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Pathogen surveillance in wild bottlenose dolphins *Tursiops truncatus*

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ABSTRACT: The number and prevalence of diseases is rapidly increasing in the marine ecosystem. Although there is an increase in the number of marine diseases observed world-wide, current understanding of the pathogens associated with marine mammals is limited. An important need exists to develop and apply platforms for rapid detection and characterization of pathogenic agents to assess, prevent and respond to disease outbreaks. In this study, a broad-spectrum molecular detection technology capable of detecting all sequenced microbial organisms, the Lawrence Livermore Microbial Detection Array, was used to assess the microbial agents that could be associated with wild Atlantic dolphins. Blowhole, gastric, and fecal samples from 8 bottlenose dolphins were collected in Charleston, SC, as part of the dolphin assessment effort. The array detected various microbial agents from the dolphin samples. *Clostridium perfringens* was most prevalent in the samples surveyed using the microarray. This pathogen was also detected using microbiological culture techniques. Additionally, *Campylobacter* sp., *Staphylococcus* sp., *Erwinia amylovora*, *Helicobacter pylori*, and *Frankia* sp. were also detected in more than one dolphin using the microarray, but not in culture. This study provides the first survey of pathogens associated with 3 tissue types in dolphins using a broad-spectrum microbial detection microarray and expands insight on the microbial community profile in dolphins.

KEY WORDS: Diagnostics · Microarray · Microbial community · Molecular detection · Marine diseases · Microbiological culture

INTRODUCTION

The number and prevalence of diseases found in the marine ecosystem is increasing (Harvell et al. 1999, Ward & Lafferty 2004, Fey et al. 2015). While several factors have been associated with this increase, the cause of this increase is not known and the pathways of disease transmission are either not known or poorly understood. An unprecedented survey of seabirds, marine mammals, and sharks on the US East Coast revealed that marine wildlife contains a wide variety of disease-causing microbes, including many that have developed resistance to antibiotics and several that can be transmitted to humans (Bogomolni et al. 2008). Increases have occurred in disease reports for marine mammals (Ward & Lafferty 2004, Gulland & Hall 2007). The incidence of these diseases is increased by (1) new combinations of hosts and pathogens creating an environment favoring the emergence of pathogens in novel species, and (2) anthropogenic factors including contaminants and human activity patterns that stress marine hosts and decrease their capacity to develop appropriate immune responses. Newly described or

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reemerging disease agents or diseases affecting marine mammals include morbilliviruses (Raga et al. 2008), papillomaviruses, dolphin poxvirus (Bracht et al. 2006), lobomycosis, and various neoplastic diseases (Bossart 2011). An estimated 75% of emerging infectious diseases are zoonotic (Chomel et al. 2007) with anthropogenic influences being a common factor in the emergence and reemergence of zoonotic pathogens (Daszak et al. 2001).

Emerging known and unknown pathogens create profound threats to wildlife. Recently, a summary of 76 pathogens that were identified in marine mammals was used to develop a risk analysis for cetaceans (Venn-Watson & Stamper 2011). The 10 highest priority pathogens among small cetaceans were morbillivirus, parapoxvirus, *Brucella* spp. anisakis, calicivirus, herpesvirus, nasitrema, *Clostridium* spp., and toxigenic *Escherichia coli.* Characterization of the bacteria and fungi isolated by culture of samples obtained from dolphins inhabiting the estuarine waters of Charleston, SC and the Indian River Lagoon, FL resulted in observations of a number of pathogenic species (Morris et al. 2011). Antibiotic-resistant bacteria have also been documented in wild dolphins (Greig et al. 2007, Stewart et al. 2014).

While molecular diagnostics have greatly improved the ability to find microbes in marine mammal samples, it has been difficult to assess the impact of pathogens on both individual and population health of marine mammals. One obstacle to better understanding cetacean pathogens is the difficulty of diagnosing the cause of disease in marine mammals. During mortality events, some pathogens can be detected in tissues although it is unknown whether the pathogen contributed to the cause of death or to an underlying condition. For wild populations, a more thorough understanding of baseline exposures to pathogenic agents would provide further understanding on the diagnostic interpretation of pathogens that may be present during mortality events. However, current dolphin health assessment studies include detection only of those microbial and viral agents for which tests are available. There is an important need for the development of rapid response capability using molecular and microbiological diagnostics to identify and study an array of pathogens to which marine mammals may be exposed. Platforms for rapid detection and characterization of pathogenic agents are critically needed to assess, prevent and respond to disease outbreaks.

