Discovery of an orthoreovirus in the aborted fetus of a Steller sea lion (Eumetopias jubatus)

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An aborted mid-gestational male Steller sea lion fetus with an attached placenta was recovered on the floor of an open floating capture trap located off Norris Rock near Denman Island, British Columbia. Viral culture of the placenta demonstrated cytopathic effect. Although no specific signal was obtained in microarray experiments using RNA obtained from viral culture, elution and sequence analysis revealed the presence of a reovirus. Complete genome pyrosequencing led to the identification of an orthoreovirus that we have tentatively named Steller sea lion reovirus (SSRV). Phylogenetic analysis revealed similarities between SSRV and orthoreoviruses of birds, bats and other mammals that suggests potential for interspecies transmission.

INTRODUCTION

Steller sea lions (Eumetopias jubatus) are large otariid pinnipeds found in the northern Pacific. The genetically distinct eastern North American population of Steller sea lions comprises animals born on rookeries from central California to northern Southeast Alaska and is listed as a threatened species (Pitcher et al., 2007).

Systematic studies of neonatal mortality of pinnipeds have focused on Antarctic fur seals (Arctocephalus gazella), northern fur seals (Callorhinus ursinus) and harbour seals (Phoca vitulina) (Baker & Doidge, 1984; Keyes, 1965; Steiger et al., 1989). Reovirus-like particles were observed in one sample from Smith Island in Puget Sound, Washington, USA, from a harbour seal pup that was emaciated even though its stomach contained fresh milk (Steiger et al., 1989). These particles were 80 nm in diameter and appeared identical to others observed in tissues of California sea lions (Zalophus californianus), Steller sea lions and northern fur seals in the northern Pacific (Steiger et al., 1989).

The virus family Reoviridae includes 15 recognized virus genera (Mertens et al., 2005), including the recently described Mimoreovirus, Cardoreovirus and Dinovernavirus, the genomes of which comprise nine to twelve linear segments of dsRNA (Attoui et al., 2005, 2006; Day, 2009; Mohd Jaafar et al., 2008). Reoviruses have been found in many organisms, including vertebrates, arthropods, protists, fungi and plants. Those that infect aquatic organisms include members of the genera Aquareovirus and Mimoreovirus, which have 11 segments, and Cardoreovirus, which have 12 segments. The International Committee on Taxonomy of Viruses (ICTV) recognizes five species in the genus Orthoreovirus (Chappell et al., 2005). One species (Mammalian orthoreovirus) includes...
all the non-fusogenic mammalian orthoreoviruses; all other species induce syncytium formation. A second species comprises the avian reoviruses (ARV), including those from chicken, Muscovy duck, turkey and goose. The third species is represented by Nelson Bay virus (NBV), an atypical syncytium-inducing mammalian reovirus, isolated from a grey-headed flying fox (*Pteropus poliocephalus*). Recently, viruses related to NBV were obtained from bats (Pulau virus; Pritchard *et al.* 2006) and humans [Melaka virus (Chua *et al.*, 2007); Kampar virus (Chua *et al.*, 2008); and HK23629/07 (Cheng *et al.*, 2009)]. Phylogenetic analyses of the few available genome segments demonstrated that, although NBV-related viruses were more closely related to ARV isolates than to other mammalian or reptilian orthoreoviruses, they represent a distinct species. The main arguments for this were: (i) the extent of sequence divergence in the σ-class core and major outer capsid protein; (ii) the absence of evidence for reassortment between the ARV and NBV isolates; and (iii) the classical notion that viruses in each orthoreovirus species correspond to a specific or related host type. The two remaining species of the genus are baboon orthoreovirus and reptilian reoviruses (Chappell *et al.*, 2005).

Here we report the isolation and characterization of an orthoreovirus recovered from the aborted fetus and associated placenta of a Steller sea lion at Norris Rock near Denman Island, British Columbia, Canada. Surprisingly, characterization of the full genome of this virus identified it as being a member of a clade that includes ARV and NBV.

