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Molecular analysis of parapoxvirus from a spotted seal *Phoca largha* in Japan

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ABSTRACT: A spotted seal *Phoca largha* with nodular and scab lesions on the whole body was brought to an aquarium in Nagoya, Japan. We extracted DNA from the lesions and used the polymerase chain reaction (PCR) method for detecting orthopoxvirus and parapoxvirus DNA. Parapoxvirus but not orthopoxvirus DNA was detected. The partial nucleotide sequence of the envelope gene was determined from the PCR product, and the sequence was seen to be closely related to 2 parapoxvirus strains from spotted seals in Alaska, showing 100% identity at the amino acid level, with one nucleotide substitution. Virus-neutralizing (VN) antibody against canine distemper virus (CDV) was not detected in the serum, indicating that this individual was not infected with CDV or phocine distemper virus (PDV), which both have a high mortality rate for marine mammals. These results suggest that the lesions were caused by infection with pinniped parapoxvirus, and that the viruses spread and are maintained within the habitat range or populations of spotted seals from the Bering Sea to the Japan Sea. This is the first report of molecular analysis of parapoxvirus in marine mammals in Japan.

KEY WORDS: Parapoxvirus · Spotted seal · Molecular analysis · Marine mammals

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INTRODUCTION

The genus *Parapoxvirus* belongs to the family *Poxviridae*; it is a large, double-stranded and enveloped DNA virus. The genus *Parapoxvirus* includes orf virus, bovine papular stomatitis virus, pseudocowpox virus, and parapoxvirus of red deer in New Zealand (Damon 2007). Parapoxvirus infections are widespread in both domestic and wild ruminants worldwide. Pox-like dermal diseases have been observed in a wide variety of marine mammals, including bottlenose dolphins *Tursiops truncatus*, Pacific white-sided dolphins *Lagenorhynchus obliquidens* (Kohyama et al. 2010), Weddell seals *Leptonychotes weddellii* (Tryland et al. 2005), grey seals *Halichoerus grypus* (Nettleton et al. 1995), harbor seals *Phoca vitulina* (Müller et al. 2003), and California sea lions *Zalophus californianus* (Nollens et al. 2006a). They involve nodular lesions (from 0.5 to 3.0 cm diam.) on the skin of the head, neck, and flippers, and on the mucosal surface of the mouth and nasal passages (Kennedy-Stoskopf 2001, Nollens et al. 2006b), similar to the lesions seen on terrestrial mammals. Although some lesions persist for months, they are usually self-limiting and regress after about 4 wk (Gulland et al. 2001). Some viruses of pinnipeds were tentatively classified as new members of the genus *Parapoxvirus*, based on the partial sequence of the viral envelope gene and on phylogenetic analysis (Kennedy-Stoskopf 2001, Becher et al. 2002, Bracht et al. 2006). In some aquaria in Japan, poxviruses have been detected, using electron microscopy, in South American sea lions *Otaria byronia* (Okada & Fujimoto 1984), bottlenose dolphins, and Pacific

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white-sided dolphins (Kohyama et al. 2010). However, the genetic relationship between parapoxviruses in both pinnipeds and cetaceans in Japan and those in other countries remains unclear. In this study, we performed sequence analysis and molecular characterization of parapoxvirus isolated from nodular lesions from a spotted seal *Phoca largha* in Japan.

MATERIALS AND METHODS

Sample collection

In March 2010, a 1-year-old, 26.5 kg female spotted seal *Phoca largha* showing nodular and scab lesions on the back and flippers was transported to an aquarium in Nagoya, Japan (Fig. 1). Three nodules on the back and flippers were collected by scratching with curettes and swabbing with a BD BBL Culture Swab kit (BD Japan). Serum was also collected for the virus-neutralizing (VN) test.

VN test

Large numbers of seals all over the world have reportedly died from infection with phocine distemper virus (PDV) or canine distemper virus (CDV; Osterhaus et al. 1990b, Have et al. 1991, Barrett et al. 1992), and thus PDV or CDV infection is considered a high-mortality threat to marine mammals. Both viruses are cross-reactive and closely related to each other. Thus the VN test against CDV was carried out using the serum sample and CDV KDK-1 strain (Mochizuki et al. 1999) as described in Nakano et al. (2009).