Recent advances in genomic-based technologies have revolutionized the study of microbial ecology and its influence on infectious diseases in humans (Shafquat et al. 2014). PCR and DNA sequencing approaches have been widely used for pathogen detection and characterization. PCR assays are limited in that only a single or few organisms can be investigated per assay. While DNA sequencing can identify a larger scope of organisms, current DNA sequence analysis methods are lengthy, costly and require significant computational time, and there is a lack of bioinformatic tools to rapidly identify and quantify abundances of species identified in a sample. In an effort to improve high-throughput analysis and detection, Gardner et al. (2010) developed an innovative microarray platform called the Lawrence Livermore Microbial Detection Array (LLMDA) to probe for all known microbiological agents for which whole genomes, segments and plasmid sequences are available. Each probe tests for a particular sequence of DNA and small groups of probes can be used to check for specific bacteria or viruses up to the species level. The microarray targets both conserved and unique genomic regions of sequenced microbial strains. Multiple microbe targets provide confirmation and can serve as an internal validation mechanism. Ease of use by diagnostic personnel is provided by an automated data analysis algorithm, Composite Likelihood Maximization Method (CLiMax), which is integrated with a web interface that enables LLMDA data analysis within 30 min.

In this study, both the microbiological culture method and the molecular detection method, the LLMDA were used to analyze the pathogens in blowhole, gastric and fecal samples collected from wild dolphins. The results from the 2 methods were compared and selected pathogens were additionally confirmed using polymerase chain reaction (PCR).

MATERIALS AND METHODS

Sample collections and assessment

Dolphin health assessments were performed in Charleston, SC as part of the Bottlenose Dolphin Health and Risk Assessment (HERA) Project. This comprehensive, integrated, multi-disciplinary research study was initiated in 2003, to assess the health status of Atlantic bottlenose dolphins *Tursiops truncatus* in 2 southeast US sites (Charleston, SC and the Indian River Lagoon, FL) and investigate associations between health status and environmental stressors. Detailed information pertaining to the study site, methods for capture, sampling and release are described by Fair et al. (2006). During the sampling

process, each animal received an entire physical examination, including full body photo-documentation, diagnostic ultrasound, blood and urine collection, blubber and lesional biopsies, and microbiologic and cytologic sampling of the blowhole, gastric fluid and feces as described (Fair et al. 2006). In August 2013, health assessments were performed on 19 dolphins from the estuarine waters of Charleston, SC under National Marine Fisheries Permit No. 14352-02 and approved by the Florida Atlantic University Institutional Animal Care and Use Committee. Swab samples were collected from blowhole, gastric fluid and anus from 8 dolphins and selected for analysis for pathogen comparisons using LLMDA and microbiological methods (Table 1). The other 11 dolphins did not have all 3 types of swabs collected, and were not included in the present study. Age was determined by counting post-natal dentine layers in an extracted tooth (Hohn et al. 1989). In addition to these pathogen tests, samples were collected to determine clinical and immune status, disease and contaminant exposure and antibiotic resistant organisms.

Lawrence Livermore Microbial Detection Array analysis

Samples were collected from blowhole, gastric fluid and anus using sterile swabs and placed in cryovials with RNA Later for the microarray analysis. The swab samples were kept cold for 24 h and then frozen and sent on dry ice to LLNL with the sample types blinded for testing using the LLMDA. This study employed the 12-plex 135 000 probe format of this array (version 5), which is restricted to pathogens associated with vertebrate infection, including 1856 viral, 1398 bacterial, 123 archaean, 48 fungal, and 94 protozoan species (current as of December 2011). Strategies for probe design and quality control have been previously published (Gardner et al. 2010). Nucleic acids from the swab samples were extracted using a modified protocol of the Qiagen QIAamp *cador* Pathogen Mini kit. Swabs were placed in 500 µl buffer ATL with 20 µl Proteinase K for 30 min at 55°C. After 30 min, 500 mg each of 106 µm and 500 µm silica/zirconia beads were added to the tubes and the samples were bead-beaten in a Biospec Mini-Beadbeater at speed 48 for 1 min. The samples were centrifuged for 5 min at 15 000 × *g* and the supernatant was collected. Extraction of the supernatant was completed using the QIAamp *cador* Pathogen Mini kit following standard manufacturer's protocols. The quantification of RNA and DNA from each sample was determined using the Life Technologies Qubit fluorimeter.