**RESULTS**

**Pathological studies**

The fetus was a mid-gestation male, in good body and post-mortem condition (code 2; Geraci & Loundsbury, 1993) with a total length of 50 cm, axillary girth of 29 cm with a mid-ster nal blubber thickness of 0.4 cm. A moderate amount of meconium was interspersed within the chorio-allantoic villi of the placenta and, microscopically, there was a necrosuppurative placentitis. There was extensive haemorrhage with variable oedema throughout the fetal mediastinum, lung, hypodermis, heart and nasal turbinates with mild, non-suppurative inflammation of the heart, adrenal gland and lungs. The haemorrhage was attributed to agonal or terminal trauma, presumably during or shortly after parturition. Moderate haemosiderosis with florid extramedul lary haematopoiesis was noted throughout the liver. There were no apparent lesions within sections of the oesophagus, larynx, trachea, peripheral nerve, rib, peripheral vasculature, large blood vessel, urinary bladder, tongue, umbilicus, salivary gland, urethra, small intestine, colon, bone, bone marrow, trachea, pancreas, peripheral ganglia, lymph node, thymus, testes, epididymis, kidney, adipose tissue, brain, spleen or thyroid gland.

Aerobic culture yielded mixed alpha-haemolytic streptococci and actinobacilli from the lung, small intestine and placenta with scant to light growth of alpha-haemolytic streptococci from the liver, brain, kidney and stomach contents. No bacteria were recovered from the spleen or gastric mucosa. Enrichment cultures of the small intestine did not yield bacteria of the genera *Salmonella or Brucella*. Based on the nature of the microbial isolates, lack of attendant inflammatory infiltrate and multiple percutaneous lacerations, the bacteria that were found were not considered pathologically significant. PCR analysis of pooled lung, spleen, lymph node and brain did not detect influenza virus, *Toxoplasma gondii*, *Brucella* spp., phocid distemper virus or canine distemper virus. *Leptospira* sequences were detected by PCR in extracts of lung, spleen, lymph node and brain, but not placenta.

**Virus isolation**

Syncytial formation and rounding of individual cells were noted in both Vero and Vero.DogSLAMtag flasks that were inoculated with clarified tissue homogenates 3–5 days after inoculation (Fig. 1). Infected cell sheets rapidly deteriorated thereafter, with cytopathic effects (CPE) detected in flasks from both cell lines from all the tissues sampled. Supernatant fluid from infected cells was aliquoted and stored at −80 °C for further testing. Typical sized reovirus particles were observed by electron microscopy (Supplementary Fig. S1, available in JGV Online).

**Molecular characterization**

PCR amplification of the infected cell extract by using the degenerate reovirus primer pair resulted in a 549 bp product, after editing out the primer sequences. BLASTN results showed the highest score with an ARV (strain 176, GenBank accession no. EU707936.1, 73% nucleotide identity). Pyrosequencing libraries yielded approximately 60 233 reads. In concert these reads represented approximately
9.2 kbp of sequence distributed along the reovirus genome scaffolds, when aligned to the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank) by using BLASTN/BLASTX (Altschul et al., 1990). The sequences comprised single and assembled-contiguous fragments, representing approximately 39% of the reoviral sequence. Gaps between fragments and the termini of gene segments were completed by PCR, cloning and sequencing. The genomic sequence was verified by classical dieoxyribonucleotide sequencing using primers designed by using the draft sequence.

Consistent with the genome organization characteristic for members of the genus Orthoreovirus, the genome of Steller sea lion reovirus (SSRV) comprises ten RNA segments (GenBank accession nos HM222971–HM222980).

Phylogenetic analysis of the polymerase in the context of representative members of the family Reoviridae (Fig. 2) and analysis of all other segments in the context of representative members of the genera Aquareovirus and Orthoreovirus (Figs 3, 4, 5 and Supplementary Figs S2–S5, available in JGV Online) indicated that SSRV showed consistent association with other ARV and with NBV. Interestingly, SSRV is most similar to an orthoreovirus detected in a captive psittacine bird in Germany [eastern rosella, Platycercus eximius, GenBank accession nos EU252582 (S1), EU189200 (S2), EU189201 (S3) and EU189202 (S4)].
identical genomic organization in the S1 segment with three ORFs called \( sC \), p10 and p17. The known exception is Muscovy duck ARV, which contains only one ORF, \( sC \). In contrast, mammalian orthoreovirus presents two overlapping ORFs in S1 (called \( s1 \) and \( s1s \)); and reptilian orthoreovirus (RRV) and baboon orthoreovirus (BRV) have two in the analogue segment, segment 4 (called p14 and \( sC \) in RRV; p16 and p15 in BRV).