DNA extraction and polymerase chain reaction

DNA was extracted from the collected nodules and swabs from 3 different lesions using a DNeasy tissue kit (QIAGEN) according to the manufacturer's instructions; it was then used for the polymerase chain reaction (PCR). The PCR primers, North American consensus sequence primers 1 and 2 (NACP1/NACP2) and Eurasian-African consensus sequence primers 1 and 2 (EACP1/EACP2) were used for the detection of the orthopoxvirus hemagglutinin (HA) gene (Ropp et al. 1995). The Pan-parapoxvirus primers 1 and 4 (PPP-1/PPP-4; Inoshima et al. 2000), as well as orf virus B2L F1 and R2 (OVB2LF1/OVB2LR2; Hosamani et al. 2006), Envelope F/Envelope R, and virus interferon resistance F and R (VIR F/VIR R; Guo et al. 2004) primer sets were used for the detection of the partial or full-length major envelope antigen gene, or virus interferon resistance (VIR) gene of parapoxvirus, respectively (Table 1).

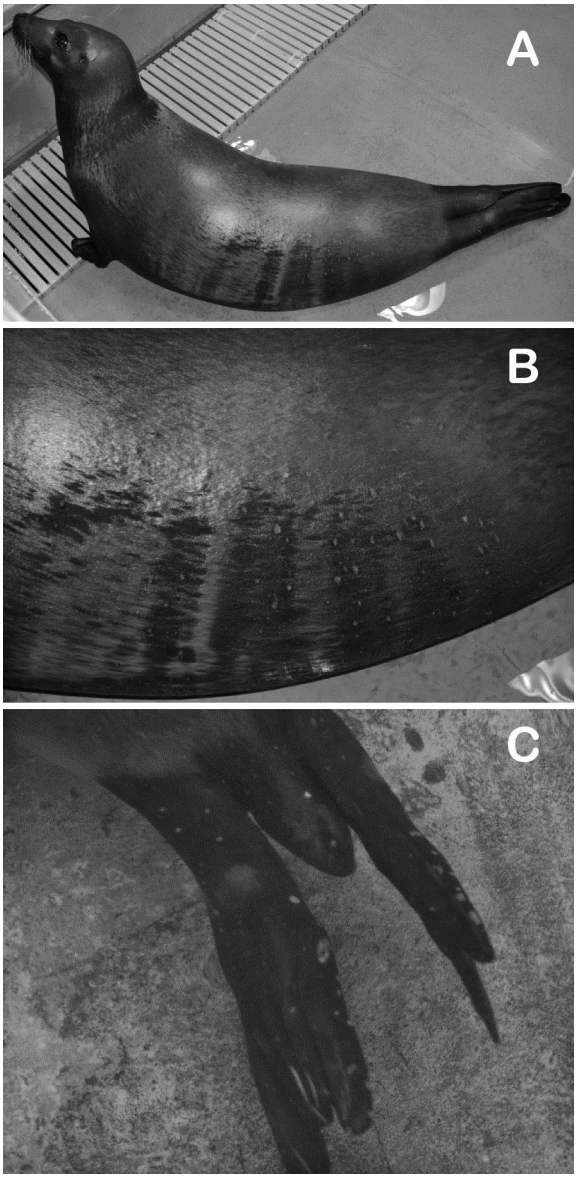

Fig. 1. *Phoca largha.* (A) Nodular lesions on body surface, (B) expanded image of body surface lesions, and (C) lesions on flippers

Sequence and phylogenetic analyses

A partial nucleotide sequence of the envelope gene (554 bp) was determined from the amplified 594 bp PCR product by direct sequencing using an ABI 310

Table 1. *Phoca largha*. Primers used for polymerase chain reaction. NACP: North American consensus sequence primer; EACP: Eurasian-African consensus sequence primer; PPP: pan-parapoxvirus primer; OVB2L: orf virus B2L; VIR: virus interferon resistance

