

Short Communication

Novel anellovirus discovered from a mortality event of captive California sea lions

Terry Fei Fan Ng,¹ Wm Kirk Suedmeyer,² Elizabeth Wheeler,³ Frances Gulland³ and Mya Breitbart¹

Correspondence

Mya Breitbart

mya@marine.usf.edu

¹University of South Florida, Saint Petersburg, FL, USA

²Kansas City Zoo, Kansas City, MO, USA

³Marine Mammal Center, CA, USA

A viral metagenomic study was performed to investigate potential viral pathogens associated with a mortality event of three captive California sea lions (*Zalophus californianus*). This study identified a novel California sea lion anellovirus (ZcAV), with 35% amino acid identity in the ORF1 region to feline anelloviruses. The double-stranded replicative form of ZcAV was detected in lung tissue, suggesting that ZcAV replicates in sea lion lungs. Specific PCR revealed the presence of ZcAV in the lung tissue of all three sea lions involved in the mortality event, but not in three other sea lions from the same zoo. In addition, ZcAV was detected at low frequency (11%) in the lungs of wild sea lions. The higher prevalence of ZcAV and presence of the double-stranded replicative form in the lungs of sea lions from the mortality event suggest that ZcAV was associated with the death of these animals.

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New diseases in marine animals are emerging at an increasing rate and the causative agents of the diseases are mostly unknown (Harvell *et al.*, 1999; Van Bresseem *et al.*, 1999). As animals are considered to be the source of >70% of emerging human infections (Cleaveland *et al.*, 2001), surveillance of pathogens in animals, especially in non-human vertebrates, is important for protecting the health of both humans and wild animal populations (Kruse *et al.*, 2004; Kuiken, 2005).

Investigating new viral infections has been difficult due to limitations of current detection methods (reviewed by Delwart, 2007). For example, degenerate PCR and pan-viral microarrays can detect viruses with close sequence similarity to known viruses, but are limited for discovering novel viruses that share limited similarity with previously sequenced virus genomes. Recent studies have demonstrated the effectiveness of virus-particle purification and shotgun sequencing (viral metagenomics) for describing novel viruses, including those that are unrelated to previously described virus families (Delwart, 2007; Edwards & Rohwer, 2005). The techniques of viral metagenomics were first applied to characterize virus communities present in the environment (Breitbart *et al.*, 2002) and have recently been applied to identify animal viruses in respiratory-tract aspirates (Allander *et al.*, 2005),

blood (Breitbart & Rohwer, 2005; Jones *et al.*, 2005) and tumours (Ng *et al.*, 2009).

One potential application of viral metagenomics is for examination of viral pathogens involved in unusual mortality events of animals. In 2005 and 2006, three California sea lions at the Kansas City Zoo, MO, USA, died in a mortality event of unknown aetiology. Necropsy and histopathology revealed granulomatous, non-suppurative mediastinitis and pleuritis in two of the sea lions, whilst the third sea lion was found to have an accumulation of lymphocytes and macrophages in the upper respiratory submucosa (W. K. Suedmeyer, unpublished results). Chemical toxicity assays using gas chromatography and mass spectroscopy did not detect pesticides, organic toxic compounds or abnormal mineral levels in pool water or gastric contents. All sea lions were negative for pathogenic bacteria, fungi and mycoplasma in culture-based assays and histopathology. The sea lions also tested negative for West Nile virus, and no viruses could be cultured using green monkey or canine cell lines (W. K. Suedmeyer, unpublished results).

This study employed viral metagenomics to investigate the DNA viruses found in the lungs of one of the captive California sea lions involved in the mortality event. Viruses were purified from frozen lung necropsy samples according to previously published methods (Breitbart & Rohwer, 2005; Ng *et al.*, 2009). Briefly, lung tissue was homogenized in sterile SM buffer (50 mM Tris, 10 mM MgSO₄, 0.1 M NaCl, pH 7.5) and host cells were removed by centrifugation.