Reovirus fusion-associated small transmembrane (FAST) proteins are non-structural, single-pass membrane proteins that induce cell–cell fusion and syncytium formation (Shmulevitz & Duncan, 2000). With the exception of the mammalian orthoreoviruses, all orthoreoviruses have a FAST protein (Clancy & Duncan, 2009). There is evidence that FAST proteins are virulence factors (Brown et al., 2009). The predicted structure of the SSRV p10 ORF is similar to the FAST proteins of other reoviruses (Fig. 6).

**DISCUSSION**

To our knowledge, this is the first characterization of an orthoreovirus in marine mammals. SSRV was isolated from all tissues submitted for isolation, indicating a pancytotropic infection within the developing fetus. Reovirus-like particles have previously been seen in pinnipeds by electron microscopy but were not further characterized (Steiger et al., 1989); a reovirus in the genus Rotavirus has been identified in Galapagos sea lions (Zalophus wollebaeki) (Cora-Galindo et al., 2009).

Reoviruses have been demonstrated to cause abortion in mice (Hassan & Cochran, 1969), hamsters (Kilham & Margolis, 1974), rats (Priscott, 1983) and swine (Kirkbride & McAdaragh, 1978). Although this precedent suggests plausibility, and we recovered SSRV from multiple tissues, we have not proven a causal relationship between SSRV infection and abortion.

Leptospira sp. was detected by PCR in pooled lung, spleen, lymph node and brain, but not in placenta. Although the site of isolation seems not to be related with the abortion, Leptospira interrogans has been associated with reproductive failure in California sea lions (Smith et al., 1974). Lesions seen in an aborted California sea lion fetus with \( L. \) interrogans included a friable liver, subcapsular haemorrhage of the liver and both kidneys and unclotted blood in the peritoneal cavity (Gilmartin et al., 1976). \( L. \) interrogans is not prevalent in Steller sea lions (Burek et al., 2005), although significant diversity exists amongst \( L. \) interrogans serotypes (He et al., 2007). Given that our findings only relate to one case, the prevalence of SSRV and its role as a potential cause of fetal loss and abortion in Steller sea lions and, more broadly, marine mammals needs to be further explored.

Virus isolation attempts from animals presenting unusual clinical signs remain a powerful tool for the discovery of new and possibly emerging viruses of importance to both human and animal health. Cell-culture propagated virus isolates also provide abundant genetic material, thereby...
facilitating the identification of, and subsequently phylogenetic relationship to, other virus family members.

Our findings may have an impact on the view of host–virus relationships of viruses in the family Reoviridae. It has previously been suggested that aquareoviruses and orthoreoviruses and their respective hosts have co-speciated (Attoui et al., 2002), which implies significant host fidelity. ARV and NBV have already been shown to form a clade (Duncan, 1999; Wellehan et al., 2009). SSVR appears to be an additional member of this clade, and represents the first complete genome available in this clade. Partial genomic information (the four small segments) indicates that a reovirus isolated from a psittacine bird in Germany (de Kloet, 2008) is very closely related to SSVR.

Viruses within the family Reoviridae have been found to cause disease in hosts from diverse taxa, illustrating their ability to replicate in cells of diverse hosts (Attoui et al., 2006; Wellehan et al., 2009). The ability of SSVR to grow efficiently in cells from African green monkey (Cercopithecus aethiops) origin, albeit in an in vitro setting, also underscores its potentially broad host range. One recent study scored the viruses infecting mammals for biological properties that were considered advantageous to host switching, and found that Reoviridae scored highest (Pulliam, 2008). The finding of closely related reoviruses in sea lions, bats and psittacine birds implies host switching and lack of host fidelity.

