Aquatic Mammals 2015, *41*(2), 149-166, DOI 10.1578/AM.41.2.2015.149

Occurrence of Human Pathogenic Bacteria and *Toxoplasma gondii* in Cetaceans Stranded in the Philippines: Providing Clues on Ocean Health Status

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Abstract

The general consensus of a rapidly changing ocean ecosystem being affected by anthropogenic activities needs to be understood in relation to both wildlife and human health. The risks and challenges for the Philippines include lack of scientific information on waterborne diseases that are potentially zoonotic. The present study fills in this knowledge gap by detecting the occurrence of bacteria, *Giardia*, and *Toxoplasma gondii* in locally found cetacean species. Cetaceans (n = 30) that stranded from January 2012 through March 2013 were appropriately responded to, and biological materials were taken whenever applicable. A total of 25 bacteria were isolated from nine stranders. Phenotypic and genotypic methods of isolate identification yielded 12 consensus genera: *Acinetobacter, Aeromonas, Burkholderia, Enterococcus, Moraxella*, *Proteus, Providencia, Rhizobium, Serratia, Sphingomonas, Staphylococcus,* and *Vibrio*. No screened strander was positive for *Giardia*. Serological assay detected antibodies for *T. gondii* in five stranders, while nested polymerase chain reaction positively amplified the B1 gene of the parasite in two stranders. This study provides the first report on bacteria and *T. gondii* in cetaceans found in the Philippines. Since the detected microorganisms include species recognized to cause new infections in marine mammals worldwide, the findings of the study underscore the potential of stranded cetaceans to serve as sentinels for studying the movement of emerging pathogens in marine habitats, provide clues on the health status of their free-ranging populations, and present the health risks available to humans who share the same water resource with them.

Key Words: cetaceans, strandings, bacteria, *Giardia*, *Toxoplasma gondii*, sentinels

Introduction

Marine mammals face the challenges of a changing environment primarily affected by human activities. Thus, they are in a good position to serve as sentinels of their habitat conditions (Bossart, 2011). Moore (2008) suggested that the utility of these animals to serve as sentinels stems from their ecological diversity as well as inherent variability in marine ecosystems. Furthermore, Stewart et al. (2008) provided that information on their emerging or recurring health problems can be used as a measure of ocean health and indicator of impending human health issues. Assessments of health status among marine mammals usually involve general pathogen screenings in stranded and free-ranging populations (Gerber et al., 1993; Calle et al., 2002; Duignan, 2003; Hanni et al., 2003; Zarnke et al., 2006; Aguirre et al., 2007; Greig et al., 2007; Bogomolni et al., 2008; Alekseev et al., 2009; Zuerner et al., 2009; Brownstein et al., 2011). Outbreaks of infectious diseases, sometimes characterized by high morbidity and mortality, were documented in several marine mammal populations worldwide, posing threats to public health (Lipscomb et al., 1996; Nielsen et al., 2001; Van Bressem et al., 2009). While it seems necessary to investigate pathogens in cases of mass mortalities or large-scale disease epidemics, knowledge of their occurrence in the absence of these occasions is urgently needed, particularly in the case of Philippine marine mammals which do not have baseline data yet.

There may already be clinical signs of infectious diseases in some marine mammal populations in the Philippines, but the lack of research attention being given to them makes them unrecognized, much less documented. Except for the reported case of aspergillosis in a melon-headed whale (*Peponocephala electra*) calf that stranded and died in Bataan (Torno et al., 2008), the occurrence of other bacterial, protozoan, or fungal pathogens causing either overt or hidden manifestations of infection is not known in any marine mammal species found in the country. The present study fills in the current knowledge gap on the occurrence of pathogenic agents among locally found cetacean species and the potential of these animals to serve as sentinels of emerging and zoonotic diseases. For such purpose, the researchers maximized the sampling opportunity provided by local stranding events in the country, observed to have an increasing trend in recent years. Of the 30 identified marine mammal species in the Philippines, 28 are cetaceans and 22 of these are reported to strand (Aragones et al., 2010), providing the much needed chance to study these difficult-to-observe albeit charismatic animals. While it is not the primary aim of the study to investigate the role of pathogen or disease occurrence in sampled stranding events, the involvement of such may be suggested as done elsewhere (Lopez et al., 2002; Kreuder et al., 2003; Gonzalez-Solis et al., 2006; Stoddard et al., 2008; Fauquier et al., 2009; Forman et al., 2009; Colegrove et al., 2010). This study generally aimed to detect the occurrence of bacteria, *Giardia*, and *Toxoplasma gondii* in cetaceans stranded in the Philippines.

