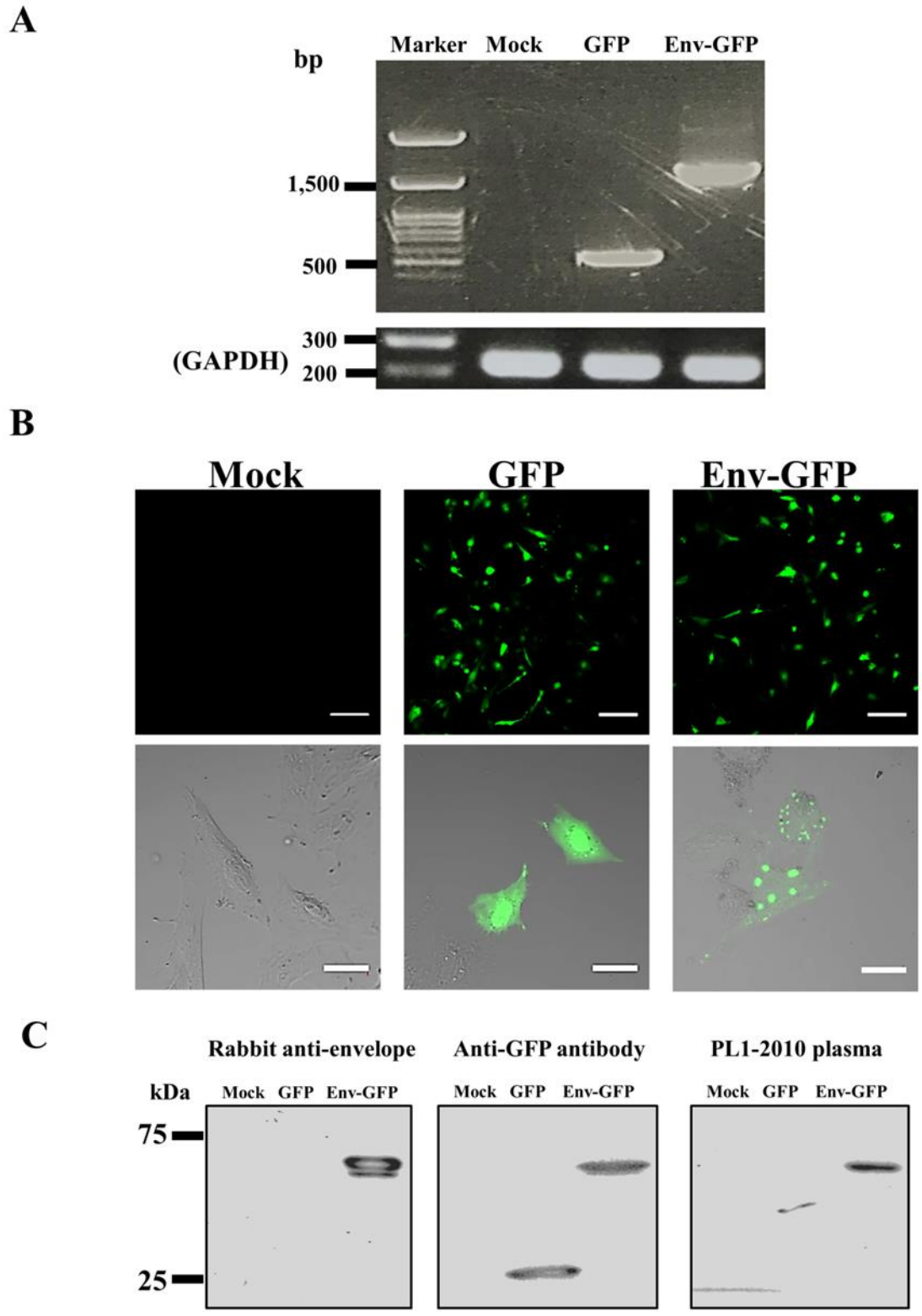

Fig. 2. Confirmation of seal parapoxvirus envelope gene cloning and expression of the green fluorescence protein (GFP) and fusion protein (Env-GFP) in transfected cells. A – Results of the PCR using DNA extracted from transfected cells (mock, empty, and cloned vectors) at 48 hours post transfection (hpt); B – Upper panels: confocal microscopic photos of transfected cells (mock, empty, and cloned vectors) at 48 hpt showing the expression of GFP and Env-GFP (low magnification, scale bar 200 μm). Lower panels: the distribution of GFP and Env-GFP inside transfected cells (high magnification, scale bar 30 μm); C – 48 hpt results of Western blotting using the transfected cell lysates (mock, empty, and cloned vectors) and the rabbit anti-envelope serum, anti-GFP antibody, or PL1-2010 plasma, illustrating the expression of GFP and Env-GFP in transfected cells by the detection of specific bands of both proteins