RNA and DNA from each sample were whole-genome amplified using a whole transcriptome amplification kit from Qiagen with modifications as described by Erlandsson et al. (2011). Following the amplification of each sample, one µg of amplified product was fluorescently labeled, purified and hybridized to the 12-plex LLMDA arrays. Data were analyzed using the automated LLMDA 'CLiMax' algorithm (Gardner et al. 2010). The log likelihood for each of the possible targets is estimated from the BLAST similarity scores of the probe and target sequences, together with the probe sequence complexity and other covariates derived from the BLAST results. Presented are the data for sequences that were equal to or greater than the 99% threshold of random control probes. A minimum of 8 probes per target sequence and greater than 20% of total probes were used to select for positive detection of target sequences.

Table 1. Dolphins *Tursiops truncatus* sampled at Charleston, SC, USA

Dolphin ID	Gender	Age (yr)
862	Male	26
863	Female	10.5
866	Male	33
886	Male	30
8F2	Male	22
8F4	Male	16
8F8	Male	17
8J2	Male	33

Microbiological assessment

Microbiological assessments of blowhole, gastric and fecal swabs were performed by Micrim Laboratories (Fort Lauderdale, FL) for aerobic and anaerobic microbes as previously described (Morris et al. 2011). Briefly, collection of samples from blowhole, gastric fluid and anus for aerobic, anaerobic and fungal cultures were made using Aimes culturettes (MML Diagnostics). Samples were stored in coolers each day and sent on cold packs overnight for analysis. As part of the comprehensive health screening of the Dolphin HERA Project, clinical approaches were used to characterize the bacteria and fungi isolated from the upper respiratory tract (blowhole), gastric fluid, and anus of wild bottlenose dolphins (*Tursiops truncatus*).

PCR analysis of *E. coli* in samples

A set of samples that had *E. coli* detected in the microbial assessment, but not on the LLMDA was tested with an *E. coli* real-time PCR assay. Pan *E. coli* PCR primers (forward: 5′-GGC GAA GCG GCA AAT TTC; reverse: 5′-CCG CAG TAA CCA CAG TAT;) and probe (5′-FAM-GGG TCG GTA CGT CAG GTC ATT GAT CTC-BHQ) were designed at LLNL as described by Gardner et al. (2009), and manufactured by Integrated DNA Technologies. For each reaction approximately 0.5–1.0 ng of extracted DNA from each tested sample was used. Each 20 µl reaction was constructed using the Life Technologies TaqMan® Fast Universal PCR Master Mix (Cat. 4352042) using the manufacturer's recommended protocols. The reactions were cycled for 1 cycle of 2 min at 50°C, 1 cycle of 10 min at 95°C, and 45 cycles of [15 s at 95°C, 1 min at 60°C]. All tested samples were run in triplicate on an Applied Biosystems 7500 Fast instrument.

RESULTS

Microbiological culture results

Table 2 summarizes the culture results of the 3 sample types from 8 dolphins. *Plesiomonas shigelloides* was identified in 7/8 individuals, from both blowhole and fecal samples. *Aeromonas hydrophila* was cultured from 6/8 individuals, in both blowhole and fecal samples. Other bacteria that were culturable from both blowhole and fecal samples are *Edwardsiella tarda*, *Klebsiella pneumoniae* and *E. coli*. Gastric samples did not grow as many bacteria as fecal or blowhole samples. *Bacillus* sp. was only identified from Dolphin 863, and a yeast *Candida galbrata* was cultured from Dolphin 886. Of the microbes cultured from the samples, only *Clostridium perfringens* in the fecal sample of Dolphin 863 was detected by both microarray and culture.