According to the ICTV, conclusive species classification requires the direct demonstration (or lack) of exchange of genetic material via reassortment of genome segments (Chappell et al., 2005). Reassortment between avian orthoreoviruses has been demonstrated (Liu et al., 2003), and further experiments are indicated to look for genetic exchange between SSVR, ARV and NBV. However, this criterion for species delineation may need to be reconsidered; evidence for genetic exchange between two distinct reoviral genera, Aquareovirus and Coltivirus, has recently been published (Mohd Jaafar et al., 2008).

Reassortment and reclassification pose challenges to viral classification; different regions of viral genomes may not share common lineages. The advent of high-throughput sequencing technologies has facilitated full-genome sequencing. Where feasible, complete genome information should be obtained to allow analysis of the evolution and relationships of all regions, thus providing greater understanding of virus ecology and behaviour.

METHODS

Tissue sampling. As part of a study on foraging behaviour, Steller sea lions were being captured using a floating trap anchored off Norris Rock, near Denman Island, British Columbia, Canada (49° 48’ N 124° 64’ W). On 25 January 2005, a dead, freshly aborted, mid-gestational male Steller sea lion fetus with an attached placenta was found on the floor of the capture trap prior to a capture event. An adult Steller sea lion, assumed to be the mother of the aborted fetus, was subsequently captured and restrained for handling and processing. Following being trapped, the adult female attempted to eat or attack the dead fetus prior to its removal from the trap, at which time the fetal abdomen and head were punctured and the placenta detached from the fetus. The aborted fetus and placenta were removed from the trap and examined.

A full necropsy of the fetus was conducted in the field and portions of placenta and of each major organ were preserved in 10% neutral buffered formalin for histopathological examination. Representative samples of placenta, brain, lung, liver, kidney, spleen, gastric mucosa and small intestine were cultured for aerobic bacteria. Tissue homogenate of pooled brain, lung, spleen and lymph node were processed for Toxoplasma gondii, generic Brucella spp., Leptospira sp., phocid distemper virus and canine distemper virus by PCR, and for virus isolation. Additional samples of lung, spleen and mesenteric lymph node were also available for virus isolation.

Cells and virus isolation. Tissues were homogenized in a MiniMix bag system homogenizer (Interscience) to give a 10% (w/v) suspension in Hanks balanced salt solution (HBSS) containing antibiotics (200 IU penicillin ml⁻¹ (Hyclone), 200 µg streptomycin ml⁻¹ (Hyclone) and 50 µg gentamicin ml⁻¹ (Mediatech). Suspensions were centrifuged at low speed (700 g) for 15 min to remove cell debris. Inocula consisted of 250 µl of each cell-free suspension added onto draining, 80% confluent cultures of African green monkey kidney cells (Vero C1008; American Type Culture Collection) and Vero.DogSLAMtag cells (Vero cells stably expressing canine signalling lymphocyte activation molecule, the morvillivirus receptor; donated by Dr Yasuke Yanagi, Kyushu University, Fukuoka, Japan), all grown in 25 cm² flasks (Corning). Adsorption was allowed to continue for 1 h at 37 °C before the medium was removed and 5 ml of fresh medium [Dulbecco’s modified Eagle’s medium/Ham’s F-12 with antibiotics (as given above) and 2% Cosmic calf serum (HyClone)] was added to each flask. Flasks were incubated at 37 °C and observed daily for signs of CPE. Flasks were subcultured at a ratio of 1:2 every week.

Molecular virus characterization. Using an RNeasy Tissue kit (Qiagen), RNA was extracted from Vero cells displaying CPE. The extracted RNA was analysed using a GreeneChip (version 1.5) (Palacios et al., 2007; Quan et al., 2007). Probe intensities were background corrected, log₂-transformed, Z-score converted and their corresponding P values calculated. Positive hybridization events were selected as those spots with log₂-fluorescence values greater than two SD above the mean signal. Candidate viruses [defined by their TaxID identifier (GenBank, NCBI)] were ranked by combining the P values for the positive probes within that TaxID by using the qFAST method of Bailey & Gribskov (1998).