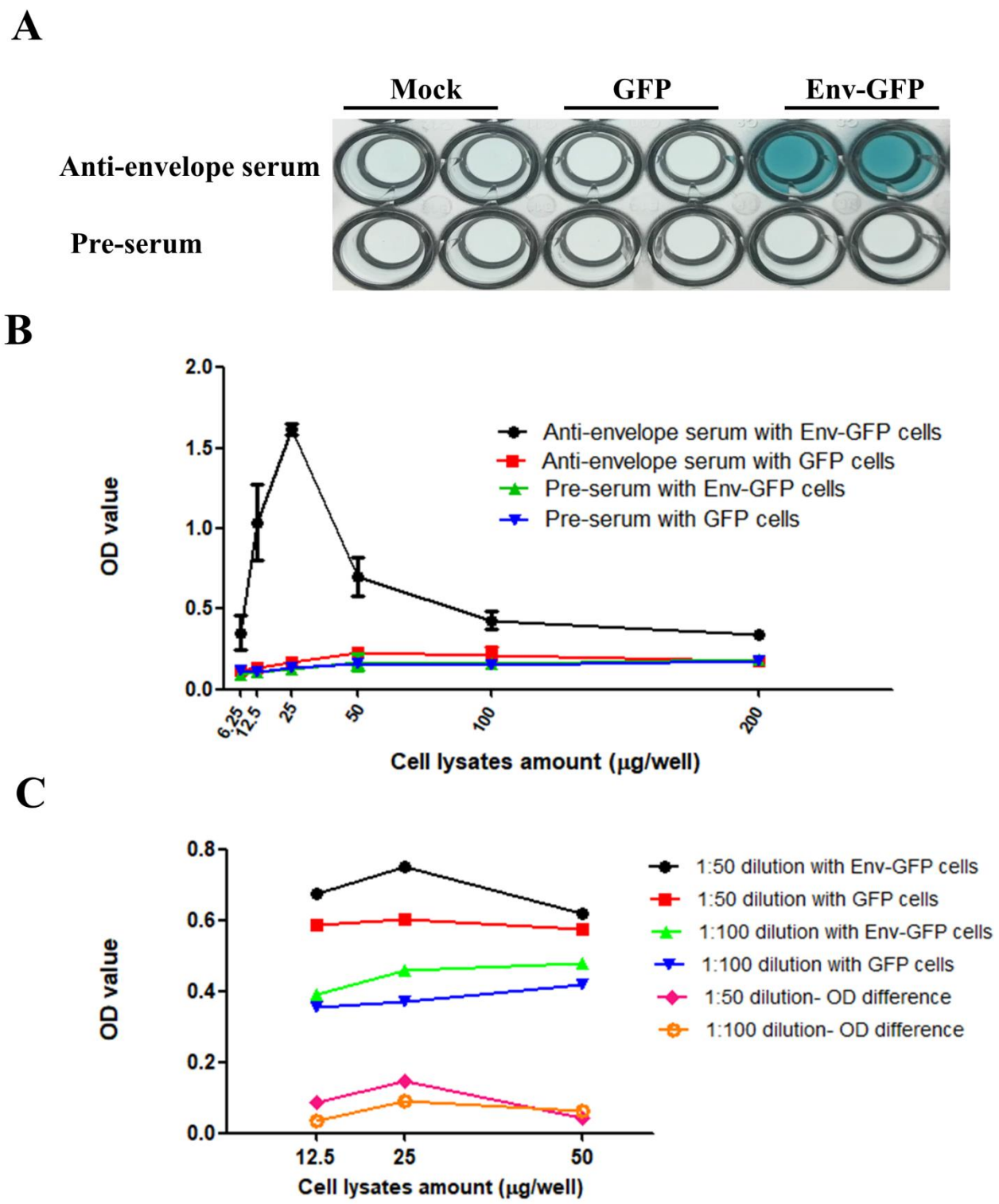

Fig. 3. Optimisation of the ELISA cell lysates and primary antibody concentrations. A – Colour reactions in the ELISA plate wells coated with mock, green fluorescence protein (GFP), or fusion protein (Env-GFP) cell lysates (25 µg/well) and incubated with either rabbit anti-envelope serum or pre-serum (1:50 dilutions); B – Optical density (OD) values detected using either rabbit anti-envelope serum or pre-serum at 1:50 dilution in either Env-GFP lysate– or GFP cell lysate–coated wells at different concentrations. Data are shown as means ± SD for two separate experiments; C – OD values detected using PL1-2010 plasma at either 1:50 or 1:100 dilution with different concentrations of Env-GFP lysate– or GFP cell lysate–coated wells, in addition to the OD difference in each case (subtracting the GFP OD value from the Env-GFP OD value)