The GenBank/EMBL/DDBJ accession number for the ZcAV genome is FJ459582; those for shorter ZcAV sequences detected in wild sea lions are FJ580971–FJ580976.

gation at 10 000 g for 10 min, followed by filtration of the supernatant through a 0.45 μm filter. Viruses in the filtrate were purified and concentrated by using a caesium chloride step gradient consisting of 1 ml each of 1.7, 1.5 and 1.2 g CsCl ml^{-1} in SM buffer. The gradient was ultracentrifuged at 61 000 g at 4 $^{\circ}C$ for 3 h and the virus fraction between 1.2 and 1.5 g ml^{-1} was collected. The virus fraction was then treated with 0.2 vols chloroform for 10 min, followed by incubation with 2.5 U DNase I μl^{-1} for 3 h at 37 $^{\circ}C$. Virus particles were further concentrated and washed twice with sterile SM buffer on a Microcon 30 column (Millipore) and DNase activity was inhibited by adding EDTA to a final concentration of 20 mM. Finally, DNA was extracted from the purified virus particles by using a QIAamp MinElute Virus Spin kit (Qiagen) and amplified with the strand-displacement method of the GenomiPhi V2 DNA Amplification kit (GE Healthcare) according to the manufacturer's instructions. A GenomePlex Whole Genome Amplification kit (Sigma-Aldrich) was then used to fragment and amplify the viral DNA, which was subsequently cloned into the pCR4 vector using TOPO TA cloning (Invitrogen). In total, 86 transformants were sequenced, from which 69 high-quality reads were obtained. Metagenomic sequences were analysed by using TBLASTX against the GenBank non-redundant database (Altschul *et al.*, 1990, 1997) and fragments with >85% identity over 20 nt were assembled into contigs (contiguous sequences) by using SEQUENCHER 4.7 (Gene Codes Corporation).

Approximately 10% of the sequences from the viral metagenome had significant BLAST similarities to feline and human anelloviruses. The majority of the remaining metagenomic sequences had no BLAST similarities to any known sequences in GenBank, and a few sequences were most similar to bacteria. Efforts were focused on the sequences with similarity to anelloviruses, as this group has not previously been described in marine animals. Viruses in the 'floating' genus *Anellovirus* contain small, negative-sense, circular, single-stranded DNA genomes (Biagini *et al.*, 2005). Anelloviruses are subgrouped into torque teno virus (TTV), torque teno mini virus (TTMV), torque teno midi virus (TTMDV) and small anellovirus (SAV), with known hosts including humans, non-human primates and domestic animals (Biagini *et al.*, 2007; Hino & Miyata, 2007; Leary *et al.*, 1999). The pathology of anelloviruses remains unknown (Davidson & Shulman, 2008; Hino & Miyata, 2007). To complete the genome of the novel California sea lion anellovirus (ZcAV), strand-displacement amplification (GenomiPhi or TempliPhi) and PCR were performed to amplify and sequence the genomic regions between the contigs until 3 \times coverage of the genome was obtained. Putative open reading frames (ORFs) were predicted by using the NCBI ORF Finder (<http://www.ncbi.nlm.nih.gov/projects/gorf>) with a minimum size of 100 aa, and the potential nuclear-localization signal was predicted by using PREDICTNLS (Cokol *et al.*, 2000). The GenBank accession number for the ZcAV genome is FJ459582.

The circular genome of ZcAV was 2140 nt long, with a G+C content of 44.1 mol%. Polarity of the packaged ZcAV genome was determined by using the strand-specific primer-extension method (Okamoto *et al.*, 2000b). Briefly, purified virus DNA was amplified linearly for 100 cycles in separate reactions using either the sense or antisense primer. The linearly amplified DNA was then used as the target in a PCR with a pair of internal primers and the PCR product intensities were compared.

