Original Contribution

Molecular Detection of Circovirus and Adenovirus in Feces of Fur Seals (*Arctocephalus* spp.)

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Abstract: In some regions, little is known about exposure to viruses in coastal marine mammals. The present study aimed to detect viral RNA or DNA in 23 free-ranging fur seals on the northern coastline of Rio Grande do Sul State, Brazil. Polymerase chain reaction was used to detect nucleic acids of circoviruses, adenoviruses, morbilliviruses, vesiviruses, and coronaviruses in the feces from twenty-one South American fur seals (*Arctocephalus australis*) and two Subantarctic fur seals (*A. tropicalis*). Adenovirus DNA fragments were detected in two South American fur seals; nucleotide sequences of these fragments revealed a high degree of similarity to human adenovirus type C. *Circovirus* DNA fragments were detected in six animals of the same species. Two were phylogenetically similar to the *Circovirus* genus, whereas the other four nucleotide fragments showed no similarity to any of the known genera within the family Circoviridae. RNA fragments indicating the presence of coronavirus, vesivirus, and morbillivirus were not detected. These findings suggest that adenoviruses and circoviruses are circulating in fur seal populations found along the coast of Rio Grande do Sul State, Brazil.

Keywords: pinniped, virus, PCR, detection

INTRODUCTION

Since the last decade of the 20th century, the detection and characterization of viruses in marine mammals have increased substantially. This is partly due to greater awareness

of morbillivirus epizootics in cetaceans and pinnipeds throughout the world's oceans. Morbilliviruses, single-stranded RNA viruses belonging to the Paramyxoviridae family, have caused several major epizootics with high mortality in many pinniped species since 1987 (Kennedy-Stoskopf 2001). In addition to morbilliviruses, other viral infections have been reported to cause a number of diseases in pinnipeds, such as adenoviruses, vesiviruses, and coronaviruses.

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Adenoviruses (family Adenoviridae) are double-stranded DNA viruses first detected in marine mammals in 1979. The virus was identified in stranded California sea lion livers (*Zalophus californianus*) with hepatitis (Britt et al. 1979; Dierauf et al. 1981). A novel adenovirus, otarine adenovirus type 1 (OtAV1), was recently associated with viral hepatitis and endothelial cell infection in the same species (Goldstein et al. 2011). Vesiviruses are small single-stranded RNA viruses, from the family *Caliciviridae*, first isolated in California sea lions from San Miguel Island in 1972. The virus was named San Miguel sea lion virus (SMSLV) (Smith et al. 1973). It causes vesicular lesions in the mouth and flippers of pinnipeds (Smith et al. 1983) and has been suggested as the possible cause of epizootic gastroenteritis reported in California sea lions in 2009 (Schmitt et al. 2009). Coronaviruses (family Coronaviridae) have also been reported in pinnipeds. These are positive single-stranded RNA viruses that have been associated with acute necrotizing enteritis in harbor seals (Bossart and Shwartz 1990).

Although most viral infections reported in pinnipeds are associated with clear clinical signs, there are many others whose impact on these animals is still unknown (Britt et al. 1979; Kennedy et al. 1988). Circoviruses (family *Circoviridae*) such as the small, circular single-stranded DNA viruses are commonly present in mammalian feces (Blinkova et al. 2010; Li et al. 2010b, c; Phan et al. 2011). Circovirus was recently identified in feces from an asymptomatic New Zealand fur seal (*A. forsteri*) (Sikorski et al. 2013).

Although there are no pinniped breeding colonies in Brazil, dozens of fur seals, sea lions, elephant seals, and Antarctic seals haul out on Brazil's southern shores every year between autumn and spring, following their post-reproductive migration through the Falkland current (Pinedo 1990; Simões-Lopes and Ott 1995; Oliveira et al. 2006). Given that these areas are densely populated with humans and that these animals often haul out on beaches, there is the potential for contact between seals and people (IBGE 2010). Thus, knowledge of pathogens that may circulate in these animals is crucial (Smith et al. 2006). The present study aimed to detect the presence of circovirus, adenovirus, morbillivirus, vesivirus, and coronavirus nucleic acid in feces from two species of fur seals (*A. australis* and *A. tropicalis*) along the southern coast of Brazil.

