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Commentary

A Retrospective Oxford Nanopore Metagenomic Investigation of Unexplained Mortality in Cape Fur Seals (*Arctocephalus pusillus pusillus*) in Namibia

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Simple Summary

Cape fur seals living along the Namibian coast occasionally experience large mortality events whose causes remain unknown. In 2020, more than 7000 seals died during one such event, but routine diagnostic investigations did not identify a clear cause. In this study, we re-examined stored lung samples collected from seals found dead between 2020 and 2022 using NGS sequencing approach that can simultaneously detect genetic material from many different microorganisms without targeting a specific pathogen in advance. Several potentially disease-associated microorganisms were identified, particularly in animals that died during the unexplained 2020 event. The most notable finding was an adenovirus previously described in sea lions, which was detected in two Cape fur seals and whose complete genetic sequence could be reconstructed. Other potentially relevant microorganisms, including bacteria, parasite, and fungi, were also detected. However, because the samples had been collected several years earlier and additional diagnostic investigations were unavailable, these microorganisms cannot be confirmed as the causes of death. Our findings demonstrate how modern sequencing methods can be applied retrospectively to stored samples to investigate unexplained wildlife mortality events and improve knowledge of infectious agents circulating in marine mammals.

Abstract

Cape fur seals (*Arctocephalus pusillus pusillus*) periodically experience large mortality events along the Namibian coast, some of which remain etiologically unresolved despite routine diagnostic investigations. This retrospective study investigated archived lung samples collected from six Cape fur seals found dead between 2020 and 2022, including five animals recovered during the major 2020 mortality event, using molecular assays and Oxford Nanopore shotgun metagenomic sequencing. A total of 1,430,814 reads were generated, of which 81.8% were taxonomically classified. Notably, the most relevant putative pathogens detected in this study were identified in animals originating from the previously unexplained 2020 mortality event. The strongest finding was the detection of California sea lion adenovirus 1 (CSLAdV-1; *Mastadenovirus otariidae*) in two seals, allowing complete viral genome reconstruction. The two genomes were highly similar to each other and to the reference CSLAdV-1 genome, retained all 30 coding sequences, and showed only limited amino-acid variation, mainly within the low-complexity region of the Fiber protein. Other potentially relevant microorganisms detected in seals from the same mortality event included *Toxoplasma gondii*,

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Clostridium septicum, *Clostridium perfringens*, *Paraclostridium sordellii*, and *Cryptococcus* spp. However, because samples were collected post-mortem and several years before metagenomic analysis, and because histopathological and targeted confirmatory investigations were unavailable, the causal contribution of these agents to mortality should not be overinterpreted. To our knowledge, this study provides the first complete-genome characterization of CSLAdV-1 in wild Cape fur seals and demonstrates the value of retrospective metagenomics for identifying plausible infectious candidates in previously unresolved wildlife mortality events.

Keywords: Cape fur seals; metagenomics; CSLAdV-1; Nanopore; Namibia; stranding

1. Introduction

The family *Adenoviridae* comprises non-enveloped viruses with linear, double-stranded DNA genomes of approximately 25–48 kbp and medium-sized icosahedral capsids, generally about 90 nm in diameter [1]. Adenoviruses replicate within the host-cell nucleus and infect a wide variety of vertebrate hosts, ranging from fish to humans. The family is currently divided into six genera. Members of the genus *Mastadenovirus* infect mammals, whereas those of the genus *Aviadenovirus* infect birds. The genus *Ichtadenovirus* currently includes a fish adenovirus, while members of the genus *Testadenovirus* infect turtles. Members of the genus *Barthadenovirus*—formerly known as *Atadenovirus*—have been detected in squamate reptiles, birds, ruminants, marsupials, and tortoises, whereas members of the genus *Siadenovirus* infect birds, turtles, and frogs. The clinical outcome of adenovirus infection may range from subclinical infection to severe or lethal disease (https://ictv.global/report/chapter/adenoviridae/adenoviridae; accessed on 26 August 2026) [1].

Adenoviruses have traditionally been considered strongly host-associated, with individual viruses generally restricted to a single host species or to a group of evolutionarily closely related hosts [2]. However, increasing evidence indicates that several adenoviruses can cross host-species barriers. For example, canine adenovirus 1 (CAdV-1) (*Mastadenovirus canidae*) has been detected not only in domestic dogs but also in numerous wild carnivore species, including wolves, foxes, raccoons, bears, and skunks [3].

Broader host ranges are particularly common among members of the genera *Barthadenovirus* and *Siadenovirus*, for which host-switching events may have played an important role in their evolutionary history. For example, snake adenovirus 1 has been detected in corn snakes, boas, pythons, and eastern indigo snakes [4–6], whereas snake adenovirus 2 has been reported in eastern corn snakes, California kingsnakes, and asp vipers [7]. Both viruses have also been detected in tortoises [8]. Similarly, the siadenovirus psittacine adenovirus 2 has been described in representatives of at least a dozen species belonging to the order *Psittaciformes*) [9–11].