Table 2. Summary of microbial organisms identified by microbiological culture. Sample sources are blowhole (B), gastric fluid (G), fecal (F). Data are from 8 dolphins

Organism	Source	No. positive
Aeromonas hydrophila	B, F	6
Bacillus sp.	G	1
Candida glabrata	G	1
Clostridium perfringens	F	1
Escherichia coli	B, F	3
Edwardsiella tarda	B, F	5
Enterobacter cloacae	B	2
Klebsiella pneumoniae	B, F	2
Plesiomonas shigelloides	B, F	7
Pseudomonas fluorescens	B	2

Microarray analysis of dolphin samples

Nucleic acids extracted from gastric, fecal and blowhole swab samples were analyzed on the LLMDA array. The pathogens detected by the array are shown in Table 3.

Clostridium was detected in 4/8 dolphins and in all 3 sample types, blowhole, fecal and gastric. All other organisms were not detected by culture. This data suggested that the organisms detected by molecular techniques could be either unculturable, or slow growing and not identifiable by standard culture techniques.

An example of the microarray data from Dolphin 886 (fecal sample) is shown in Fig. 1, including log-odds ratios and the array probes detected vs. expected for the 4 bacterial and 1 viral families. Targets are grouped by taxonomic family and are listed within families in decreasing order of conditional log-odds scores.

Table 3. Summary of microbial signatures detected by the Lawrence Livermore Microbial Detection Array. Sample sources are blowhole (B), gastric fluid (G), fecal (F). Data are from 8 dolphins

Organism	Sample source	No. positive
Brevibacillus brevis	G	1
Campylobacter lari 300 plasmid pCL300	B, G, F	2
Clostridium perfringens	B, G, F	4
Clostridium phage phiSM101	F	1
Cyanothece sp. *CCY0110*	B	1
Erwinia amylovora IL-5 plasmid	B, G	2
Exiguobacterium sp. *AT1*	B	1
Frankia sp. *EUN1f*	B, G	3
Helicobacter pylori plasmid	F	2
Lyngbya majuscula 3L	G	1
Photobacterium damselae	F	1
Planococcus donghaensis MPA1U2	G	1
Propionibacterium phage PA6	G	1
Staphylococcus sp. plasmid	G	2
Tursiops truncatus papillomavirus type 1	F	1
Vibrio phage Vf12	F	1