Primers	Target	Sequence (5′ to 3′)	Source
Orthopoxvirus			
NACP1	Hemaggulutinin	ACG ATG TCG TAT ACT TTG AT	Ropp et al. (1995)
NACP2		GAA ACA ACT CCA AAT ATC TC	
EACP1	Hemaggulutinin	ATG ACA CGA TTG CCA ATA C	Ropp et al. (1995)
EACP2		CTA GAC TTT GTT TTC TG	
Parapoxvirus			
PPP-1	Envelope	GTC GTC CAC GAT GAG CAG CT	Inoshima et al. (2000)
PPP-4		TAC GTG GGA AGC GCC TCG CT	
OVB2LF1	Envelope	TCCCTGAAGCCCTATTATTTTTGTG	Hosamani et al. (2006)
OVB2LR2		GCTTGCGGGCGTTCGGACCTTC	
Envelope F	Envelope	TTAATTTATTGGCTTGCAGAACTCCGAGCGC	Guo et al. (2004)
Envelope R		ATGTGGCCGTTCTCCTCCATC	
VIR F	Virus interferon resistance	TTAGAAGCTGATGCCGCAG	Guo et al. (2004)
VIR R		ACAATGGCCTGCGAGTG	

DNA sequencer (Applied Biosystems) with a BigDye Terminator ver. 3.1 Cycle Sequencing kit (Applied Biosystems). The sequence was obtained from both strands for verification. Phylogenetic analysis was performed using the neighbor-joining method, and a tree was constructed using MEGA5 software (Tamura et al. 2011) with bootstrap values calculated from 1000 replicates.

RESULTS

VN test

VN antibody against CDV was not detected in the serum (data not shown), indicating that this individual was not infected with CDV or PDV.

PCR

No HA gene-specific fragment was amplified by the NACP1/NACP2 primers or the EACP1/EACP2 primers (data not shown), indicating that the nodules and scab lesions were not caused by an orthopoxvirus. In addition, specific DNA was not amplified either by the Envelope F/Envelope R primers for the full-length envelope gene or by the VIR F/VIR R primers for the VIR gene of parapoxvirus (data not shown). Only the primers for the partial envelope gene of parapoxvirus, PPP-1 and PPP-4, amplified DNA from 2 of the 3 nodule samples and one of the 3 swab samples (data not shown).

Sequence and phylogenetic analyses

The sequence of the PCR product from the PPP-1 and PPP-4 primers was then determined. The determined sequence was seen to be closely related to 2 parapoxvirus strains from spotted seals *Phoca largha* (AY780676 and DQ073805), showing 99.8% and 100% homology at the nucleotide and amino acid levels, respectively (Figs. 2 & 3, Table 2). Thus both the sequence and phylogenetic analyses revealed that the viral DNA in the lesions originated from pinniped parapoxvirus, and that infection with this virus was likely responsible for the lesions. We designated the virus as pinniped parapoxvirus strain Nagoya. The sequence has been deposited in DDBJ/EMBL/GenBank under accession no. AB571081.

DISCUSSION

Of the various sets of primers for parapoxvirus, only the pair of primers for the partial envelope gene (PPP-1 and PPP-4) proved capable of amplifying an appropriately sized DNA fragment from the lesions. The primers for the full-length envelope and VIR genes proved incapable of amplifying any fragments. These primers were designed for the detection of orf virus, a member of the genus *Parapoxvirus* (Guo et al. 2004, Hosamani et al. 2006), which is the prototype of parapoxvirus and maintained mainly in sheep and goats. In contrast, the PPP-1/PPP-4 primer pair was designed for the detection of all parapoxvirus members (Inoshima et

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Spotted seal Nagoya (AB571081)   1:TGGSLATIKNLGVYSTNKHLAVDLMNRYNTFSSMVVDPKQPFTRFCCAMITPTATDFHMNHSGGGVFFSDSPERFLGFYRTLDEDLVLHRIDAAENSIDL 100
Spotted seal        (AY780676)   1:.................................................................................................. 100
Spotted seal        (DQ073805)   1:.................................................................................................. 100
Harbor seal         (DQ273136)   1:.................................................................................................. 100
Harbor seal         (DQ273135)   1:..................................................................................................K..... 100
Harbor seal         (DQ219804)   1:..................................................................................................K..... 100
Harbor seal         (AY952937)   1:..................................................................................................K..... 100
Harbor seal         (AF414182)   1:.....................................S............................................................K..... 100
Grey seal           (DQ273134)   1:.................................................................................................. 100
Steller sea lion    (AY952946)   1:.....................................A.....................H.............................K..... 100
Steller sea lion    (AY952940)   1:.....................................I.....................H.............................K..... 100
Steller sea lion    (AY952943)   1:.....................................I.....................H.............................K..... 100
California sea lion (DQ163058)   1:.................................................................................................. 100
California sea lion (DQ273138)   1:.....................................I.....................H.............................K..... 100
California sea lion (DQ273137)   1:..................R..F.........G..........A.L...I.....................................S....... 100