Mastadenoviruses are associated with a broad clinical spectrum, ranging from asymptomatic or persistent infections to diseases characterised by high morbidity and mortality [12,13]. In humans, severe and disseminated adenovirus infections occur predominantly in immunocompromised patients and may manifest as pneumonia, fulminant hepatitis, encephalitis, haemorrhagic cystitis, or multiorgan disease [14]. In animals, mastadenoviruses have been associated with respiratory disease in horses [15], cattle [16], pigs, sheep, and goats [12]. Among canids, CAdV-1 is the causative agent of infectious canine hepatitis, a potentially life-threatening systemic disease that is particularly severe in young dogs. The virus has also been associated with encephalitis in foxes. The closely related canine adenovirus 2 (CAdV-2) primarily causes infectious canine laryngotracheitis and contributes to the canine infectious respiratory disease complex, commonly known as kennel cough,

particularly in kennelled or densely housed dog populations [17]. Nevertheless, subclinical mastadenovirus infections are also common in both domestic and wild animal hosts. In otters, canine adenovirus 1 has been identified in captive Eurasian otters (*Lutra lutra*), whereas a distinct novel adenovirus has been reported in southern sea otters [18,19]. Other Mastadenoviruses have also been identified in marine mammals, and novel viruses and species continue to be reported [20]. Marine mammal adenoviruses belong to the genus *Mastadenovirus* and have been detected in cetaceans (bottlenose dolphins, sei whales, bowhead whales, beluga whales, and harbour porpoises) pinnipeds (northern elephant seals and Pacific harbour seals), and otters [13,18,20–29].

California Sea lion adenoviruses-1 (CSLAdV-1) (*Mastadenovirus otariidae*) were originally isolated from California sea lions [23], while closely related adenoviruses have subsequently been detected in liver or faecal samples collected from several pinniped species, including South African and South American fur seals, South American sea lions hosted in a Japanese aquarium, and Hawaiian monk seals [20,26]. Adenovirus-associated lesions in pinnipeds include acute necrotising hepatitis, ulcerative keratitis, corneal oedema, iridocyclitis, arteritis, and conjunctivitis. In ocular diseases, viral infection of endothelial cells has also been documented [30,31].

Between 2020 and 2022, the Central Veterinary Laboratory (CVL) in Windhoek, Namibia, received tissue samples collected from 6 Cape fur seals (*Arctocephalus pusillus pusillus*) found dead along the Namibian coastline. Despite the routine diagnostic investigations performed at the time, the cause of death could not be established for any of the animals included in the study. A retrospective investigation was therefore conducted on archived samples, taking advantage of recent advances in molecular diagnostics and sequencing technologies.

The present study describes the first detection and complete genome characterisation of a CSLAdV-1 in lung tissue collected from a Cape fur seal. In addition, potential co-infecting microorganisms were investigated using metagenomic sequencing performed with Oxford Nanopore Technologies.

2. Materials and Methods

2.1. Study Population

The Cape fur seal, *Arctocephalus pusillus pusillus*, also known as the South African fur seal, is the only pinniped taxon endemic to the African continent and represents one of the most abundant marine mammals along the south-western African coastline. Its distribution extends from southern Angola and Namibia to the western and southern coasts of South Africa, with breeding colonies located mainly along the Atlantic coast of the Benguela Current system. The total population of the subspecies has been estimated at approximately 1.7 million individuals, distributed across several breeding colonies in Angola, Namibia and South Africa [32,33]. Namibia supports a particularly large proportion of this population, with an estimated 1.2 million seals occurring in 26 colonies, highlighting the country's importance for the conservation and ecological monitoring of this species [33].

Cape fur seals are highly gregarious otariids that form dense breeding aggregations on beaches, rocky shores and coastal peninsulas. Adult males arrive at breeding colonies from October onwards to establish territories, while females give birth mainly between November and January and subsequently alternate periods ashore for nursing with foraging trips at sea [34,35]. This life-history strategy makes breeding colonies key locations for studying population dynamics, reproductive ecology, foraging behaviour and exposure to anthropogenic pressures.

In Namibia, several colonies are of particular ecological, economic and conservation relevance. Cape Cross, located north of Swakopmund along the Skeleton Coast, is widely

recognised as one of the largest breeding colonies of Cape fur seals worldwide. The Cape Cross Seal Reserve is described by the Namibian Ministry of Environment, Forestry and Tourism (https://www.meft.gov.na/national-parks/cape-cross-seal-reserve/214/; accessed on 12 August 2026) as a sanctuary for the world's largest breeding colony of Cape fur seals, with up to approximately 210,000 animals present during the breeding season, particularly in November and December. Other important Namibian colonies include Pelican Point near Walvis Bay, a readily accessible colony that has become highly relevant for tourism, behavioural studies and rescue activities related to marine-debris entanglement; Atlas Bay and Wolf Bay in the Lüderitz area, historically associated with commercial seal harvesting; Van Reenen Bay, which has been included in ecological and dietary studies; and more remote colonies distributed along the central and northern Skeleton Coast [33,35].

2.2. Archive Samples

Between 2020 and 2022, the Central Veterinary Laboratory (CVL), Windhoek, Namibia, received tissue samples from six Cape fur seals found dead along the Namibian coastline between Walvis Bay and Cape Cross, including Henties Bay, in the Erongo Region (Table 1). Five seals were recovered during the major mortality event that occurred in late 2020 along the central Namibian coast, with its epicentre at Pelican Point, near Walvis Bay, during which overall mortality was estimated to exceed 7000 animals. The sixth carcass was recovered in 2022 from the Bird Island guano platform at Walvis Bay following a suspected case of avian influenza. Available tissue samples included lung, liver, spleen and brain and were submitted to the CVL for diagnostic investigation according to the clinical and epidemiological context.