MATERIALS AND METHODS

Sample Collection and Preparation

In June and July of 2012, 23 fur seals of the genus *Arctocephalus* were found dead with no apparent cause of death. The stage of decomposition of all carcasses was Geraci code 2 (Geraci and Lounsbury 1993). Fecal samples were collected from 21 South American fur seals (*A. australis*) and two Subantarctic fur seals (*A. tropicalis*). The coastal area of approximately 300 km where the animals were found is located between the cities of Torres (coordinates: 29°20'31"S, 49°43'47"W) and Mostardas (coordinates: 31°6'25"S, 50°55'15"W), Rio Grande do Sul State, Southern Brazil (Fig. 1). The area was monitored weekly for pinnipeds and other marine animals on shore. Fecal samples were collected from the intestine using sterile collection materials, kept on ice, sent to the laboratory, and stored at -80°C . At the time of processing, samples were thawed and approximately 5 g of fecal material were resuspended in 10 mL of Hank's balanced salt solution (HBSS, pH 7.2). The slurry was removed by pelleting at $10,000\times g$ for 10 min. The supernatants were sequentially transferred to fresh tubes, and DNA and RNA were extracted.

DNA and RNA Extraction and Internal Control

Total fecal DNA was extracted from 400 μL of the supernatants with buffered phenol (InvitrogenTM). The extracted DNA was eluted in 50 μL of TE (Tris-HCl, pH 8.0, 1.0 mM EDTA), treated with RNase, and stored at -80°C . Total fecal RNA was extracted from 100 μL of the supernatants with TRIzol (InvitrogenTM), as recommended by the manufacturer. The extracted RNA was eluted in 50 μL of RNase-free water and stored at -80°C . To ensure that the extracted DNA and RNA were suitable for amplification, the ribosomal gene 16S of Gram-positive bacteria was amplified as internal control. Synthesis of cDNAs was performed in 10 μL of the extracted RNA suspensions with Moloney's murine leukemia virus reverse transcriptase (InvitrogenTM) and random hexamers of oligonucleotides (InvitrogenTM). Reactions were carried out in 20 μL , following the manufacturer's instructions. Extracted DNA and cDNAs were then used for 16 S PCR with primers FC27 (5'-AGAGTTTGATCCTGGCTCAG-3') and R530 (5'-CCGCGGCTGCTGGCACGTA-3') (Gontang et al. 2007) in

Figure 1. Map of the coastal region of Rio Grande do Sul, South Brazil, where the 23 samples were collected from carcasses of fur seals between June and July of 2012.

25 μL reactions containing 20–50 ng of DNA, 1 mM of MgCl_2 , 0.2 μM of each primer (IDT), 1.5 U Taq DNA polymerase (InvitrogenTM), 10% PCR buffer (InvitrogenTM), and 0.6 mM dNTP (ABgene). Cycling conditions consisted of an initial 5 min denaturation step at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C, and a final 5 min extension step at 72°C. Five microliters of the amplicons obtained (approximately 500 bp) were electrophoresed in 1.5% agarose gels and visualized under UV light after staining with ethidium bromide.

Viral Nucleic Acid Detection

All samples tested positive for the 16s gene used for adenovirus, vesivirus, circovirus, coronavirus, and morbillivirus nucleic acid amplification using PCR or nested-PCR. Specificity of the sets of primers varies in each virus family. The primers used are listed in Table 1.