Methods

Stranded Cetacean Samples

Cetaceans stranded in Philippine waters from January 2012 through March 2013 were opportunistically sampled. Live or dead individuals were characterized in terms of species, sex (i.e., based on genital and/or mammary slits), length (i.e., tip of the snout to the tip of the tail or notch of the flukes), age class (inferred from length based on species), and type of stranding (e.g., single or mass; live or dead). The chance of responding to a stranded cetacean is affected by the proximity of the stranding site, and so the researchers collaborated with some stakeholders (i.e., Philippine Marine Mammal Stranding Network [PMMSN]; Bureau of Fisheries and Aquatic Resources [BFAR]) for monitoring stranding events all over the country. The Philippines is an archipelagic country with three major islands: (1) Luzon, (2) Visayas, and (3) Mindanao. To respond to strandings reported within Luzon, the researchers travelled by land (i.e., at least 5 h and at most 12 h) or water (i.e., 6 h) immediately after a report was made. On the other hand, Visayas and Mindanao can be reached by plane or ship. Biological materials were collected from stranded cetaceans considering the (1) animal disposition (e.g., if it was a live or dead strander); (2) physical preservation based on the expanded version (Geraci & Lounsbury, 2005) of the code system established by the Smithsonian Institution's Marine Mammal Events Program (e.g., bacteriological samples were only collected from live or freshly dead animals in order to minimize, if not avoid, contamination); and (3) sampling conditions (e.g., handling limitations resulting to contamination of body parts to be sampled).

Detection of Pathogens

The detection of potentially emerging and infectious zoonotic pathogens was limited to bacteria (with *Vibrio* spp. as preliminary targets) and protozoa (*T. gondii* and *Giardia* spp.). Screening of cetaceans for these microorganisms proceeded depending on the type of biological material(s) obtained from each stranded sample (see Table 1). The laboratory work was performed at the Microbiological Research and Services Laboratory (MRSL) and the Molecular Protozoology Laboratory (MPL) of the Natural Sciences Research Institute (NSRI), University of the Philippines, Diliman as well as at the Biological Sciences Department, College of Science and Computer Studies, De La Salle University, Dasmariñas, Cavite, Philippines.

Phenotypic Methods for Bacteria Screening— Specimens were obtained from routine and nonroutine sites using sterile rayon swabs with transport medium (i.e., Stuart). For routine sites, swab samples were collected from the blowhole and anus of cetaceans (Buck et al., 1991, 2006; Miller et al., 2010; Morris et al., 2011). For the blowhole area, swabs were inserted into the hole during a breath, gently moved along the wall, and removed during the next breath in live stranders. Another method is by lowering a sterile Petri dish directly over the blowhole to collect the exhaled breath condensate (blow) and then swabbing afterwards. Anal swabs were collected by inserting rayon swabs into the anal orifice and gently swabbing the area. For freshly dead individuals, swab samples were also taken from blowhole and anal areas. Considering the physical condition and disposition of sampled cetaceans, the actual number of swab samples collected from the blowhole and anus varied, and so these samples were pooled for each body site and stranded individual. Whenever applicable, swab samples from nonroutine sites (e.g., lesions, organs, and abdominal or thoracic fluid) were also obtained from both live and dead animals (Bogomolni et al., 2008), especially in relation to

Table 1. Stranded cetaceans responded for sampling from January 2012 through March 2013