Table 1. Metadata relative to samples included in the study.

Subject	Date	Location	Sex	Age
F13	September 2020	Pelican point—Walvis bay	Female	Adult
F62	October 2020	Pelican point—Walvis bay	Male	Juvenile
F75	November 2020	Henties bay	Female	Juvenile
F76	October 2020	Cape cross	Female	Adult
F78	February 2022	Bird Island—Walvis bay	Female	Juvenile
F79	November 2020	Cape cross	Male	Juvenile

Samples from all animals had been previously tested for rabies virus, canine distemper virus, avian influenza virus, *Brucella* spp. and *Mycoplasma* spp., with negative results in all cases. Consequently, the individual cause of death had not been established for any of the seals included in the study. At the population level, severe nutritional stress associated with reduced or not constant prey availability was considered the most likely underlying factor in the 2020 mortality event, although a definitive aetiological diagnosis was not established.

The aim of this study was to retrospectively investigate, several years after sample collection, the possible causes of death of Cape fur seals found dead along the Namibian coastline. To this end, archived lung samples stored over the years in the virology biobank of the Central Veterinary Laboratory were analysed using a metagenomic approach. Only lung tissues were included in the study, as other tissues from the same animals had been used for previous diagnostic investigations and were no longer available in the laboratory archives. This strategy was intended not only to explore potential viral agents associated with the unexplained mortality of these animals, but also to assess the presence of unconventional or previously undetected viruses harboured by Cape fur seals.

2.3. Sample Processing

For each animal, lung samples were processed for combined DNA and RNA extraction. Briefly, tissues were homogenised in 1 mL of sterile phosphate-buffered saline using a TissueLyser LT system (Qiagen, Hilden, Germany). After DNA digestion using the HL-SAN nuclease kit (ArcticZymes Technologies ASA, Tromsø, Norway) to deplete host genome, genetic material was extracted from 200 µL of tissue homogenate using the High Pure Viral Nucleic Acid Kit (Roche, Basel, Switzerland), according to the manufacturer's instructions, and eluted in a final volume of 100 µL of the elution buffer provide by the kit.

2.4. Oxford Nanopore Shotgun Metagenome Library Preparation and Reads Processing

The metagenomic composition of the samples was investigated using a rapid metagenomic sequencing workflow. Total RNA was reverse-transcribed using random nonamer primers (RLB RT 9N) and a template-switching oligonucleotide (TSO), enabling the generation of cDNA with universal primer-binding sequences at both ends. The resulting cDNA was subsequently amplified and barcoded by PCR using the Rapid PCR Barcoding Kit 24 V14 (SQK-RPB114.24) (Oxford Nanopore Technologies, Oxford, UK), followed by sequencing adapter attachment and sequencing on an R10.4.1 MinION flow cell (FLO-MIN114) using a MinION Mk1B device (Oxford Nanopore Technologies, Oxford, UK), as described by Claro et al. [36], with modifications. Two negative controls, constituted by molecular biology grade water, were processed as diagnostic samples form extraction phase onwards and included in the analysis.

Microbial taxa were preliminarily identified using the *wf-metagenomics* workflow (v2.14.2) implemented in the EPI2ME platform (v5.4.0). Sequencing reads in FASTQ were analysed against the PlusPF-8 reference databases (1 January 2025). Taxonomic classification was performed using Kraken2 [37], with the confidence score threshold set to 0.01, according to the workflow recommendations. The resulting taxonomic profiles were inspected using the interactive HTML report generated by the workflow. To further validate taxonomic assignments, representative reads from the most relevant potentially pathogenic taxa and those supported by a limited number of reads were subjected to BLASTn analysis against the NCBI nucleotide database (nt).

2.5. Reference-Based Genome Reconstruction

Considering the high number of reads classified as CSLAdV-1 by Kraken analysis, a reference-guided mapping approach was performed to reconstruct the complete viral genome. Sequencing reads were aligned against the reference sequence using minimap2 [38] with the map-ont preset. The resulting SAM files were converted into BAM format, filtered to retain mapped alignments, coordinate-sorted, and indexed using SAMtoolsv1.24 [39].

A global consensus sequence was generated from the sorted BAM file using SAMtools consensus. A minimum depth of 10 reads was required for base calling. Positions with coverage below this threshold were explicitly replaced with ambiguous nucleotides (N).

To investigate the presence of co-infection with member of the same species, within-sample viral haplotypes were reconstructed using HaploDMF (https://github.com/dhcai21/HaploDMF; accessed on 24 November 2026) [40]. Only mapped alignments were provided to the program. To limit computational demand and reduce differences associated with highly heterogeneous sequencing depths, a maximum of 5000 mapped alignments per sample was randomly selected using reservoir sampling. When fewer than 5000 mapped alignments were available, all alignments were retained. The complete reference region was considered, and the minimum expected haplotype abundance was set to 0.05. SNV detection was performed using an assumed sequencing error rate of 0.10, a significance level of 0.05, an SNV-frequency parameter of 0.80, minimum read-support parameters of 10 and

5 reads, a maximum gap parameter of 15, and a minimum SNV number of 20. Deep matrix factorisation model training was conducted using a weight parameter of 0.2, a learning rate of 0.001, 10 epochs, and a batch size of 512. Haplotype clustering and reconstruction were performed using Ward's hierarchical clustering algorithm, a depth parameter of 5, clustering thresholds of 0.95 and 0.90, and a maximum of 10 iterations. As for the global consensus sequence, reconstructed haplotypes were conservatively masked by replacing positions covered by fewer than ten reads with N.