For adenoviruses, a 261 bp fragment corresponding to the DNA polymerase (*pol*) gene was targeted. According to BLASTN analysis, primer coverage included all members of *Mastadenovirus* genus. Amplification was performed by nested-PCR as previously described (Li et al. 2010a). For the first reaction, the 25 μL reaction mix contained 20–50 ng of extracted DNA, 1 mM MgCl_2 , 2.5 μL PCR buffer, 0.2 μM of each primer, *pol*-F and *pol*-R, 0.2 mM dNTP, and 0.5 U Taq DNA polymerase (PromegaTM). The cycling conditions were 5 min at 94°C, 30 cycles of 30 s at 94°C,

30 s at 48°C, 30 s at 72°C, and final extension of 10 min at 72°C. The second reaction was performed using the same cycling parameters with 1 μL of the first reaction amplicon as DNA template, the forward primer *pol*-nF, and the reverse primer *pol*-nR (Li et al. 2010a).

PCR to detect vesivirus RNA targeted a 350 bp fragment along the viral helicase (*hel*) gene. Primers *hel*-1 and *hel*-2 were designed to detect the genome of all members of *Vesivirus* genus (Neill et al. 1995). The 25 μL reaction mix contained 2 μL of cDNA, 1 mM MgCl_2 , 2.5 μL PCR buffer, 0.2 μM of each primer, 0.2 mM dNTP, and 0.5 U Taq DNA polymerase (PromegaTM). The cycling conditions were 5 min at 95°C, 35 cycles of 45 s at 94°C, 45 s at 59°C, 45 s at 72°C, and final extension step of 5 min at 72°C.

To detect circovirus nucleic acids, a nested-PCR with the following degenerate primers was used: CV-F1, CV-R1, CV-F2, and CV-R2 (Li et al. 2010b). These were expected to amplify ~400 bp segment of the viral *rep* gene conserved in *Circovirus* and in the tentatively named *Cyclovirus* genera (Li et al. 2010b). The first reaction was performed in 25 μL consisting of 20–50 ng of DNA, 1 mM MgCl_2 , 2.5 μL PCR buffer, 0.2 μM of each primer, 0.2 mM dNTP, and 0.5 U Taq DNA polymerase (PromegaTM). The cycling conditions were 5 min at 95°C; 40 cycles of 1 min at 95°C, 1 min at 52°C, 1 min at 72°C, and a final extension at 72°C for 10 min. For the second reaction, 1 μL of the amplicon from first reaction was used as a template with the same reaction parameters as the first round. The cycling conditions were 5 min at 95°C; 40 cycles of 1 min at 95°C, 1 min

Table 1. Primer Pairs Used to Detect Each Virus.

Virus	Corresponding genome region	Primer name	Sequence (5'–3')	Amplicon size	References
<i>Adenovirus</i>	DNA polymerase	pol-F	CAGCKCKGTTTRTGYAGGGT	261 bp	Li et al. (2010a)
		pol-R	GCHACCATYAGCTCCAACCTC		
		pol-nF	GGGCTCRTTRGTCCAGCA		
		pol-nR	TAYGACATCTGYGGCATGTA		
<i>Vesivirus</i>	Helicase	hel-1	GTCCCAGTATTCGGATTTGTCTGCC	350 bp	Neill et al. (1995)
		hel-2	AGCGGGTAGTTTCAGTCAAGTTCACC		
<i>Circovirus</i>	Replication initiator protein	CV-F1	GGIAYICCCICAYYTICARGG	400 bp	Li et al. (2010b)
		CV-R1	AWCCAICCRTARAARTCRTC		
		CV-F2	GGIAYICCCICAYYTICARGGITT		
		CV-R2	GYTGYTCRTAICCRTCCCACCA		
<i>Coronavirus</i>	RNA polymerase	1b-F	GGTTGGGACTATCCTAAGTGTGA	440 bp	Woo et al. (2005)
		1b-R	CCATCATCAGATAGAATCATCATA		
<i>Morbillivirus</i>	Phosphoprotein	pho-F	ATGTTTATGATCACAGCGGT	429 bp	Barret et al. (1993)
		pho-R	ATTGGGTTGCACCACTTGTC		

Both the corresponding genome region targeted and the sequences are shown.

at 56°C, 1 min at 72°C, and a final incubation at 72°C for 10 min.

