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NOTE

Marine Mammal Science

Detection of cetacean morbillivirus in dolphin feces and the potential application for live cetacean health monitoring

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Cetacean mortality resulting from infectious disease is significant. Viral, bacterial, fungal, and parasitic infections have been identified as causes of death from many regions of the world, with infectious disease responsible for up to 60% of deaths in stranded cetaceans when quantified according to natural and anthropogenic causes from isolated areas (Cuvertoret-Sanz et al., 2020; Diaz-Delgado et al., 2018). Cetacean morbillivirus (CeMV) has been a leading cause of death in single and mass stranding events occurring worldwide since the 1980s (Cunha et al., 2021; Cuvertoret-Sanz et al., 2020; Marutani et al., 2022). All that is known of CeMV infections in free-ranging cetaceans comes from necropsy and cause of death investigations on stranded animals (Van Bressem et al., 2014), sampling of cetaceans harvested by indigenous hunters (Nielsen et al., 2000), serology on odontocetes sampled during capture release studies (Black et al., 2019; Bossart et al., 2017; Stone et al., 2005), and from the exhale of two live humpback whales (*Megaptera novaeangliae*; Groch et al., 2020).

Investigations of stranded cetaceans are limited by carcass recovery and condition. Carcass recovery rates of cetaceans irrespective of condition are low, estimated to range between 2% and 25% (Caretta et al., 2017; Williams et al., 2011). Autolysis often precludes histopathological investigation and the identification and isolation of pathogens in many stranded animals that may be in moderate to advanced states of decomposition. Stranding location or difficulty of terrain may also limit accessibility for necropsy and sample collection. Even when carcasses are

recovered fresh and full necropsy and histopathology are feasible, postmortem diagnostics provide only limited information about the spread of pathogens in live populations and may miss subclinical infections or the identification of individuals recovering from prior infection. Additionally, epidemiological analysis of the emergence and spread of infectious agents across populations and species requires monitoring of populations to determine incidence and prevalence. Live cetacean health monitoring and postmortem-based disease investigations of stranded or bycaught animals are complementary to understanding disease threats to protected species.

Health assessments of free-ranging cetaceans are limited by the challenges associated with biological sample collections from live animals that spend much of their lives below the ocean surface. On a global scale, minimal epidemiological data have been obtained from live cetacean populations with the exception of temporary capture and release efforts for near-shore resident common bottlenose dolphins (*Tursiops truncatus*; Barratclough et al., 2019; Bossart et al., 2017; Schwacke et al., 2014), beluga whales (*Delphinapterus leucas*; Thompson et al., 2022), Hector's dolphins (*Cephalorhynchus hectori*; Stone et al., 2005), and narwhal (*Monodon monocerus*; Black et al., 2019). Bottlenose dolphin health assessments in Florida and South Carolina detected infections with CeMV, dolphin papilloma and herpes viruses, fungal disease, and other pathogens (Bossart et al., 2017). However, the success of such extensive capture-release programs requires coordination of large numbers of highly trained personnel and multiple vessels, which can be cost prohibitive and involves procurement of permits and consideration for animal welfare. The logistics involved in capture release efforts to conduct health monitoring are magnified when managing larger cetacean species that are only found offshore. Consequently, remote collection of samples such as a skin/blubber biopsy, exhalation or fecal plume of free-ranging cetaceans has been more widely applied across cetacean species and locations.

Biopsy samples of skin and blubber are routinely collected from both large and small cetaceans and have been utilized for a wide range of applications. Biopsy sampling of epidermal lesions in large whales for molecular characterization has been suggested (Hunt et al., 2013), but to our knowledge has not yet been applied to screen for specific pathogens or the presence of disease in free-ranging cetaceans. Cetacean morbillivirus RNA was detected by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) in a frozen skin lesion sample from a fin whale (*Balaenoptera physalus*) live-stranded in Scotland, by reverse transcriptase polymerase chain reaction (RT-PCR) in skin samples from four of five striped dolphins (*Stenella coeruleoalba*) that stranded in the Mediterranean between 2011 and 2015, and in Guiana dolphins (*Sotalia guianensis*) from Brazil where histopathology and immunohistochemistry confirmed systemic morbillivirus infection in all cases (Dagleish et al., 2021; Groch et al., 2020; Rubio-Guerri et al., 2018). RT-qPCR demonstrated viral loads that were lower in skin when compared to other organ samples from the stranded fin whale (Dagleish et al., 2021) and it is currently unknown if highly sensitive RT-qPCR techniques would detect CeMV presence in skin samples of free-ranging animals with early or subclinical infection.