Microarray analysis using GreeneLAMP yielded no statistically significant viral signal. Nonetheless, nucleic acid bound to the array was eluted with the intent of enriching for cryptic hybridized viral sequences. One hundred microlitres of water at 90 °C were added to the array and mixed ten times. Eluate was recovered and reamplified by PCR. The library of DNA obtained was cloned into a plasmid vector (TOPO-TA; Invitrogen). After transformation into Escherichia coli, colonies were screened by sequencing, thus revealing the presence of reovirus nucleic acid. This finding was subsequently confirmed by RT-PCR of tissue-culture extracts by using consensus primers for orthoreoviruses and aquareoviruses (Wellehan et al., 2009). The protocol was modified to use the primer 1607F (5’-CARMCNGCNS-CHMGHTCHATHATGGC-3’) as a forward primer and 2200R (5’-CCRTCRTCWCYTGRCACAGRTT-3’) as a reverse primer in the second round (Landolfi et al., 2010), thus yielding a 549 bp product.

Viral genome sequencing and analysis of ORFs. RNA extracts from virus supernatant were amplified and prepared for unbiased high-throughput pyrosequencing. Total RNA extracts were treated with DNase I (DNA free; Ambion) and cDNA was generated by using
a Superscript II system (Invitrogen) for reverse transcription primed by random octamers that were linked to a defined 17-mer primer sequence (Palacios et al., 2007). The resulting cDNA was treated with RNase H and then randomly amplified by PCR (Palacios et al., 2008). Products of >70 bp were selected by column purification (MinElute) and ligated to specific linkers for sequencing using a 454 Genome Sequencer FLX (454 Life Sciences) without fragmentation of the cDNA (Cox-Foster et al., 2007; Margulies et al., 2005; Palacios et al., 2008). Removal of primer sequences, redundancy filtering and sequence assembly were performed with software programs accessible through the analysis applications at the GreenePortal website (http://tako.cpmc.columbia.edu/portal).

Sequence gaps between the aligned fragments were filled by specific PCR amplification with primers designed by using the data from pyrosequencing. Terminal sequences were generated by ligation (Potgieter et al., 2009). Sequences were verified by classicalideoxynucleotide sequencing, using primers designed based on the draft sequence.

Phylogenetic analysis. The initial sequences were compared to those in GenBank [National Center for Biotechnology Information (NCBI), Bethesda, Maryland, USA], EMBL (European Molecular Biology Laboratory, Cambridge, UK) and Data Bank of Japan (Mishima, Shizuoka, Japan) databases by using BLASTN (Altschul et al., 1990).

A set of sequences of viruses of the family Reoviridae was used to assess the phylogenetic history of SSRV. RNA polymerase amino acid sequences of reoviruses were aligned using the programs PROMALS3D (Pei et al., 2008) and 3DCoffee (O’Sullivan et al., 2004), with the purpose of obtaining an alignment that not only considered primary sequence data but also the secondary structure of the protein. To evaluate the robustness of the approach, the ability to find and align motifs that had previously been identified as being conserved amongst the family Reoviridae was also used as a maker. Phylogenetic analysis was performed using p-distance as a model of amino acid substitution, as accepted by the ICTV for analysis of the family Reoviridae (Attoui et al., 2006; Mertens et al., 2005). MEGA (Kumar et al., 2004) was used to produce phylogenetic trees, reconstructed through the NJ method (Saitou & Nei, 1987). The statistical significance of a particular tree topology was evaluated by bootstrap resampling of the sequence 1000 times. Identical results were obtained by Bayesian phylogenetic analyses using the BEAST, BEAUti and TRACER analysis software packages (Drummond & Rambaut, 2007) (data not shown). All other orthoreovirus and aquareovirus segment sequences were aligned by using a similar approach. The evolutionary distances were computed by using the Poisson correction method and are in the units of number of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons.

Sequence analysis. Programs of the GENEIOUS package (Biomatters) were used for sequence assembly and analysis; p-distances were calculated by using MEGA3. Topology and targeting predictions were generated by employing SignalP, NetNGlyc, TMHMM (http://www.cbs.dtu.dk/services), the web-based version of TopPred2 (http://bioweb2.pasteur.fr/seqanal/interfaces/toppred.html) and PHOBUS (http://phobius.sbc.su.se/) (Bendtsen et al., 2004; Claros & von Heijne, 1994; Käll et al., 2004; Krogh et al., 2001).

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Mortality of harbor seal pups at different sites in the inland waters of Washington.