Coronavirus PCR screening was carried out with primers targeting a 440 bp segment of the RNA-dependent RNA polymerase (*1b*) gene, 1b-F and 1b-R (Woo et al. 2005). Although these primers were originally designed to detect human coronaviruses, BLASTN analysis showed coverage of representative sequences of all genera within the *Coronavirinae* subfamily. The 25 µL reaction mix contained 2 µL of cDNA, 1 mM MgCl₂, 2.5 µL PCR buffer, 0.2 µM of each primer, 0.2 mM dNTP, and 0.5 U Taq DNA polymerase (PromegaTM). The cycling conditions were 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 49°C and 1 min at 72°C, and final extension of 5 min at 72°C.

The detection for morbillivirus RNA was conducted by targeting a 400 bp segment of the viral phosphoprotein (*P*) gene, using the primers pho-F and pho-R (Barret et al. 1993). The 25 µL reaction mix contained 2 µL of cDNA, 1 mM MgCl₂, 2.5 µL PCR buffer, 0.2 µM of each primer, 0.2 mM dNTP, and 0.5 U Taq DNA polymerase (PromegaTM). The cycling conditions were 5 min at 95°C, 35 cycles of 45 s at 94°C, 45 s at 49°C, 45 s at 72°C, and final extension step of 5 min at 72°C.

PCR products were electrophoresed in 1.5% agarose gel, and amplicons were visualized under UV light after staining with ethidium bromide. Standard precautions were taken to avoid PCR contamination; blank controls

without template were included in every set of five assays. The following controls were used for each PCR protocol: an autochthonous bovine adenovirus type 2 isolate, the porcine circovirus type 2 15/5P strain, a commercial vaccine for canine distemper virus and canine coronavirus (VencofarmaTM), and a synthetic gene fragment corresponding to the expected vesivirus PCR product (InvitrogenTM). Analytical sensitivity was verified under our laboratory conditions. Protocols for circovirus and adenovirus can detect ten molecules per reaction and 100 per reaction for morbillivirus, coronavirus, and vesivirus.

Sequencing and Phylogenetic Analysis

Amplicons were cloned using a pCR[®]2.1-TOPO[®] cloning kit (InvitrogenTM) before being submitted for nucleic acid sequencing. Samples were sequenced with the Big Dye terminator cycle sequencing ready reaction (Applied BiosystemsTM) in an ABI-PRISM 3100 Genetic Analyzer (ABITM), according to the manufacturer's protocol. Sequence analyses were performed with BLASTN and BLASTX software (<http://www.ncbi.nlm.nih.gov/blast/>). Nucleotide sequences were aligned and compared to human-, animal-, and sewage-associated virus sequences available in the GenBank database using ClustalX 2.0 (Larkin et al. 2007). The alignments were performed with the BioEdit Sequence Alignment Editor Program, version 7.0.9 (Hall 1999). The algorithm to generate the phyloge-

netic trees was selected using Modeltest 3.7 software (Posada and Crandall 2001). The phylogenetic tree of the deduced amino acid sequences of the partial *rep* gene sequence was constructed using the Neighbor-Joining method (Saitou and Nei 1987). Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2013). The amplified circovirus DNA fragments were provisionally named fur seal circovirus (FSCV) and fur seal feces-associated circoviridae (FSfaCV), followed by the last two digits of the sample number.

RESULTS

Molecular Detection of Viral DNA in Feces

Six of the 21 fecal samples from the South American fur seals examined were positive for circoviruses (G1508, G1516, G1518, G1520, G1527, and G1546). Fragments of

adenovirus DNA polymerase gene fragments were obtained from two of the 21 fecal samples (G1517 and G1526) of the same species. The samples from the two Subantarctic fur seals were not PCR positive for any of the targeted viruses. None of the 23 samples was positive for morbillivirus, vesivirus, or coronavirus nucleic acids (Table 2).