Strander code and number	Cetacean species	Stranding location	Stranding date	Sex/ age class	Condition code at the time of sampling[b]	Biological materials for pathogen screening	
						Swab specimen for bacteria isolation	Specimen for protozoa detection
S1	*Kogia sima* Dwarf sperm whale	Sto. Tomas, La Union	7 February 2012	Male/ adult	2	Blowhole Anus Abdominal fluid Genital area	
S2	*Stenella attenuata* Pantropical spotted dolphin	Bolinao, Pangasinan	9 February 2012	Male/ subadult	3		
S3	*Stenella longirostris* Spinner dolphin	Pier, Legaspi City	9 March 2012	Male/ subadult	3		
S4	*Globicephala macrorhynchus* Short-finned pilot whale	Lubang Island, Occidental Mindoro	16 March 2012	Female/ subadult	1	Blowhole Anus Lung[d] Abdominal fluid[d] Skin lesion	Feces Blood
S5	*Globicephala macrorhynchus* Short-finned pilot whale	Claveria, Cagayan	9 April 2012	Male/ adult	2	Blowhole Anus Liver	Feces Blood
S6	*Stenella attenuata* Pantropical spotted dolphin	Alaminos, Pangasinan	29 April 2012	Female/ subadult	3		
S7	*Steno bredanensis* Rough-toothed dolphin	Cabangan, Zambales	7 June 2012	Male/ subadult	1	Blowhole Anus	Feces Blood Serum
S8	*Tursiops aduncus* Indo-Pacific bottlenose dolphin	Bigaa, Legazpi City	11 June 2012	Female/ adult	3		
S9	*Tursiops truncatus* Common bottlenose dolphin	Talisay, Camarines Norte	27 July 2012	Female/ subadult	1[c]		

S10	*Stenella attenuata* Pantropical spotted dolphin	Cruz, Davao City	28 July 2012	Female/ adult	1		Blood
S11	*Stenella attenuata* Pantropical spotted dolphin	Cruz, Davao City	28 July 2012	ND[a]/ calf	1		Blood
S12	*Kogia breviceps* Pygmy sperm whale	Capalonga, Camarines Norte	8 August 2012	Female/ adult	3		
S13	*Stenella attenuata* Pantropical spotted dolphin	Bacarra, Ilocos Norte	30 August 2012	Male/ subadult	2		Feces
S14	*Kogia breviceps* Pygmy sperm whale	Santa Ana, Cagayan	12 September 2012	Female/ adult	1	Blowhole Anus	Feces Blood Serum
S15	*Kogia breviceps* Pygmy sperm whale	Santa Ana, Cagayan	12 September 2012	ND[a]/ calf	1		Feces
S16	*Kogia breviceps* Pygmy sperm whale	San Antonio, Zambales	16 September 2012	Male/ adult	1	Blowhole Anus	Feces Blood Serum
S17	*Kogia breviceps* Pygmy sperm whale	San Fernando, La Union	17 September 2012	Male/ adult	1	Blowhole Anus Heart[d] Lung[d] Liver[d]	Feces Blood Serum
S18	*Stenella longirostris* Spinner dolphin	Tiwi, Albay	5 November 2012	Female/ subadult	2		Blood
S19	*Stenella attenuata* Pantropical spotted dolphin	Palauig, Zambales	7 November 2012	Male/ subadult	1	Blowhole Anus	Blood Serum
S20	*Stenella longirostris* Spinner dolphin	San Jose, Camarines Sur	16 November 2012	Male/ adult	2		Blood
S21	*Physeter macrocephalus* Giant sperm whale	San Antonio, Pilar, Sorsogon	30 December 2012	Male/ subadult	3		
S22	*Mesoplodon* sp. Beaked whale	Lupi, Prieto Diaz, Sorsogon	4 January 2013	Female/ adult	2		Feces Blood Serum

S23	*Steno bredanensis* Rough-toothed dolphin	Candelaria, Zambales	14 January 2013	Male/ adult	1	Blowhole Anus	Feces Blood Serum
S24	*Globicephala macrorhynchus* Short-finned pilot whale	Pasuquin, Ilocos Norte	25 January 2013	Male/ adult	2		Feces Blood Serum
S25	*Kogia breviceps* Pygmy sperm whale	San Antonio, Zambales	14 February 2013	Male/ adult	3		
S26	*Lagenodelphis hosei* Fraser's dolphin	Pamplona, Cagayan	21 February 2013	Female/ subadult	1[c]		
S27	*Tursiops aduncus* Indo-Pacific bottlenose dolphin	Morong, Bataan	24 February 2013	Female/ adult	2		Blood
S28	*Globicephala macrorhynchus* Short finned pilot whale	Buguey, Cagayan	25 February 2013	Male/ adult	3		
S29	*Steno bredanensis* Rough-toothed dolphin	Bauang, La Union	5 March 2013	Male/ subadult	3		
S30	*Tursiops truncatus* Common bottlenose dolphin	Anda, Pangasinan	2 March 2013	Male/ adult	3		