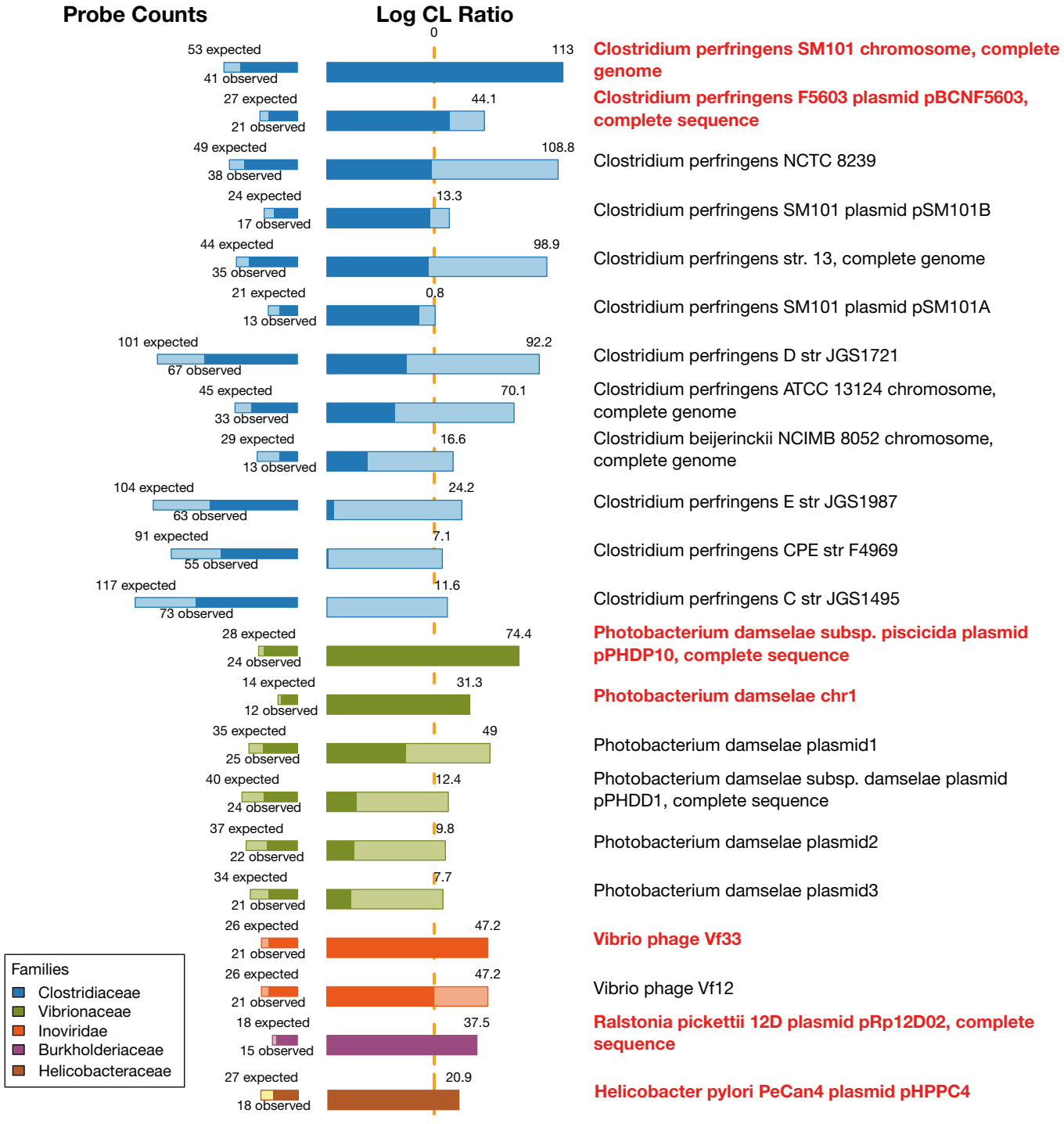

Fig. 1. Microarray results of Dolphin 886 fecal sample, analyzed using the CLiMax analysis. Red text: Targets predicted as likely to be present. Log CL ratio bar graphs: log-odds scores for corresponding predicted target genome; darker and lighter portions represent the conditional and unconditional scores, respectively (conditional score shows the contribution from a target that cannot be explained by another, more likely target above it; unconditional score illustrates that some very similar targets share a number of probes, so that multiple targets may be consistent with the hybridization signals). Probe count bar graphs provide some additional guidance for interpreting the prediction results: larger 'expected' score is obtained by summing the conditional detection probabilities for all probes; smaller 'observed' score (darker portion) is derived by limiting this sum to probes that were actually detected. Because probes often cross-hybridize to multiple related genome sequences, numbers of expected and detected probes often greatly exceed the number of probes actually designed for a given target organism

Clostridium perfringens, *Photobacterium damselae*, Vibrio phage Vf12 and *Helicobacter pylori* plasmid were detected by microarray in this individual. The microarray data from the other dolphins are provided in the Supplement (www.int-res.com/articles/suppl/d116p083_supp.pdf); no microorganisms were detected from Dolphin 8F2 using the microarray.

PCR analysis of *E. coli*

To further confirm the different results from culture and microarray, we performed PCR testing of *E. coli* on selected dolphin samples. *E. coli* was found in 3 samples (from Dolphins 863, 866 and 886) by culture, but not detected by array. PCR results are summarized in Table 4. A gastric sample from Dolphin 8F8 which was negative by culture was also included as a negative control. All samples were negative by PCR, suggesting that the results agree with the microarray results, but not with culture.