Strandedness of the ZcAV genome, both in the purified virus particles and in various tissue samples, was determined by using the methods of Okamoto *et al.* (1998). To determine strandedness, DNA was digested with either *EcoRI* or mung bean nuclease (New England Biolabs), followed by PCR with the ZcAV-1 primers (described below), which flank the *EcoRI* restriction site. Samples resistant to *EcoRI* and sensitive to mung bean nuclease contain single-stranded DNA, whereas samples sensitive to *EcoRI* but resistant to mung bean nuclease contain double-stranded DNA. Samples containing DNA resistant to both separate treatments were considered to contain both single- and double-stranded DNA. Polarity and strandedness experiments demonstrated that the ZcAV particles contain a negative-sense, single-stranded genome, which is consistent with the characteristics of anelloviruses.

The genome organization of ZcAV was similar to that of other anelloviruses, with three ORFs located on the negative-stranded genome. ORF1 was the largest ORF, and two smaller ORFs (ORF2 and 3) were located in different reading frames (Fig. 1). ORF1 encodes a protein of 469 aa and has weak amino acid level similarity to

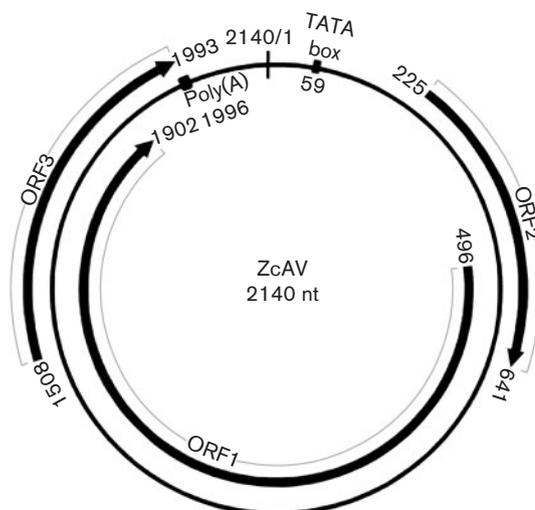


Fig. 1. Genomic organization of the California sea lion anellovirus (ZcAV). Genome annotation is based on sequence data only; the potential second splicing event is not shown. The purified ZcAV particle contains a negative-sense genome.

feline anelloviruses PRA1 (33%), PRA2 (35%), and Fc-TTV4 (34%) (Biagini *et al.*, 2007; Okamoto *et al.*, 2002). The beginning of ORF1 contained an arginine-rich region, with 22 arginine residues in the first 40 aa (55%). Arginine-rich regions are common in single-stranded DNA animal viruses, including anelloviruses, circoviruses and gyroviruses. A strong nuclear-localization signal was predicted for ORF1, due to the arginine-rich sequences 'RNRHWWGRRRR' near the beginning of the ORF and 'LKSRKRKRP' near the end. ORF2 encodes a protein of 140 aa and ORF3 encodes a protein of 163 aa. Neither ORF2 nor ORF3 has any amino acid identity to proteins in GenBank. ZcAV contains a TATA box (TATAAA) at position 59 (which is 166 nt upstream of ORF2) and a poly(A) signal (AATAAA) immediately after ORF3.

Based on the entire nucleotide sequence of ORF1 (Biagini *et al.*, 2007), a neighbour-joining phylogenetic tree was created to determine the relationship of ZcAV to other anelloviruses (Fig. 2). Although quite divergent in sequence, ZcAV clusters with feline anelloviruses on a branch that is distinct from TTV, TTMDV and SAV. In contrast to the relatedness of ZcAV to feline anelloviruses, the sea lion host is phylogenetically related more closely to dogs than to cats (Flynn & Nedbal, 1998). As other animal anelloviruses also do not cluster according to host phylogeny, the evolution of animal anelloviruses appears to be more complex than solely virus–host co-divergence.