2.6. Phylogenetic Analysis of Members of the Mastadenovirus

Reference sequences of the genus *Mastadenovirus*, whose accession numbers are available from the ICTV (https://ictv.global/report/chapter/adenoviridae/adenoviridae/mastadenovirus; accessed on 12 June 2026), were downloaded from GenBank and aligned with those obtained in the present study using MAFFTv7.526, [41]. Thereafter, a phylogenetic tree was reconstructed using the maximum-likelihood approach implemented in IQ-TREE v3.1.3 [42]. The best-fitting substitution model was selected based on the Bayesian information criterion (BIC) calculated using the same software and was identified as GTR + G + I. Branch support was assessed using the approximate likelihood-ratio test (aLRT) with 1000 replicates.

3. Results

3.1. Shotgun Metagenome Analysis

Overall, 1,430,814 reads were generated, of which 1,170,360 (81.8%) were taxonomically classified by the Kraken2-based workflow, while 260,454 (18.2%) remained unclassified. A summary of per-sample quality is reported in Supplementary Table S1. Bacterial and viral assignments accounted for 306,428 (21.4%) and 126,844 (8.9%) reads, respectively. Viral reads were almost exclusively assigned to California sea lion adenovirus 1, which accounted for 126,803 reads and 99.97% of the entire viral dataset. The negative controls yielded a total of 3998 reads were obtained from the two negative controls, including 1892 reads from N1 and 2106 reads from sample N2. Taxonomic classification identified 142 reads assigned to the host (Homo sapiens), corresponding to 3.6% of the total, and 396 reads (9.9%) assigned to fungal species, mainly Malassezia restricta, Rhizoctonia solani, and Zymoseptoria tritici. All remaining reads were grouped into an unclassified category, accounting for 3460 reads (86.5% of the total).

The distribution of viral reads was highly heterogeneous among samples and different microorganisms; potential pathogens displayed a variable presence in the samples (Table 2). Sample F75 showed the strongest viral signal, with 121,586 adenovirus reads, corresponding to 85.4% of all reads generated from this sample and 99.97% of its viral reads. Adenovirus was also clearly detected in F76, although at a lower abundance, with 3496 reads. Only trace of adenovirus assignments was observed in the remaining samples. A low-abundance signal corresponding to *Sigmatorquevirus otari1* was also detected in F75 but was supported by only 35 reads and was therefore considered preliminary. No meaningful viral signal was identified in F13, F62, F78 or F79.

The bacterial profiles differed markedly among samples. F62 was almost entirely dominated by *Clostridium septicum*, which accounted for 247,761 reads, corresponding to 97.4% of the bacterial reads and 95.2% of the total reads generated from this sample. A further clostridial signal was observed in F75 and F76, although in F76 most reads could not be confidently resolved below the genus level. F79 was primarily characterised by *Paraclostridium sordellii*, represented by 2817 reads and accounting for 33.2% of the bacterial reads. In contrast, the bacterial profiles of F13 and F78 were dominated by the genus *Psychrobacter*, with 16,083 and 2530 reads, respectively.

Table 2. Selected microorganisms of potential biological relevance detected by metagenomic sequencing.

Sample	Main Candidate	Other Candidates
F13	*Toxoplasma gondii*—1507 reads	Cryptococcus spp.—736 reads
F62	*Clostridium septicum*—247,761 reads	*C. perfringens*—791 reads
F75	*Mastadenovirus otariidae*—121,586 reads	*C. septicum*—1729 reads
F76	*Mastadenovirus otariidae*—3496 reads	No convincing bacterial co-agent
F78	No candidate	*Psychrobacter sanguinis*—620; *T. gondii*—65; *Campylobacter jejuni*—37
F79	*Paraclostridium sordellii*—2817 reads	*C. septicum*—207; *Vagococcus lutrae*—140

A limited number of reads were assigned to potentially parasitic eukaryotes. Apicomplexan reads were primarily detected in samples F13 and F78, accounting for 4993 and 278 reads, respectively. In F13, 1507 reads were assigned to *Toxoplasma gondii*, 829 to *Plasmodium* spp., and 311 to *Babesia* spp. Similar but substantially weaker signals were observed in F78, where 65 reads were assigned to *T. gondii*, 125 to *Plasmodium* spp., and 18 to *Babesia* spp. Other potentially parasitic protozoa were detected at considerably lower abundance across the entire dataset, including *Naegleria* spp. (65 reads), *Giardia duodenalis* (46 reads), Microsporidia (21 reads), *Spironucleus salmonicida* (21 reads), *Leishmania* spp. (9 reads), and *Trypanosoma* spp. (1 read).