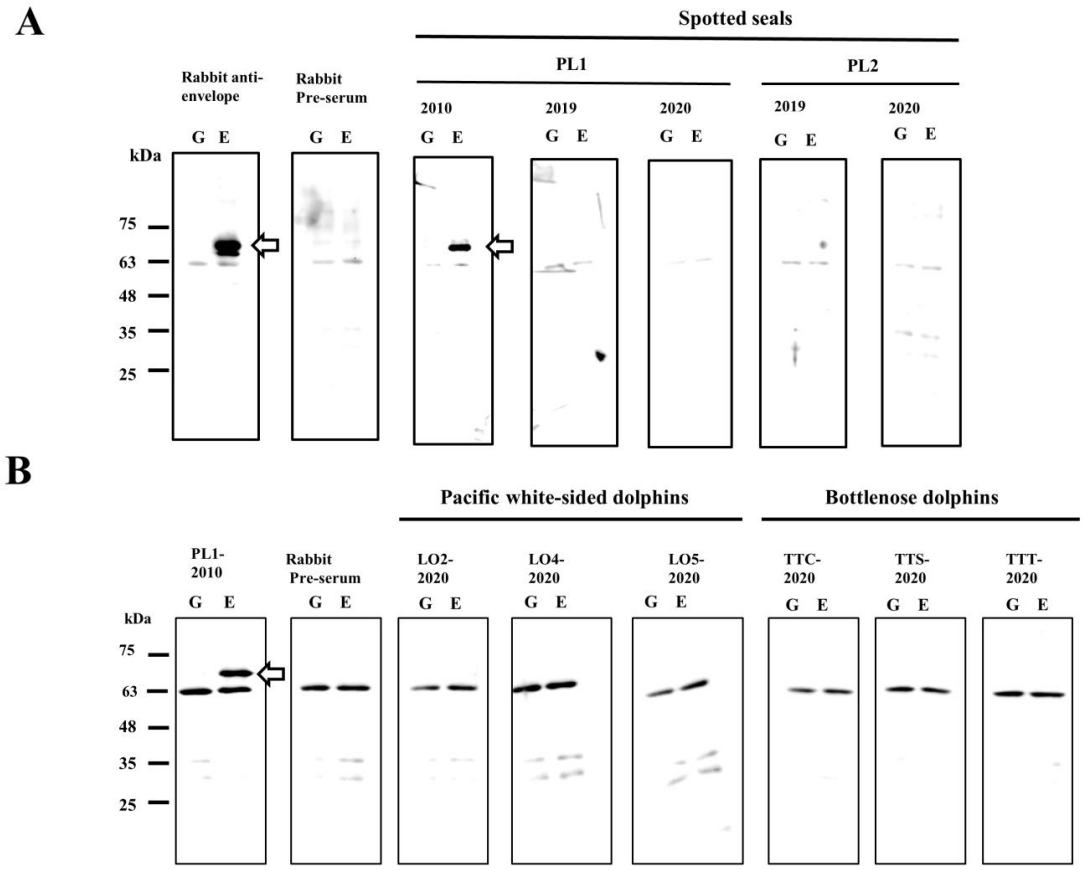

Fig. 4. Western blotting assay to examine the reactivity of selected serum samples. Transfected cell lysates, green fluorescence protein (GFP) (G lane) and fusion protein (Env-GFP) (E lane), were loaded as the antigens, horseradish peroxidase-conjugated protein A/G was used in place of the secondary antibody, and sera from rabbit (anti-envelope and/or pre-serum) were used as primary antibodies with A – Primary antibodies from all spotted seals or B – Primary antibodies from selected cetaceans. Arrows – Specific bands in the E lanes at approximately 70 kDa indicating positive reactions only with the rabbit anti-envelope serum and PL1-2010 plasma. A protein ladder showing different sizes is provided on the left

Expression of Env-GFP and GFP was confirmed in transfected cells by confocal microscopy. Inside transfected cells, Env-GFP was distributed differently than the native GFP. In a similar experiment using the F13L protein (p37K) of vaccinia virus (the homologue protein of the PPV major envelope protein), a cloned vector expressing the F13L-GFP fusion protein was transfected into cells; the fusion protein was found to accumulate in the Golgi areas and post-Golgi vesicles in the cytoplasm (13). This association with Golgi-derived membranes is related to tight membrane trafficking signals that are also found in the PPV major envelope protein (40); therefore, it is assumed that homologous proteins, namely the F13L protein of the of vaccinia virus and the major envelope protein of SPPV, behaved similarly inside transfected cells.

The difference in results between rabbit anti-envelope serum and PL1-2010 plasma may be explained by differences in the biochemical composition of both materials. Marine mammals' blood and serum have a high lipid content (25), which together with other particulate matter present in marine mammal serum or plasma may induce non-specific reactions in some immunological diagnostic assays (4). Another contributing factor may be a higher anti-SPPV envelope antibody titre in rabbit serum: WB results showed stronger bands for rabbit anti-envelope serum than for PL1-2010 plasma (Figs 2C and 4A).