For human TTV, three distinct species of mRNA have been observed through transcriptional studies (Kamahora *et al.*, 2000). Likewise, three splicing events could be predicted in

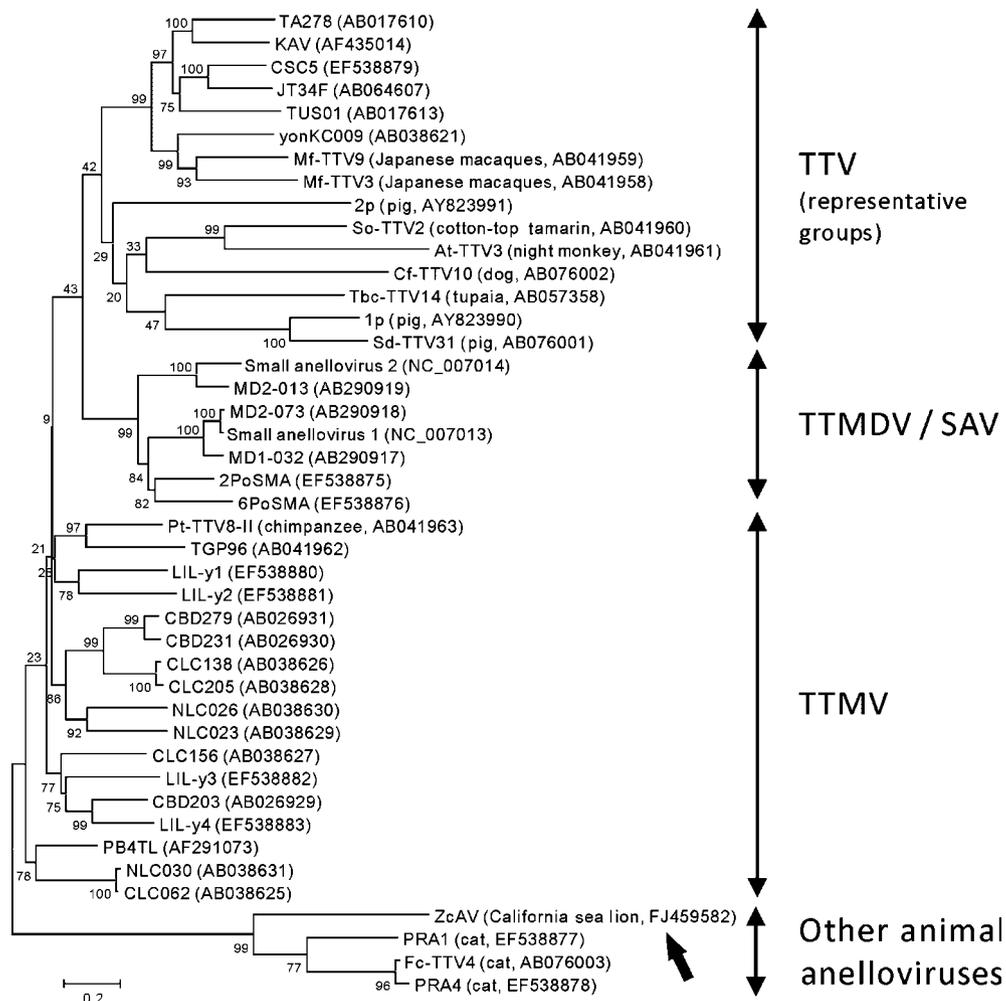


Fig. 2. Neighbour-joining phylogenetic tree constructed by using the entire nucleotide sequence of ORF1, as described by Biagini *et al.* (2007). A CLUSTAL W multiple alignment was created in BioEdit (Hall, 1999) and bootstrapping (percentages of 1000 replicates are shown) was performed in MEGA4 (Tamura *et al.*, 2007). The newly discovered sea lion anellovirus (ZcAV) is indicated by an arrow. ZcAV clusters most closely with feline anelloviruses and is distinct from torque teno virus (TTV), torque teno midi virus (TTMDV), small anellovirus (SAV) and torque teno mini virus (TTMV). In parentheses, the host is indicated for any non-human sequences and GenBank accession numbers are shown.

many animal counterparts by using consensus motifs of donor and acceptor sites (Breathnach *et al.*, 1978; Mount, 1982; Okamoto *et al.*, 2001b). The ZcAV genome was analysed for potential donor and acceptor sites by using GeneSplicer (http://www.tigr.org/tdb/GeneSplicer/gene_spl.html) and compared manually with the splicing events known for other anelloviruses. With the ZcAV genome, only the second splicing event was recognized. Three splicing events could be recognized in most of the animal anelloviruses, but only the second splicing event was detected in the tupaia TTV (Okamoto *et al.*, 2001b).