Fungal reads were detected in all samples but were predominantly concentrated in F13 and F78, accounting for 9433 and 1392 reads, respectively. Considerably lower numbers were observed in F79 (272 reads), F62 (171 reads), F75 (155 reads), and F76 (31 reads). At the phylum level, 4067 reads were assigned to *Ascomycota* and 3380 to *Basidiomycota*. The most represented fungal genera included *Cryptococcus* spp. (786 reads), *Fusarium* spp. (625 reads), *Puccinia* spp. (499 reads), *Brettanomyces* spp. (446 reads), *Vanrija* spp. (296 reads), *Alternaria* spp. (237 reads), *Candidozyma* spp. (207 reads), *Colletotrichum* spp. (192 reads), *Sporisorium* spp. (186 reads), and *Malassezia* spp. (175 reads). Most reads assigned to *Cryptococcus* spp. occurred in F13 (736 reads), with a substantially weaker signal in F78 (37 reads), and included assignments to the *Cryptococcus gattii* species complex (429 reads) and the *Cryptococcus neoformans* species complex (356 reads). Other potentially relevant fungi were detected at considerably lower abundance, including *Aspergillus* spp. (23 reads), *Penicillium* spp. (17 reads), *Candida* spp. (7 reads), and *Saccharomyces* spp. (2 reads).

3.2. Sea Lion Adenoviruses Complete Genome

3.2.1. Reference-Based Genome Annotation and Comparison

Reference-guided annotation was successfully performed for both reconstructed genomes using NC_024150 as the reference sequence. Both genomes showed limited divergence from the reference, and no haplotypes were detected within samples. F76 (Acc.number PZ851658) was 31,706 nt long, three nucleotides shorter than the 31,709-nt reference genome, while the obtained F75 (Acc.number PZ851659) had the same length as the reference genome. The two viruses have a percentage of identity of 99.9527% among each other, while the reference genome was 99.9495% and 99.9748% identical to F75 and F76, respectively.

All 30 reference coding sequences were successfully transferred to both genomes and passed the implemented structural quality-control criteria. No frameshifts, internal stop codons, ambiguous nucleotides, or alterations of the expected reading frames were detected, indicating that the coding capacity of both genomes was substantially preserved.

In F76, 29 of the 30 predicted proteins were identical to their reference counterparts. The only protein-level differences involved the Fiber protein, which increased from 610 to 612 amino acids. In the selected alignment, the changes affected the glutamate- and proline-rich low-complexity region between approximately residues 388 and 442 of the reference protein. Comparison of the repeat blocks indicated that the replacement of the EPEPA motif by EPEKVEP could be represented as two amino-acid substitutions together with a two-residue insertion. However, the exact positions of these changes were alignment-dependent because of the repetitive and low-complexity nature of the region.

F75 showed a slightly higher degree of protein-level variability, although all predicted proteins remained structurally intact (Table 3). Twenty-five of the 30 proteins were identical to the reference. Three single amino-acid substitutions were detected: V104A in E1A, S262T in pIIIa, and T259S in the 100-kDa protein. The E3 37.9-kDa protein contained an additional glutamate residue inserted between E234 and Y235 (p.E234_Y235insE) and consequently increased from 320 to 321 amino acids.

Table 3. Amino-acid differences detected in the predicted proteins of F75 and F76 compared with the reference sequence NC_024150.

Sequence	Gene/Protein	Reference Length (aa)	Query Length (aa)	Amino-Acid Difference(s)	Net Effect
F76	Fiber protein	610	612	The replacement of the EPEPA motif by EPEKVEP.	Net increase of 2 aa
75	E1A	245	245	V104A	Single amino-acid substitution
	pIIIa	570	570	S262T	Single amino-acid substitution
	100-kDa protein	686	686	T259S	Single amino-acid substitution
	E3 37.9-kDa protein	320	321	Insertion of E between E234 and Y235 (*p.E234_Y235insE*).	Net increase of 1 aa
	Fiber protein	610	612	Replacement of the AEPEP motif by AEPEKVE	Net increase of 2 aa

The F75 Fiber protein also increased from 610 to 612 amino acids. The differences were confined to the glutamate- and proline-rich low-complexity region and included the substitutions P403N and P437A. In addition, replacement of the AEPEP motif at positions 418–422 by AEPEKVE could be parsimoniously represented as one amino-acid substitution and an in-frame insertion of two residues. Because of the repetitive and low-complexity nature of this region, the precise identity and positional assignment of the substitution and insertion within the repeat block were alignment-dependent.

3.2.2. Phylogenetic Analysis

Phylogenetic analysis of *Mastadenovirus* genus clearly confirmed the clustering of the obtained sequences with the *Mastadenovirus otariidae*. The clade containing F75 and F76 comprised mastadenoviruses associated exclusively with bats and marine mammals among the reference sequences with available accession numbers. Bat-associated sequences originated from Egyptian fruit bats (MG551742), Schreibers's bent-winged bats (KT698852 and KT698856), straw-coloured fruit bats (AP018374), and Leschenault's rousettes (KX961095, KX961096, and OR998962). Marine mammal-associated sequences originated from a California sea lion (KJ563221), common bottlenose dolphins (LT841149 and KR024710), and a harbour porpoise (KY352473). Within this clade, F75 and F76 were most closely related

to the California Sea lion adenovirus sequence KJ563221, forming a strongly supported subclade (node support = 100) (Figure 1).