Nucleotide Sequences and Phylogenetic Analyses

Nucleotide sequences corresponding to part of the circovirus *rep* gene obtained from six *A. australis* fecal samples were submitted to GenBank (accession numbers KF712527 to KF712532). A local alignment performed with BLASTX showed that sequences FSCV-20 and FSCV-46 were similar to *rep* sequences of members of the *Circovirus* genus. These sequences displayed approximately 69% amino acid identity with the closest mammal circovirus sequence found in GenBank (SFbeef/USA/2010, ADV37434).

Table 2. PCR Results Showing the Presence or Absence of Viral RNA/DNA Fragments.

Species	Sample	Geraci code*	PCR resulting fragments presence/absence				
			<i>Circovirus</i>	<i>Adenovirus</i>	<i>Vesivirus</i>	<i>Coronavirus</i>	<i>Morbillivirus</i>
<i>A. australis</i>	G 1507	2	—	—	—	—	—
<i>A. australis</i>	G 1508	2	+	—	—	—	—
<i>A. australis</i>	G 1510	2	—	—	—	—	—
<i>A. australis</i>	G 1514	2	—	—	—	—	—
<i>A. australis</i>	G 1515	2	—	—	—	—	—
<i>A. australis</i>	G 1516	2	+	—	—	—	—
<i>A. australis</i>	G 1517	2	—	+	—	—	—
<i>A. australis</i>	G 1518	2	+	—	—	—	—
<i>A. australis</i>	G 1519	2	—	—	—	—	—
<i>A. australis</i>	G 1520	2	+	—	—	—	—
<i>A. australis</i>	G 1521	2	—	—	—	—	—
<i>A. australis</i>	G 1524	2	—	—	—	—	—
<i>A. australis</i>	G 1525	2	—	—	—	—	—
<i>A. australis</i>	G 1526	2	—	+	—	—	—
<i>A. australis</i>	G 1527	2	+	—	—	—	—
<i>A. australis</i>	G 1529	2	—	—	—	—	—
<i>A. australis</i>	G 1534	2	—	—	—	—	—
<i>A. tropicalis</i>	G 1535	2	—	—	—	—	—
<i>A. tropicalis</i>	G 1537	2	—	—	—	—	—
<i>A. australis</i>	G 1544	2	—	—	—	—	—
<i>A. australis</i>	G 1545	2	—	—	—	—	—
<i>A. australis</i>	G 1546	2	+	—	—	—	—
<i>A. australis</i>	G 1549	2	—	—	—	—	—

* According to Geraci and Lounsbury (1993).

The sequences FSfaCV-16, FSfaCV-18, and FSfaCV-27 differed from *Circovirus* and *Cyclovirus rep* sequences, originating a separate branch and an uncultured marine *rep* encoding virus sequence (AGA18314). The sequence obtained from FSfaCV-08 could also not be classified as any of

the known *Circoviridae* genera and was distant from the other five sequences described in this study. FSfaCV-08 clustered in a separate branch in phylogenetic analysis, clustering with *rep* encoding sequences from viruses detected in humans (ADF80731) and in starfish (KR186219)

Figure 2. Phylogenetic tree constructed with the deduced amino acid sequences of the partial *rep* gene sequence of *Circoviridae* members using the Neighbor-Joining method (Saitou and Nei 1987). Percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) is shown next to the branches (Felsenstein 1985a). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed using the p-distance method (Felsenstein 1985b) and are in the units of the number of amino acid differences per site. Sequences detected in this study are marked in blue. Accession numbers are indicated in figure. Chicken anemia virus (AB027470) was used as outgroup. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2013) (Color figure online).

Table 3. Comparison Between Partial Sequences of Adenovirus *pol* Gene (261 nt) Identified in Samples G1526 and G1517, and Human Adenovirus Type C (HAdV-C).