To investigate the prevalence and tissue specificity of ZcAV, total DNA was extracted from different tissues by using a DNeasy Blood and Tissue kit (Qiagen). Specific PCR to detect ZcAV was executed by using the primers ZcAV-1F (5'-TTCTAGAAGGATGGTATCTTACCG-3') and ZcAV-1R (5'-GTCTCGCATGGACATTGTTG-3'). The PCR [containing 1 μ M each primer, 200 μ M dNTPs, 1 U REDTaq DNA polymerase (Sigma-Aldrich), 1 \times REDTaq reaction buffer (Sigma-Aldrich) and 5 μ l target DNA in a 50 μ l reaction] was amplified as follows: 95 °C for 5 min; 45 cycles of 94 °C for 1 min, 56 °C –0.2 °C per cycle for 1 min, 72 °C for 1 min; followed by 72 °C for 10 min. Assay sensitivity was determined by ZcAV-1 PCR on a dilution series of positive-control DNA; this demonstrated that this assay was capable of detecting 10 targets.

Lung tissue from all three of the sea lions involved in the mortality event tested positive for ZcAV by specific PCR (Table 1). A lung pleural sample that was available from one of these sea lions also tested positive for ZcAV. ZcAV was not present in any of the other sample types (abdominal fluid, $n=1$; blood, $n=2$) from these animals. Strandedness could only be characterized in the lung tissue sample from the sea lion from which the viral metagenome was constructed. In the purified virus particles, the ZcAV genome was found in a single-stranded form. However, in the lungs of this sea lion, ZcAV was present as both single- and double-stranded DNA, suggesting that the lungs are a site of active replication for this virus. Double-stranded replicative forms of single-stranded DNA viruses have also

been observed in human anelloviruses (Okamoto *et al.*, 2000b) and chicken anemia virus (Noteborn *et al.*, 1991).

As ZcAV was consistently found in the lungs, lung samples from three other captive sea lions that died of unrelated causes (cancer or kidney failure) at the same zoo were tested for ZcAV. ZcAV was not detected in any of these sea lions (Table 1), further supporting the involvement of ZcAV in the mortality event.

To determine the prevalence of ZcAV amongst wild sea lion populations, lung samples were collected from necropsies of 54 wild sea lions from the California coast that died of a range of causes (domoic acid toxicity, pyothorax, pleuritis and pneumonia) (Greig *et al.*, 2005). Eleven per cent of these lung samples were positive for ZcAV (Table 1) and the double-stranded replicative form was detected in one of the three lung samples where strandedness was determined. Positive PCR products were nearly identical in sequence to the corresponding region from the original ZcAV genome (a maximum of 1.2% nucleotide difference in the 235 nt region of ORF2 amplified by the ZcAV-1 primers, GenBank accession numbers FJ580971–FJ580976). It is possible that there is sequence variability in the remainder of the ZcAV genome outside the amplicon in the wild sea lions, as sequence heterogeneity is well-known in anelloviruses (reviewed by Davidson & Shulman, 2008).

When available, other tissue samples were collected from the wild sea lions. In addition to being present in 11% of the lung samples ($n=54$), ZcAV was detected in 20% of tonsil samples ($n=5$), 12% of lymph-node samples ($n=25$) and 40% of liver samples ($n=5$) (Table 1). With only one exception, ZcAV was always found in the lungs of wild sea lions when it was found in other tissue types. Although the sample sizes are small, it is noteworthy that the double-stranded replicative form of ZcAV was detected in one of the three wild sea lion lung samples assayed, but not found in the lymph-node ($n=1$) or liver ($n=1$) samples.