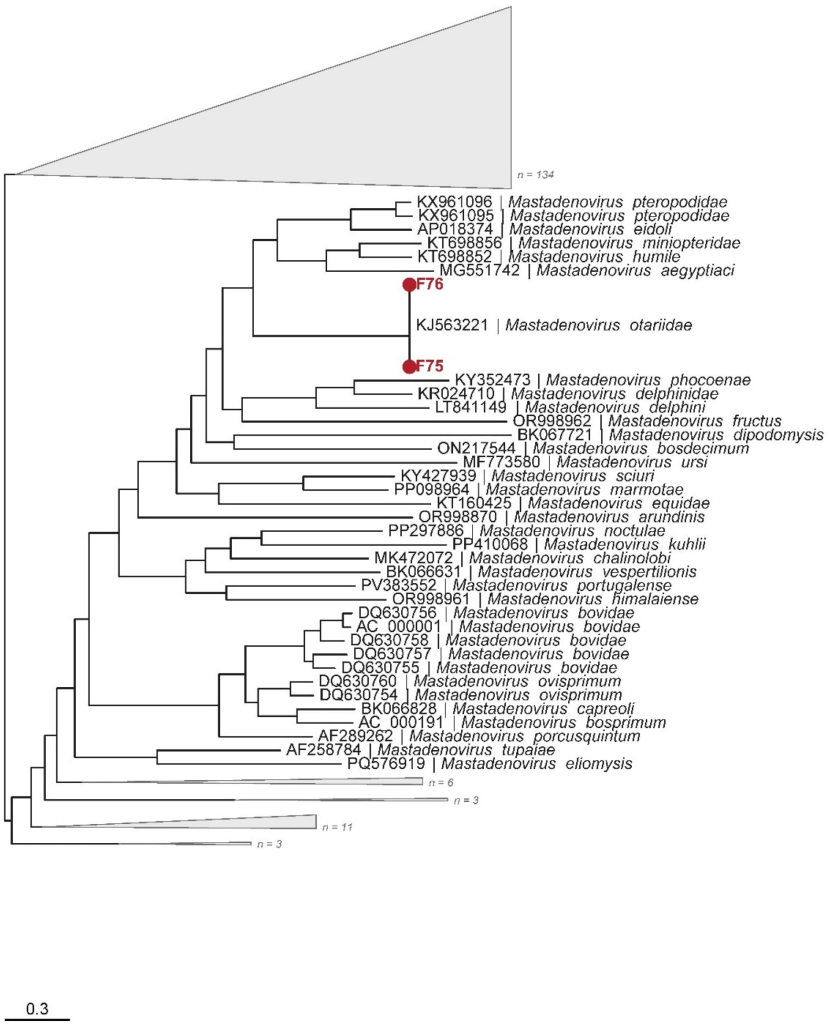


Figure 1. Maximum-likelihood phylogenetic tree based on the complete genome alignment of *Mastadenovirus* genus members. Branches leading to sequences obtained in this study are colored in red. More distant clades have been collapsed for graphical clarity. Branch support values are reported near the corresponding nodes.

4. Discussion

Cape fur seals (*Arctocephalus pusillus pusillus*) form large, densely populated colonies at discrete coastal sites. Their highly gregarious behaviour may facilitate pathogen transmission, while seasonal, foraging, and breeding-related movements among colonies and marine areas may contribute to pathogen dispersal. Historically, infectious disease outbreaks have been documented in Cape fur seals. A major mortality and abortion event occurred at Cape Cross, Namibia, in 1994, involving approximately 10,000 deaths and 15,000 abortions. Although the event was primarily associated with severe starvation, *Streptococcus phocae* was isolated from adult seals, a pup, and aborted fetuses, suggesting that bacterial respiratory and systemic infections contributed to mortality [43]. More recently, a sustained rabies outbreak has emerged in Cape fur seals along the southern African coastline. Retrospective testing of archived samples confirmed infections dating back to at least August 2022, while epidemiological and molecular evidence supports sustained seal-to-seal transmission within the population [44,45]. In Namibia, the first confirmed case was detected in a Cape fur seal at Walvis Bay Lagoon in June 2025 [46]. By July 2026, more than 100 cases had

been confirmed in South Africa and Namibia, providing evidence of sustained seal-to-seal transmission [45]. In addition, HPAI H5N1 was detected for the first time in a Cape fur seal in South Africa in 2026, although sustained transmission within the species has not yet been demonstrated [47]. Additionally, several other pathogens have been reported to be responsible for severe outbreaks in related species and in other countries [31]. However, in several instances, the observed mortality peaks remained unexplained. In 2020, another major mortality event was recorded in Namibia. Despite efforts to identify potential infectious agent responsible for the stranding event using traditional, pathogen-specific assays, no etiological agent could be identified. The present study aimed to preliminarily evaluate, through an untargeted metagenomic approach, the presence of unexpected or previously overlooked pathogens that are not typically investigated during routine diagnostic activities.

Notably, despite the extremely limited sample size imposed by the logistical constraints associated with sampling these animals in Namibia and the potential sensitivity loss due to nucleic acid, particularly RNA, degradation before necropsies, potential pathogens belonging to all major categories—parasites, bacteria, and viruses—were detected and identified as plausible candidates contributing to the observed mortality. It should be emphasized that the detection of a limited number of reads assigned to a given taxon cannot be considered robust evidence of its biological relevance, owing to the inherent uncertainty of taxonomic assignment and the absence of a demonstrated causal association. More generally, the detection of microbial nucleic acids alone should be interpreted with caution when inferring an etiological role. Additionally, although BLASTn analyses largely confirmed the initial Kraken2 classifications, particularly for the most abundant taxa, some ambiguous assignments were observed among taxa supported by a limited number of reads, highlighting the uncertainties associated with species-level identification from metagenomic data. Therefore, low-abundance detections should be interpreted with particular caution, and greater emphasis was placed on microorganisms supported by higher read counts and with previously documented pathogenic potential. For these reasons, particular attention was given to microorganisms supported by a higher number of sequencing reads and for which pathogenic potential has previously been documented, at least in other host species. Furthermore, the availability of only lung samples in the laboratory archives represents an unavoidable limitation of the study, as pathogens exhibiting strict tissue tropism may have gone undetected. Consequently, the involvement of other infectious agents potentially contributing to the observed mortality cannot be excluded. Access to a broader range of tissue samples would undoubtedly have allowed a more comprehensive investigation and strengthened the overall completeness of the study.