[a] ND = not determined

[b] Physical condition at the time of sampling based on Smithsonian Institution Condition Codes (Geraci & Lounsbury, 2005): 1 – live; 2 – fresh/carcass in good condition; 3 – fair/decomposed but organs basically intact; 4 – poor/advanced decomposition; and 5 – mummified/skeletal remains

[c] Sampling for biological collection was not done at the time of response due to animal stress; cetaceans were thoroughly investigated in relation to human interaction, the results of which are discussed in another report

[d] Additional swab samples taken right after the strander died

suspected infection. The swab samples were stored in coolers (about 7° to 10° C) and transported to the laboratory within 24 h for processing. Those inoculated to media upon collection were directly incubated 18 to 24 h at 35 ± 2° C. For the enrichment of target *Vibrio* spp., swabs were inoculated immediately into APW (alkaline peptone water) with 2 to 3% sodium chloride (NaCl) broth or streaked onto TCBS (Thiosulfate Citrate Bile Salts Sucrose) or TSA (Trypticase Soy Agar with 2 to 3% NaCl) plates when transit time is < 8 h (Elliot et al., 1998). Swabs from transport medium were placed in APW and incubated 12 to 24 h at 35 ± 2° C prior to subculture on TCBS plates. The plates were incubated 18 to 24 h at 35 ± 2°C. Suspect isolates were confirmed for Gram and cytochrome oxidase reactions, microscopically observed for cellular morphology, and identified using the rapid diagnostic kit Analytical Profile Index system (API 20 NE). Distinctive colonies primarily characterized by color and identified by the API system were streaked and cultured on new TCBS or TSA (with 2 to 3% NaCl) plates several times to purify bacterial isolates until pure cultures were obtained. Presumptive non-vibrios were further examined for other phenotypic characteristics before confirming identification using API 20 NE or VITEK 2 systems (BioMerieux SA, France).

Serological Method for Toxoplasma *Screening*—Blood extraction proceeded from the fluke vasculature of live animals or vena cavae from freshly dead stranders (Geraci & Lounsbury, 2005). To recover serum, whole blood was either placed in serum separator tubes or kept warm for 30 min until clotted and centrifuged at 1,500 rpm for 1 min. Sera were stored at 4° to 8° C and processed within 48 h. Antibodies against *T. gondii* were detected using Toxocell Latex Agglutination Test (LAT: BIOKIT Manufacturing Company, Barcelona, Spain). Serum recovery depended on the amount and quality of blood obtained.

Molecular Methods for Giardia, Toxoplasma, *and Bacteria Screening*—For *Toxoplasma* screening, blood was obtained as described above. For *Giardia* screening, fecal samples were collected from live cetaceans by enema with 50 to 100 ml sterile saline (R. Fayer, pers. comm., 6 May 2011). The liquid feces was placed in sterile plastic cups for ≥ 7 d (Fayer et al., 2008) or fixed in 10% formalin or 70% ethanol until processing. When available, swabbed fresh feces from the rectal area of live individuals (Buck et al., 2006) and solid feces (5 to 10 g) from necropsied individuals (Geraci & Lounsbury, 2005) were also collected and suspended in a buffer (e.g., PBS) or fixated and then resuspended prior to DNA extraction. All samples were held at 4° to 5° C until processing. Bacteria that were isolated and identified through phenotypic methods were pretreated for DNA extraction. Pellets (maximum 2 × 10^9 cells) from 18 to 24 h pure cultures were harvested by centrifugation at 5,000 × g (7,500 rpm) for 10 m and then resuspended in 180 µl tissue lysis buffer. Extraction of DNA was done using the QuickgDNA™ Blood MiniPrep kit (Zymo Research) for blood samples, Fecal DNA Miniprep™ kit (Zymo Research) for fecal samples, and DNeasy® Blood & Tissue kit (Qiagen) for bacteria cultures following manufacturer's instructions.