The high prevalence of ZcAV in the lungs of sea lions is similar to the situation observed for pig anelloviruses, which were found most frequently in lung (11%),

Table 1. Prevalence of sea lion anellovirus (ZcAV) amongst sea lions from the mortality event, other captive sea lions and wild sea lions

Values are number of ZcAV-positive samples/total number of samples (%).

| Tissue sampled | Sample origin | | |
|----------------|---------------------------|-------------------------|-----------------|
| | Mortality event ($n=3$) | Other captive ($n=3$) | Wild ($n=54$) |
| Lung | 3/3 (100%) | 0/3 (0%) | 6/54 (11%) |
| Lung pleura | 1/1 (100%) | – | – |
| Blood | 0/2 (0%) | – | 0/24 (0%) |
| Tonsil | – | – | 1/5 (20%) |
| Lymph node | – | – | 3/25 (12%) |
| Liver | – | – | 2/5 (40%) |

compared with inguinal lymph node (8%), mesenteric lymph node (6%), tonsil (4%) and ileum (3%) (Bigarre *et al.*, 2005). The double-stranded replicative form of human TTV has been detected in lung, bone marrow, lymph node, muscle, thyroid gland, liver, spleen, pancreas and kidney (Bando *et al.*, 2001; Okamoto *et al.*, 2000a, b, 2001a), suggesting that human TTV can replicate in all of these tissue types. Although larger sample sizes are needed in order to assess definitively the tissue tropism of ZcAV, this study has confirmed that ZcAV can replicate in the lungs.

Despite detection of ZcAV in numerous tissue types, ZcAV was not present in blood samples from any of the sea lions ($n=24$) (Table 1). This is interesting because other known anelloviruses are typically found in blood or plasma samples (Jones *et al.*, 2005; Ninomiya *et al.*, 2007; Nishizawa *et al.*, 1997; Takahashi *et al.*, 2000). Further investigation is needed to explain the absence of ZcAV in sea lion blood.

Two results suggest that ZcAV was associated with the sea lion mortality event. First, all three of the California sea lions from the mortality event were positive for ZcAV in the lungs (100%), whereas the other three sea lions from the same zoo were negative for ZcAV (0%). Although ZcAV was also found in wild sea lions, it was present at a much lower prevalence (six of 54, 11%). Second, the double-stranded replicative form of ZcAV was found in lung tissues – one from a sea lion involved in the mortality event and one from a wild sea lion – confirming that this virus was replicating actively in the lungs. This is significant because the sea lions involved in the mortality event demonstrated histological evidence of lung disease.

Currently, the role of ZcAV in the mortality event is unknown. As ZcAV was present in 11% of wild sea lions investigated, the virus was not isolated to the mortality event. No consistent findings were noted in necropsies of the ZcAV-positive wild sea lions, so it is also unknown whether ZcAV was involved in their deaths. It has been suggested that TTV can potentially enhance the negative effects of other pathogens in humans (Davidson & Shulman, 2008). Based on extrapolation from human anelloviruses, it is possible that ZcAV was not directly responsible for the mortality event, but made the sea lions more susceptible to the effects of another unidentified pathogen.

In conclusion, this study demonstrated the effectiveness of viral metagenomics for examining viruses associated with a marine animal mortality event of unknown aetiology. Virus purification successfully separated virus particles from background host DNA, allowing viral sequence to be obtained with minimal sequencing effort. In this study, viral metagenomics enabled the first discovery and genome sequencing of an anellovirus from a marine animal, and subsequently demonstrated the prevalence of this virus in wild California sea lions. The significantly higher prevalence of ZcAV in the lungs of sea lions from the mortality

event than in the wild population suggests that ZcAV was associated with the death of these sea lions. However, future research will be needed to understand the ecology and pathogenesis of the sea lion anellovirus ZcAV.

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