As a final consideration when interpreting these findings, differences in sequencing read abundance across samples and microorganisms should be interpreted with caution, as they may be influenced by several technical and biological factors, including sequencing depth, sample preservation conditions, and post-mortem microbial changes. More generally, as with any molecular biology-based technique, the detection of potentially pathogenic microorganisms, even when supported by a relatively high number of sequencing reads, does not necessarily indicate an active infection or establish a causal relationship with the observed mortality. Sample F13, and to a much lesser extent F78, contained a high number of reads matching *Toxoplasma gondii*. Ingestion of sporulated oocysts is considered the most likely source of infection; however, consumption of infected paratenic hosts and vertical transmission may also occur. Evidence of *T. gondii* infection obtained through serological surveys and direct detection methods is extensive and confirms its broad host range, including infections associated with clinical disease. *T. gondii* infection has been associated with mortality in several species [48]; pathological findings attributable to toxoplasmosis have also been reported in species belonging to the same genus as the host investigated here,

including the New Zealand fur seal (*Arctocephalus forsteri*), California sea lions (*Zalophus californianus*) and the South American fur seal (*Arctocephalus australis*) [49–51]. In those species, lesions in the brain, heart, eye, and pituitary gland were reported. Although in most instances animals are immunocompetent enough to control the infection, few might experience overt disease [48–50]. Additionally, evidence indicates that sea otters affected by protozoal encephalitis may display abnormal behaviour and become more vulnerable to predation or other risky situations, suggesting that toxoplasmosis may also impair animal health through indirect pathways [52]. Other potentially parasitic organisms, like Babesia spp., were also detected; however, they were represented by only a few sequencing reads. Although the same pathogens were not detected in the tested negative controls, considering both the very limited read support and the lack of meaningful biological evidence supporting the susceptibility of this host species to these infections, such findings should be interpreted with particular caution and should not be considered sufficient evidence of true infection since they are more likely to reflect spurious taxonomic assignments.

On the other hand, reads assigned to *Cryptococcus* spp. may be of potential biological relevance, since clinical cryptococcosis has previously been documented in pinnipeds. *Cryptococcus albidus* was associated with pulmonary and lymph-node lesions in a California sea lion [53], whereas disseminated *C. gattii* infection was reported in harbor seals. However, to the best of our knowledge, cryptococcosis has not been reported in Cape fur seals yet [54]. Therefore, metagenomic detection alone cannot confirm infection or a causal role, and targeted molecular, histopathological, or culture-based confirmation would be required.

Clostridium perfringens has previously been associated with enterotoxemia, necrotizing enteritis, myositis, and wound-associated infections in pinnipeds [55]. *C. perfringens* is, however, regarded as a common microorganism in marine environments [56], and it has also been frequently isolated from the intestinal microbiota of apparently healthy seals and sea lions, indicating that its detection alone does not demonstrate a pathogenic role [55]. However, different case reports also indicate their pathogenic potential in marine mammals. *C. perfringens*-septicaemia was reported in a stranded common dolphin in California [57]; *C. perfringens*-induced colonic rupture in a captive Californian sea lion [58] and cases of myositis and skin lesions in different marine mammals were also documented [59,60]. Thus, a role in seal mortality cannot be disregarded.

Conversely, although *C. septicum* is a recognized cause of severe histotoxic and systemic disease in several mammalian species, convincing evidence of disease caused by this bacterium in pinnipeds appears to be lacking. Therefore, particularly considering the post-mortem origin of the samples, these metagenomic assignments should be regarded as potentially relevant but unconfirmed.

The detection of *Paraclostridium sordellii* in lung tissue represents, to our knowledge, the first metagenomic report in *Arctocephalus pusillus*. Also in this case, given the well-documented capacity of Clostridium species to rapidly colonize tissues post-mortem, it is not possible to determine from sequencing data alone whether this finding reflects an ante-mortem infection or post-mortem bacterial proliferation. In all these instances, further investigation (targeted histopathology, toxin-gene PCR, culture) is needed to establish the clinical significance of this detection.

Finally, *Psychrobacter* spp. dominated the bacterial profiles of samples F13 and F78. Members of this genus are commonly associated with marine environments and have been detected as components of the normal skin, respiratory, gastrointestinal, and genital microbiota of marine mammals. Although some species, including *P. sanguinis*, have occasionally been associated with opportunistic infections [61], their detection alone does not demonstrate a pathogenic role. Therefore, these findings may reflect commensal colonization, environmental contamination, or post-mortem proliferation. Of note, subject F78, the

only sampled in the context of a sporadic stranding event, was also the one for which no consistent evidence of an infectious cause could be identified.