More recently, exhale (blow) samples have been used to measure hormones, to identify respiratory pathogens, and to study the microbiome of the respiratory system (e.g., Apprill et al., 2017; Burgess et al., 2018; Groch et al., 2020; Raverty et al., 2017). Blow examination assesses respiratory and metabolic functions, as whales and dolphins forcefully exhale an air/water mixture combined with mucus secreted by mucus glands in the bronchial mucosa. Meta-transcriptomic analysis identified six novel viruses in Eastern humpback whale blow collected by drone, and morbillivirus gene sequences were successfully detected by RT-qPCR in blow samples of humpback whales in Brazil (Geoghegan et al., 2018; Groch et al., 2021). Respiratory disease is commonly identified in stranded cetaceans (e.g., Diaz-Delgado et al., 2018) and blow sample collection offers significant promise for disease monitoring of free-ranging cetaceans.

Fecal plumes of wild cetaceans can be identified by observation when following behind pods of cetaceans, by using specially trained scent detection dogs to find floating whale feces and through the use of drones (Ayres et al., 2012; Baird et al., 2022; Rolland et al., 2006). Fecal samples are typically collected with a dip net, container, or bag and contain significant amounts of seawater (Hunt et al., 2013). Steroid hormones have been characterized by measuring fecal metabolites in free-ranging killer whales (*Orincus orca*), right whales (*Eubalaena glacialis*), and gray whales (*Eschrichtius robustus*) (Ayres et al., 2012; Lemos et al., 2020; Rolland et al., 2012). Fecal samples have been

utilized to study diet and prey preferences (Dunshea et al., 2013; Ford et al., 2016; Smith & Whitehead, 2006) as well as to identify parasites, microbiome communities, and organic pollutants, but the identification of fecal pathogens in cetaceans is limited to enteric pathogens when surveying the gut microbiome of fin and sperm whales (*Physeter macrocephalus*; Hunt et al., 2013; Marangi et al., 2021).

Analysis of feces for presence of pathogens by polymerase chain reaction (PCR), RT-PCR, or RT-qPCR may provide an opportunity to detect emerging infections and to monitor for established enteric disease agents (including viruses, bacteria, parasites, and other pathogens) that cause systemic infections. The objective of this work was to determine if it is possible to detect CeMV by RT-qPCR in cetacean fecal samples from live animals, where dilution by natural seawater at the time of collection is expected. To investigate this question, we conducted a simulated seawater dilution experiment with feces sampled from the gastrointestinal tract of a stranded Fraser's dolphin (*Lagenodelphis hosei*) that was positive for CeMV. The strain of morbillivirus in this animal was determined to be novel (Fraser's dolphin morbillivirus) and dissimilar to the beaked whale morbillivirus previously identified in Hawai'i (West et al., 2013, 2021).

The gastrointestinal tracts of two Fraser's dolphins that stranded in 2018 and in 2022 off Maui, Hawai'i, were excised at the time of necropsy and frozen intact. During a later examination, gastrointestinal tracts were thawed, and fecal samples were obtained directly from the rectum prior to freezing in sterile specimen containers. A 30-mg fecal sample of the CeMV positive Fraser's dolphin that stranded in 2018 was used to prepare five 10-fold serial dilutions in simulated sea water. Seawater was simulated by dissolving 35 g of sodium chloride in 965 g of distilled water (35‰). Brain tissue from the same animal was used as a positive control (West et al., 2021). Distilled water and feces from the Fraser's dolphin that stranded in 2022 and tested negative for morbillivirus in a suite of organ samples were used as no template and negative controls. Total RNA was extracted using RNeasy Plus Mini Kits (Qiagen, Germantown, MD). Manufacturer protocols were followed with the following adaptation: fecal samples were agitated on a plate shaker during lysing for 10 min to promote homogenization. A Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, MA) was used to confirm that RNA was successfully extracted. cDNA synthesis and RT-PCR were performed using the iTaq Universal SYBR Green One-Step kit (Bio-Rad, Hercules, CA). The RT-qPCR protocol was developed using primers and thermocycler settings adapted from Barrett et al. (1993) and the kit manufacturer's protocols (Bio-Rad, Hercules, CA). Primers included CeMV forward primer 5′ATGTTTATGATCACAGCGGT 3′ and CeMV reverse primer 5′ATTGGGTTGCACCACTTGTC 3′. The assay was performed at 50°C for 10 min, 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 60 s, with a final extension at 72°C for 10 min in the Applied Biosystems QuantStudio3 real-time PCR system (Thermo Fisher Scientific). Amplified products were cleaned using QIAquick Gel Extraction Kits (Qiagen, Germantown, MD) and then sequenced at the Advanced Studies in Genomics, Proteomics, and Bioinformatics lab at the University of Hawai'i at Mānoa. Sequencing results were processed using BioEdit sequence alignment editor and analyzed in NCBI Nucleotide BLAST.