Sequences	HAdV-C	G1526	G1517
HAdV-C	ID	0.994	0.954
G1526	0.994	ID	0.948
G1517	0.954	0.948	ID

(Fig. 2). The alignment showed identical deduced amino acid sequences for FSCV-20 and FSCV-46, which aligned with *Circovirus* genus sequences. The same was observed for FSfaCV-16, FSfaCV-18, and FSfaCV-27 (data not shown).

Adenovirus sequences were also obtained and submitted to GenBank. BLASTX analysis and global alignment indicated that these sequences were closely related to the *pol* gene sequence of human adenovirus C (HAdV-C), showing high nucleotide identity (Table 3) and amino acid similarity (data not shown) with the HAdV-C strain (KF429754).

DISCUSSION

Marine mammals such as pinnipeds can often be food sentinels in aquatic and coastal environments because they have long life spans and share similar environments and foods with humans (Reddy et al. 2001; Conrad et al. 2006). In Brazil, although there are no breeding colonies of pinnipeds, many animals haul out on the shores of Rio Grande do Sul between autumn and spring, during their post-reproductive migration (Pinedo 1990; Oliveira et al. 2006).

In the present study, adenovirus and circovirus DNA segments were detected in pinniped feces. Phylogenetic analyses revealed circulation of three potential new circoviruses in South American fur seals. The deduced amino acid sequences of circoviruses detected in samples FSCV-20 and FSCV-46 were identical to each other and highly similar to other sequences of members in the *Circovirus* genus known to infect multiple animal species (Li et al. 2010b, 2011).

In addition, the sequences from FSfaCV-16, FSfaCV-18, and FSfaCV-27 were identical to each other, but completely distinct from sequences in any of the genera within *Circoviridae*. These fragments branched separately and exhibited a low degree of similarity in their deduced amino

acid sequence when compared to the others, being only distantly related to *rep* encoding uncultured marine virus sequences.

Finally, the sequence from FSfaCV-08 was also quite distinct from any *Circoviridae* genus, but was similar to *rep* encoding viruses found in humans, starfish, bats, and mosquitoes. This strongly suggests that FSfaCVs represent viruses that might belong to different unclassified genera. Further studies are needed to investigate their ability to infect other mammals and viral epidemiology related to ingested food.

South American fur seals have mainly pelagic feeding habits, but also feed in shallower waters (Ponce de León 2000; Franco-Trecu 2010). Under conditions of poor health or starvation, these animals may be forced to search for hydration in freshwater sources, such as coastal effluents, possibly coming into contact with, or drinking polluted water (Connell et al. 2012). The adenovirus fragments detected in samples G 1517 and G 1526 were similar to human adenovirus C, suggesting the potential for exposure of fur seals to water contaminated with human feces.

No viruses were detected in the Subantarctic fur seals. This may be due the small sample size (2) that was tested. No RNA virus was detected in any of the samples collected. Some of the assays used were designed to detect specific animal viruses and may therefore have been unable to detect distinct and new strains of viruses within *Coronaviridae* and *Caliciviridae*. It is also important to note that RNA is generally less stable than DNA and more susceptible to degradation, decreasing the potential for recovering good quality RNA for testing. Thus, further studies using broader assays would be needed to confirm the absence of coronavirus, morbillivirus, and vesivirus in these samples.

Even though some viral infections reported in pinnipeds are associated with clinical signs, there are many whose impact on animal health has yet to be determined (Britt et al. 1979; Kennedy et al. 1988). Our findings

indicate the occurrence of adenoviruses and circoviruses in fur seals found along the coast of Southern Brazil. Additionally, the segments unrelated to other viruses in the *Circovirus* genera suggest these are new viruses perhaps belonging to a new *Circoviridae* genus.

CONCLUSIONS

Further studies to detect viral agents in pinnipeds may provide a better understanding of what viruses may be present which will allow us to monitor for the emergence of new viruses and the potential for viral sharing between wildlife, domestic animals, and humans. Our study indicated circovirus and an adenovirus related to Human Adenovirus C were present in South American fur seals. These results may be useful in improving our knowledge of the host range of these viruses.

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