Table 4. *E. coli* real-time PCR data to verify the negative detection of *E. coli* by microarray. Sample sources are blowhole (B), gastric fluid (G), fecal (F). nd: *E. coli* not detected; na: not applicable

Sample	Type	Concentration (ng reaction^{-1})	PCR Ct Mean	SD
Dolphin 863	F	1.0	nd	na
Dolphin 866	F	0.5	nd	na
Dolphin 886	B	1.0	nd	na
Dolphin 886	F	1.0	nd	na
Dolphin 8F8	G	0.6	nd	na
E. coli strain ATCC 43895	Positive control	1.0	24.03	0.08
Water	Negative control	na	nd	na

DISCUSSION

Effective surveillance of both wild and managed-care bottlenose dolphins is important to assess the general health of marine mammals and the environmental conditions in the surrounding coastal ecosystem. Classification of the health status of bottlenose dolphins revealed a high prevalence of diseased dolphins, with less than 50% of bottlenose dolphins inhabiting the estuarine waters of Charleston classified as healthy (Reif et al. 2008). Analysis of the microbiome of marine mammals could provide information on the potential association of pathogens with diseases (Nelson et al. 2015).

In this study, we assessed the microbial profiles associated with blowhole, gastric, and fecal samples from 8 wild dolphins using both culture and culture-independent methodologies. By coupling pathogen measurements using traditional microbiological tests with the broad-spectrum pathogen detection technology, LLMDA, we were able not only to test for those organisms previously known to infect marine mammals, but also to survey for additional pathogens that could be associated with these animals. The molecular analysis revealed a bacterial community very different from that detected in culture-based studies. This community included plant, marine, and dolphin infecting microbes that one may not expect to be detectable by common culturing methods.

The bacterium that was detected by both microarray and culturing methods, *Clostridium perfringens*, causes death and disease in dolphins (Buck et al. 1987). *C. perfringens* is a Gram-positive bacterium that exists in spore form in the environment and can cause serious infection in dolphins via skin wounds. The other microorganisms detected by the microarray included a wide diversity of bacteria, viruses, and phages. *Brevibacillus brevis*, *Erwinia amylovora*, and *Frankia* sp. are plant- or fungal-infecting pathogens that have not been implicated in dolphin health. In addition, there were a number of marine-related bacteria that do not appear to be linked to dolphin disease. These include the nitrogen-fixing *Cyanothece* sp., *Exiguobacterium* sp., *Planococcus donghaenisis*, and the blue alga *Lyngbya majuscule*.

In addition to *C. perfringens*, the microarray identified several bacterial and viral species that have been associated with dolphin and/or mammalian disease. *Campylobacter lari* is found in the gastrointestinal tracts of mammals and birds and causes several gastrointestinal associated diseases in humans (Broczyk et al. 1987, Otaševic et al. 2004). *Campylobacter* species have been found in marine mammals, e.g. *Campylobacter insulaenigrae*, a near neighbor of *C. lari*, in rectal swabs of 2 different marine mammals: common seals *Phoca vitulina* and harbor porpoise *Phocoena phocena* (Foster et al. 2004). *Photobacterium damselae* was also identified by the microarray. This bacterium has been found in ocean water, seaweed, and has been isolated as a pathogen in dolphins (Rivas et al. 2013). *P. damselae*, also known as *Vibrio damselae*, has been associated with wound infections in humans: *P. damselae* caused fulminant septicemia in a man after filleting bluefish (Perez-Tirse et al. 1993); and fatal renal failure

occurred due to *P. damselae* infection of the hand of a fisherman (Yamane et al. 2004). Thus, this marine pathogen could potentially impact human health. Lastly, the microarray identified the virus *Tursiops truncatus* papillomavirus in one of the sampled animals. This papillomavirus has been associated with oral and genital papillomas in dolphins, and identified in free-ranging dolphin populations (Rehtanz et al. 2012). Papillomavirus is a common infection in dolphins (Bossart 2011) and first reported in wild dolphins inhabiting the estuarine waters of Charleston, SC and the Indian River Lagoon, FL (Rehtanz et al. 2006). The detection of *Tursiops truncatus* papillomavirus type 1 from a fecal sample of Dolphin 8F8 was even more interesting because a biopsy sample collected from this dolphin was confirmed for genital papilloma (G. D. Bossart, data not shown).