A 248 bp partial *P* gene sequence from positive RT-qPCR products demonstrated a 98.8% match to the Fraser's dolphin morbillivirus strain described by West et al., 2021 (GenBank accession no. MZ485915.1). RT-qPCR amplification of CeMV was successful up to a dilution of 1:1,000 (Figure 1).

We demonstrated amplification of Fraser's dolphin morbillivirus RNA in simulated seawater dilutions by RT-qPCR in a fecal sample from an infected cetacean that had previously stranded. Morbillivirus RNA was previously detected in cerebellum, lung, liver, spleen, and lymph node tissues of the stranded Fraser's dolphin during the initial screening for CeMV. The dolphin was diagnosed with interstitial pneumonia, meningitis, and lymphoid depletion characteristic of acute CeMV disease (West et al., 2021). CeMV is typically associated with respiratory and neurological disease, but the presence of the virus has been demonstrated in multiple organs during systemic infections (e.g., Van Bressem et al., 2014). Morbillivirus antigen was detected in salivary glands of Guiana dolphins (Groch et al., 2020), in the intestinal epithelium of a bottlenose dolphin stranded in the Canary Islands in 2005 (Sierra et al., 2014) and in the intestine of one of the striped dolphins with systemic morbillivirus infection examined by Rubio-Gerri et al. (2018). RT-PCR testing of intestinal tract samples of two pilot whales (*Globicephalus*

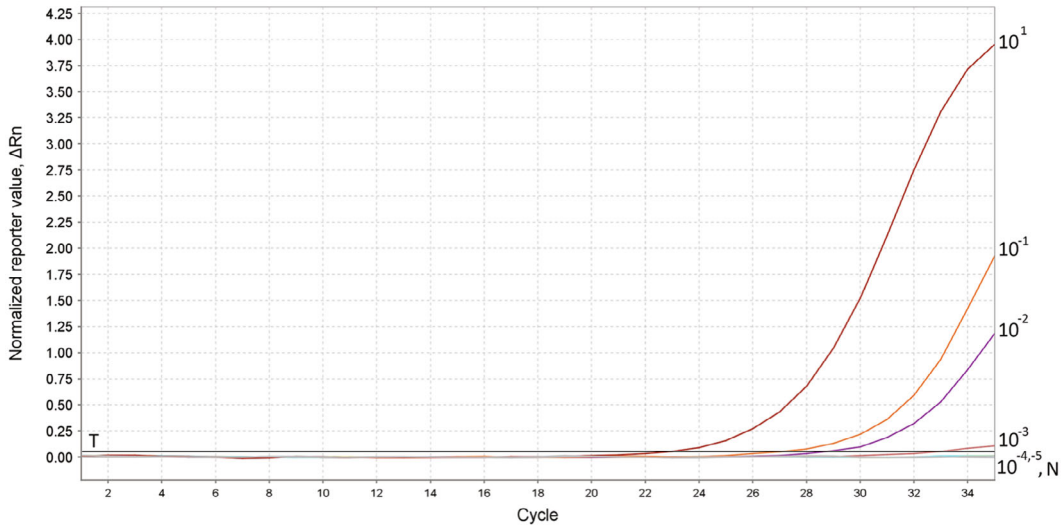

FIGURE 1 Detectability of CeMV in diluted feces using RT-qPCR (QuantStudio Design and Analysis Software). Amplification plot of *P* gene sequence following 5-fold serial dilution of CeMV positive Fraser's dolphin feces. Negative control (N) from CeMV negative Fraser's dolphin. Detectable amplification was not observed in $10^{-4,-5}$ following 35 cycles using the threshold, T = 081834 ΔRn.

macrorhynchus) with systemic morbillivirus infection detected viral RNA (Sierra et al., 2016). The presence of the virus in glands and tissues associated with the digestive tract in previously stranded cetaceans with systemic morbillivirus disease combined with the detection in feces of the Fraser's dolphin strongly suggests viral shedding by feces.