Spotted seal Nagoya (AB571081) 101:SLLSMLPVVRSGSEVHYWPLVMDALLRAAINRSVRVRIIISQWRNADPLSVAAVRALDNFGVGHVDVTARWFAVPGRDDASNNT 184
Spotted seal        (AY780676) 101:.................................................................................... 184
Spotted seal        (DQ073805) 101:.................................................................................... 184
Harbor seal         (DQ273136) 101:...................I.....................................................A... 184
Harbor seal         (DQ273135) 101:.....V......G..Y....I.................V.........................I......I.......... 184
Harbor seal         (DQ219804) 101:.....V......G..Y....I.................V.........................I......I.......... 184
Harbor seal         (AY952937) 101:.....V......G..Y....I.................V.........................I......I.......... 184
Harbor seal         (AF414182) 101:.....V......G..Y....I.................V.........................I......I.......... 184
Grey seal           (DQ273134) 101:.....V.........Y....I.................V.........................I.I......I.......... 184
Steller sea lion    (AY952946) 101:.....................I.....................................I.I......I.......... 184
Steller sea lion    (AY952940) 101:.....V......G..Y....I.........VD.....................................I.I......I.......... 184
Steller sea lion    (AY952943) 101:.....V......G..Y....I.........VD.....................................I.I......I.......... 184
California sea lion (DQ163058) 101:.....V.........Y....IT.................V.........................I.I......I.......... 184
California sea lion (DQ273138) 101:.....V......G..Y....I.........VD.....................................I.I......I.......... 184
California sea lion (DQ273137) 101:.....V.......A......I.........D.........................D.....I.I.................. 184
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Fig. 2. *Phoca largha.* Comparison of deduced amino acid sequence of the viral envelope gene of the Nagoya strain with corresponding sequences of the parapoxviruses from various seal and sea lion species. Dots = consensus amino acids. Amino acid residues that are different from the Nagoya strain are shown

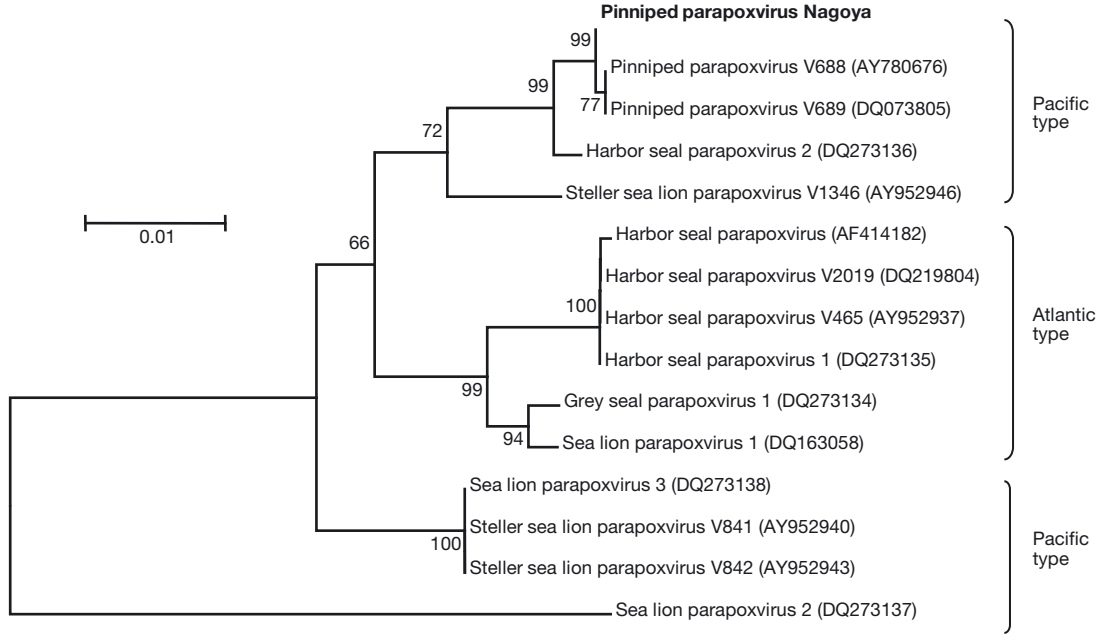

Fig. 3. *Phoca largha.* Phylogenetic relationships based on nucleotide sequence of the viral envelope gene of the Nagoya strain with corresponding sequences of the parapoxviruses. Tree was constructed by neighbor-joining method using MEGA5 software, with bootstrap values calculated from 1000 replicates. All bootstrap values are displayed above the tree branches. The Nagoya strain is shown in **bold**