Clostridium perfingens was the only bacterium detected by both culture and molecular analyses. Culture-based approaches tend to overestimate bacteria that grow easily, and underestimate those that are slow-growing or that need a specific growth medium (Fuhrman et al. 1993, Amann et al. 1995, Davies et al. 2004). Johnson et al. (2009) investigated upper respiratory samples from dolphins (n = 4) using bacterial 16S rDNA, and detected almost no overlap with the taxa found via culture-based methods, except for *Bacteroides fragilis*. Comparative analysis of microarray vs culture in human wound samples also showed discrepancy between the 2 techniques (Be et al. 2014); LLMDA detected at least one pathogen in 34% of the samples that were culture-negative, demonstrating that it was more sensitive in detecting pathogens from wound samples.

While microarray analysis detected some microbial organisms that were negative by culture, the microarray did not detect some organisms that were culture-positive. One explanation of the detection discrepancy is that the sensitivity of the microarray towards different species varies depending on the availability of the genomic data. The array has higher probe coverage for the microbial species that have more genomic sequence data available; therefore the sensitivity for detection of these species will be higher, while the microbial species with fewer known genomic sequences will have fewer probes and lower sensitivity of detection by array (Gardner et al. 2010, Be et al. 2014). Another possibility that could contribute to the detection differences between culture and molecular analyses is that different aliquots of swabs from a given bottlenose dolphin sample were used by the 2 methods. If a pathogen was not homogenously distributed in the samples, the analyses would yield different results. Using the 16S rRNA gene sequencing approach, Johnson et al. (2009) assessed microbial diversity of 4 bottlenose dolphins: an average of 200 16S rRNA sequences was obtained per dolphin, many of which belonged to 3 dominant bacterial phyla: *Proteobacteria*, *Bacteroidetes* and *Firmicutes*. In another study with 24 dolphins also using the 16S rRNA sequencing method, 19 bacterial phyla were observed in exhaled breath samples from the blowhole (Lima et al. 2012).

The present study revealed lower numbers of taxa as compared to previous studies (Johnson et al. 2009, Lima et al. 2012). In this study, the LLMDA version used included probes to detect sequenced vertebrate-infecting pathogens, with limited ability to detect novel or unknown organisms. Additionally, LLMDA was designed to detect unique genomic regions of sequenced microbes, instead of the conserved 16S rRNA sequence only. LLMDA achieves better specificity than 16S-based technologies, but detection sensitivity is lower due to the broader coverage of genomic targets. When compared with standard PCR methods on pig clinical samples, the LLMDA was estimated to be about 2 orders of magnitude less sensitive (Jaing et al. 2015). The level of sensitivity was consistent regardless of sample source. Therefore, the array is likely to deliver a positive result of pathogens when relatively large quantities of microbe nucleic acid are present. Larger sample size and a more complete microbiome study will be necessary to further validate data obtained from this study and reveal the microbiome associated with wild bottlenose dolphins. These observations also demonstrated that a combination of clinical, microbiological and molecular approaches will provide more comprehensive information on the diversity of the dolphin microbiota.

This study provides the first survey of pathogens associated with 3 tissue types (blowhole, gastric, fecal) from these bottlenose dolphins using a broad-spectrum microbial detection microarray technology. LLMDA provides a good platform for rapid and comprehensive detection of all known viruses, bacteria and fungi present in the array. Most existing detection technologies are based on antibody tests or nucleic acid amplification of sequences from one or a small set of organisms. While they are able to rapidly identify selected pathogens at the species or strain level, they cannot be multiplexed to the degree required to broadly detect different organisms. However, it should be noted that microarray results do not imply health status or presence of disease. The microarray provides a surveillance of the various microorganisms, and may provide insight on the

potential association of these organisms with certain diseases and hence, those microorganisms can then be targeted for the development of testing methods. This new technology will allow us to broaden the diagnostic disease base, validate previous findings and provide a comprehensive profile of the microbial community present in a sample. This can then lead to better management of land-based sources of microbial pollution for wild dolphins and potential sources for managed-care dolphins. Future studies will potentially expand our insight into pathogens in dolphins in relation to their overall health as well as ecological health.

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