Probably the most relevant finding is the identification and complete genome characterization of two *Mastadenovirus otariidae* strains. Although episodes of hepatitis attributed to adenovirus have been recorded in sea lions since 1978 [62], when five stranded California sea lions along the Los Angeles coast developed a fatal disease in the presence of large intranuclear inclusions in hepatocytes observed by light microscopy and adenovirus-like virions demonstrated by electron microscopy, it was only in 2011 that molecular methods allowed the identification of a distinct adenovirus [23]. Thereafter, it was described in other otariid species, including the South American sea lion and the South African fur seal, which died acutely from fulminant necrotizing hepatitis [26]. However, those animals were captive individuals housed in the same aquarium as a California sea lion showing the same clinical presentation. A later captive Hawaiian monk seal case in Hawaii yielded a partial polymerase sequence 100% identical to CSLAdV-1, extending confirmed sequence-based detection into a phocid species [29]. In contrast, the present study represents the first report of CSLAdV-1 in wild South African fur seals stranded during a major mortality outbreak.

The previous studies suggest a potential role of CSLAdV-1 as the etiological agent responsible for seal mortality and warrant particular consideration of the role that this pathogen might play in the health and conservation of this and other species, also considering its apparently less restricted host tropism compared with other adenoviruses. However, further investigations involving a larger number of animals, different tissue types, pathological examinations and independent molecular confirmation should be necessary clarify the possible involvement of the detected microorganisms in this mortality event. Also, in hypothesising CSLAdV-1 as a plausible etiological agent, not all sampled animals were positive for CSLAdV-1. Therefore, an exclusive role of this agent in disease development and mortality cannot be claimed, despite its previously demonstrated pathogenic potential. Secondary infections or opportunistic pathogens may also have benefited from a transiently weakened host or altered immune response, potentially contributing to mortality after the viral infection had already been cleared. Although immunomodulatory and immune-evasion mechanisms are well documented among mastadenoviruses, particularly through proteins encoded by the E3 region [29], no direct evidence is currently available demonstrating systemic immunosuppression associated with CSLAdV-1 infection in pinnipeds. Nevertheless, concomitant infections and the detection of CSLAdV-1 together with other viral agents have occasionally been reported [63]. Therefore, a potential contribution of adenovirus-induced modulation of host immune responses to the occurrence or severity of concurrent infections cannot be excluded, although this hypothesis remains to be experimentally demonstrated. The two Namibian strains, collected approximately one month apart at two different locations, showed very high nucleotide identity with each other and with the CSLAdV-1 reference genome obtained from a California sea lion. Despite the substantial geographical and temporal separation between the Namibian viruses and the reference strain, the remarkably high sequence conservation supports the presence of a highly conserved CSLAdV-1-like virus in free-ranging southern African otariids. Despite the overall viral genome structure appears conserved, some non-synonymous mutations in specific proteins were observed leading to single amino-acid substitutions or amino acids insertions. Particularly, in the Fiber protein changes affecting the glutamate- and proline-rich low-complexity region were observed, leading to a net increase of 2 amino acids. The C terminal region of the Fiber is highly atypical with similarity to non-adenoviral adhesive proteins, like adhesive proteins such as *Trypanosoma cruzi* trans-sialidase and *Streptococcus pneumoniae* CbpA and latency-associated nuclear antigen (LANA) of some gammaherpesviruses [28]. However, the role of this protein region and its variability have

not yet been elucidated. Based on this evidence, adenovirus testing, at least by molecular assays, and further strain characterization should be encouraged during the routine processing of samples from seals, and ideally from other species, involved in unexplained stranding events.

5. Conclusions

This retrospective study demonstrates the value of metagenomic sequencing for investigating unexplained mortality in wild marine mammals, particularly when conventional diagnostic testing has failed to identify an etiological agent. The most relevant result was the detection and complete genome characterization of CSLAdV-1 in two wild Cape fur seals during a major, previously unexplained, stranding event. The high sequence identity between the two strains and the reference virus indicates substantial genomic conservation and supports the circulation of this adenovirus in wild southern African otariids. Nevertheless, in the absence of histopathological, immunohistochemical, or targeted molecular evidence linking the virus to compatible lesions, its causal contribution to mortality cannot be definitively established and present findings should be regarded as preliminary evidence whose potential clinical and epidemiological significance remains to be determined.

The detection of *C. septicum*, *P. sordellii*, *T. gondii*, *Cryptococcus* spp., and other microorganisms identifies additional potential health threats, but it should be interpreted cautiously because of the post-mortem origin of the samples, possible bacterial overgrowth, limited read support for some taxa, and the absence of confirmatory testing. These findings should therefore be considered hypothesis-generating rather than diagnostic. Expanded surveillance based on freshly collected tissues, standardized necropsies, histopathology, targeted PCR, culture, toxin-gene detection, and whole-genome sequencing will be required to clarify the epidemiology, pathogenic potential, host range, and conservation relevance of CSLAdV-1 and other infectious agents circulating in Cape fur seals. More broadly, the timely integration of metagenomic sequencing into wildlife mortality investigations, particularly during large-scale mortality events, could facilitate the early detection of potentially relevant microorganisms and guide subsequent targeted diagnostic investigations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani16233788/s1, Supplementary Table S1. Summary of sequencing output and Kraken2 taxonomic classification by sample. Percentages are calculated relative to the total reads in each sample.

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