Another morbillivirus, peste de petits ruminants virus (PPRV) clinically causes lymphoid tissue necrosis, pneumonia, and systemic viremia in infected sheep and goats (Begum et al., 2021) and demonstrates 75% genetic similarity to the Fraser's dolphin morbillivirus described from our study animal (West et al., 2021). Bataille et al. (2019) detected PPRV viral RNA in fecal samples from Saanen goats that were experimentally infected beginning at day 5, up to day 14, post infection. This suggests that early viremia and gastrointestinal viral shedding could occur in cetaceans after infection by morbillivirus and viral RNA may be detectable in fecal samples from infected wild animals. We demonstrated viral RNA in a fecal sample by RT-qPCR in serial dilutions of seawater to a factor of 10^{-3} (Figure 1). In this case, the fecal sample was tested from an individual with known acute disease, and we are uncertain if RT-PCR would be as effective in detecting early or subclinical infections in fecal samples from free-ranging individuals. More sensitive techniques, such as RT-qPCR, may be ideal for fecal sample screening from live animals where sea water dilution is a factor and detecting lower loads of viral RNA is important.

With the exception of severe parasitosis, little is known about gastrointestinal disease of cetaceans because of the difficulty involved in obtaining well-preserved samples. Common parasites isolated and identified in fecal samples of cetaceans include *Cryptosporidium* spp., *Microsporidia*, and *Giardia* (Altieri et al., 2007; Fayer, et al., 2008). The gastrointestinal tract degrades quickly after death and poor preservation limits pathological evaluation of the enteric mucosa for disease in animals with intervals of 24 hr or more between death and necropsy. Information about bacterial and viral gastrointestinal disease is largely limited to captive and experimental studies of cetaceans. Rubio-Guerri et al. (2015) identified a novel adenovirus by PCR in fecal samples from four captive dolphins with self-limiting gastroenteritis. Adenovirus was believed to be the etiologic agent because it was only detected during the time of illness in the dolphins and other pathogens were not identified. A real-time PCR assay successfully detected 1–5 bacteria/μl in cetacean feces experimentally spiked with marine *Brucella* bacteria (Sidor et al., 2013). *Brucella* has also been demonstrated in fecal samples of captive aborted fetuses with systemic infection (Meegan et al., 2012), and it is

possible that during early infection and active spread of the bacterium it may be detectable in intestinal epithelia and feces.

Microbiome surveys of feces from free-ranging baleen and toothed whales have described microbial communities shared among marine mammals and identified distinct communities among host species. These studies often list common pathogenic bacterial and fungal genera that represent potential threats to human health in accordance with the One Health Principle (Atlas, 2013; Glaeser et al., 2022; Li et al., 2019). Microbiome surveys do not typically target pathogens known to cause disease in cetaceans and the significance of bacterial detection for cetacean health is unknown. An exception is fecal examination for parasitic, fungal, and bacterial pathogens causing infections in marine mammals from two sperm whales and two fin whales in the Pelagos Sanctuary (Northern-Western Mediterranean Sea; Marangi et al., 2021). *Erysipelothrix* spp. and *Helicobacter* spp. were found in fin whales, and *Mycobacterium* spp. and *Fusobacterium* spp. were found in sperm whale fecal samples. Samples were negative for *Brucella* spp., *Staphylococcus* spp., *Leptospira* spp., *Nocardia spp*., and *Actinomyces* spp. by PCR in both species of whale (Marangi et al., 2021).

Fecal samples can be collected relatively easily without causing disturbance to free-ranging cetaceans when using a boat to trail animals or using dogs trained in scent detection. While the collection and examination of feces offer opportunities for the health monitoring of populations, the individual origin of fecal samples may not be known when groups of animals are traveling together, limiting the understanding of pathogen spread. Additionally, fecal samples from some cetacean species may not float. Fecal samples from individuals with low viral load may be highly diluted with seawater, and filtration or centrifugation upon collection and prior to freezing or preservation has the potential to increase the concentration of fecal components. Best practices for fecal sample collection and preservation in the field could be developed in conjunction with laboratory experiments targeted at detecting fecal pathogens in wild cetaceans.

We report on the successful detection of Fraser's dolphin morbillivirus in the feces of an infected animal where CeMV was amplified by RT-qPCR up to a simulated seawater dilution of 1:1,000. This demonstrates the potential for disease screening of wild cetacean fecal samples that could be used to study the spread of pathogens and serve as an important diagnostic tool to assess cetacean health.

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AUTHOR CONTRIBUTIONS

Kristi West: Conceptualization; formal analysis; funding acquisition; investigation; project administration; resources; software; validation; writing – original draft; writing – review and editing. **Ilse Silva-Krott:** Formal analysis; investigation; methodology; validation; writing – original draft; writing – review and editing. **Cody W. Clifton:** Methodology; validation; writing – review and editing. **Conner Humann:** Data curation; resources; validation; visualization; writing – original draft; writing – review and editing. **Nicole Davis:** Resources; writing – review and editing.

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