al. 2000). Although the combination of PCR analysis and serological tests with virus isolation and/or electron microscopy would be a more accurate and reliable method for diagnosis, our results suggest that the PPP-1/PPP-4 primer pair is extremely useful for the PCR diagnosis of parapoxvirus infection in not only domestic and wild ruminants but also in marine mammals. Importantly, PCR analysis can be rapidly and easily performed for a wide variety of animal species in zoos and aquariums.

Table 2. *Phoca largha*. Sequence identity with Nagoya strain at nucleotide and amino acid levels

Host	Habitat area	Accession no.	Sequence identity (%) with Nagoya strain (AB571081) Nucleotide levels (554 bp)	Amino acid levels (184 aa)
Spotted seal *Phoca largha*	Pacific Ocean	AY780676	99.8	100.0
		DQ073805	99.8	100.0
Harbor seal *P. vitulina richardii*	Pacific Ocean	DQ273136	98.7	98.9
Harbor seal *P. vitulina concolor*	Atlantic Ocean	DQ273135	92.7	95.6
		DQ219804	92.7	95.6
		AY952937	92.7	95.6
Harbor seal *P. vitulina vitulina*	Atlantic Ocean	AF414182	92.5	95.1
Grey seal *Halichoerus grypus*		DQ273134	93.6	96.1
Steller sea lion *Eumetopias jubatus*	Pacific Ocean	AY952946	95.6	96.1
		AY952940	90.7	93.4
		AY952943	90.7	93.4
California sea lion *Zalophus californianus*	Pacific Ocean	DQ163058	93.5	95.6
		DQ273138	90.7	93.4
		DQ273137	84.4	92.3

Two parapoxviruses from spotted seals *Phoca largha* in Alaska (AY780676 and DQ073805) showed 100% homology with this new Nagoya strain at the amino acid level. The spotted seal habitat extends from the Bering Sea to the Japan Sea, and genetically closely related parapoxviruses may therefore spread or be maintained in this habitat range or population of spotted seals. Phylogenetic analysis demonstrated that 3 parapoxviruses from spotted seals were part of the same cluster, with one parapoxvirus (DQ273136) from the harbor seal and one (AY952946) from the Steller sea lion. The parapoxviruses in this cluster are from pinnipeds, whose habitat is the Pacific ocean. Other clusters were mostly constructed by viruses detected from pinnipeds from either the Pacific or the Atlantic Oceans. Therefore, it is conceivable that although sea lion parapoxvirus 1 (DQ163058) was categorized into the same cluster as grey seal parapoxvirus 1 (DQ273134), parapoxviruses in pinnipeds may be genetically classified into 2 types related by their habitat, i.e. Pacific or Atlantic types. As mentioned by Nollens et al. (2006a, 2006b), Atlantic-type parapoxvirus may be introduced into Pacific pinnipeds, and vice versa, via indirect contact with infected Arctic ice seals, including ringed seals *P. hispida* and bearded seals *Erignathus barbatus*, which are found in both the Atlantic and Pacific oceans. Further analysis is needed to clarify this issue.

Some parapoxviruses from seals and sea lions are zoonotic, giving rise to cutaneous infections on the fingers and hands of people who handle infected animals (Hicks & Worthy 1987, Clark et al. 2005), and necessitating the use of gloves in during such handling (Kennedy-Stoskopf 2001).

To date, veterinarians and staff at aquariums have called the pox-like disease in pinnipeds 'sealpox'. However, the disease, as in the present case, is caused by parapoxvirus, not orthopoxvirus. There is only one report of skin lesions caused by an orthopoxvirus infection in pinnipeds, although no sequence characterization has been reported (Osterhaus et al. 1990a). A poxvirus from a Steller sea lion was found to be distinct from the genus *Parapoxvirus*; phylogenetic analysis found it to be more closely related to, but clearly distinct from, *Orthopoxvirus* (Burek et al. 2005, Bracht et al. 2006). Therefore, although the causative virus, parapoxvirus or orthopoxvirus, cannot be identified until virological tests are carried out, 'sealpox' would be most likely caused by parapoxvirus.

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