Similarly to our study, previous researchers using an analogous procedure for the establishment of an ELISA for the serological diagnosis of severe fever with thrombocytopenia syndrome successfully used a cut-off value of OD > 0.1 after OD adjustment (18, 22). However, a 0.1 OD difference between antigen-containing and antigen-free cell lysates is small and such a result may be affected by other factors that may occur during the assay; therefore, efforts to improve the ELISA methodology should be undertaken.

None of the marine mammals currently inhabiting the Port of Nagoya Public Aquarium showed positive reactions in either ELISA or WB assays, which implies a lack of exposure to new SPPV infections since the last reported infection (29). Additionally, in respect of seal PL1 (from which the positive plasma sample had been collected approximately 10 years previously), its current serum (as of 2020) was not reactive; this result indicated that production of anti-SPPV antibodies after infection wanes over time. Cetacean species were included in the current study – although they are mostly infected with an *Orthopoxvirus*-related *Cetaceanpoxvirus*, not PPV (6) – because a previous study in Japan found that two of these species, bottlenose and Pacific white-sided

dolphins, were infected by a PPV (19). Nevertheless, the genetic relationship between PPVs of pinnipeds and cetaceans is unclear and warrants further studies.

Sera from different locations should be examined to draw firm conclusions about the seroprevalence of SPPV infection in marine mammals in Japan. Additionally, known positive and negative sera for SPPV antibodies from different species are lacking or very limited, and consequently sera from animals having confirmed or suspected clinical poxvirus infections are acutely needed. These samples will be highly valuable in validating and improving the ELISA systems developed for the serological diagnosis of SPPV infections.

ELISA provides the advantage of high throughput methodology; therefore, if a large number of samples are to be processed, we advocate using this ELISA for the initial screening, followed by WB as a confirmatory test for ELISA-positive samples. The ELISA developed here could be helpful in detecting anti-SPPV antibodies in serum samples of susceptible animals.

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