Procedures for polymerase chain reaction (PCR) were performed for amplifications of target pathogen gene fragments. For *Giardia*, 18S-rDNA at fragments ~130 bp (base pair) or 170 bp was the target size using the primer pairs RH11 and RH4 for first step PCR, and GiarF and GiarR for second step (McGlade et al., 2003; Szénási et al., 2007; Lim et al., 2009). The thermocycler conditions were three cycles of 94° C for 2 min, 53° C for 1 min, 72° C for 2 min followed by 50 cycles of 94° C for 30 s, 53° C for 20 s, 72° C for 30 s, and an extension of 72° C for 7 min (McGlade et al., 2003).

For *Toxoplasma*, amplification yielding a final nested product of 96 bp directed against the B1 gene was carried out using the primer pairs Outer Sense strand and Outer Nonsense strand for first step PCR, and Inner Sense strand and Inner Nonsense strand for second step. The thermocycler conditions were (1) for first step, denaturing at 94° C for 2 min followed by 40 cycles of 93° C for 10 s, 57° C for 10 s, 72° C for 30 s, and an extension of 72° C for 30 s; and (2) for second step, denaturing at 94° C for 2 min followed by 40 cycles of 93° C for 10 s, 62.5° C for 10 s, 72° C for 15 s, and an extension of 72° C for 15 s (Jones et al., 2000; Pretti et al., 2010).

For bacteria, the conserved regions 8 and 10 of the 16S rDNA were amplified by forward primer 1169U20 and reverse primer 1521L19 (Jin et al., 2005). The reactions were carried out with initial denaturation at 95° C for 5 min, followed by 35 cycles of 94° C for 25 s, 55° C for 30 s, and 72° C for 25 s as modified from Jin et al. (2005).

Reactions were performed in 25 µl volume with the following final concentrations of components: 1X PCR Master Mix (Promega, USA), 0.1 to 1.0 µM assigned upstream primer (AITbiotech, Singapore), 0.1 to 1.0 µM assigned downstream primer (AITbiotech, Singapore), < 250 ng DNA template, and nuclease-free water adjusted accordingly. Negative controls consisted of PCR reagents excluding DNA template. Positive controls included DNA samples of *Giardia* sp. (Lim, Department of Parasitology, University of Malaya, Malaysia), *T. gondii* (Ishida, University of Tokyo, Japan; Lim, Department of Parasitology, University of Malaya,

Malaysia), *Vibrio parahaemolyticus* (Philippine National Collection of Microorganisms [PNCM], National Institute of Molecular Biology and Applied Microbiology [BIOTECH], University of the Philippines, Los Baños), and *Escherichia coli* (Microbiological Research and Services Institute [MRSL], Natural Sciences Research Institute [NSRI], University of the Philippines, Diliman). Electrophoresis of PCR products in TAE (Tris-acetate-EDTA) buffer was performed on 1 to 2% agarose gel at 100 V with ethidium bromide (0.5 mg/ml) staining. A 100 bp molecular mass ladder (Vivantis) was included in each gel. For bacteria, PCR-positive samples were processed for purification, DNA quantification, and sequencing (1st Base, Malaysia). Molecular analyses were performed using software programs *Bioedit*, Version 7.0.5.3 (Hall, 1999) for editing and aligning of sequences, and *MEGA* 5 for phylogenetic analyses (Tamura et al., 2011). Sequence homologies were determined based on parameters suggested by Hall (2011).

Results

Stranded Cetaceans

A total of 30 cetaceans that stranded in the Philippines were responded to from January 2012 through March 2013. Cetacean samples were confirmed to be from 11 species: *Globicephala macrorhynchus* (short-finned pilot whale), *Kogia sima* (dwarf sperm whale), *Kogia breviceps* (pygmy sperm whale), *Lagenodelphis hosei* (Fraser's dolphin), *Mesoplodon* sp. (unidentified beaked whale), *Physeter macrocephalus* (giant sperm whale), *Stenella attenuata* (pantropical spotted dolphin), *Stenella longirostris* (spinner dolphin), *Steno bredanensis* (rough-toothed dolphin), *Tursiops aduncus* (Indo-Pacific bottlenose dolphin), and *Tursiops truncatus* (common bottlenose dolphin). As to age class, 53% of the stranders were adults (n = 16), 40% were subadults (n = 12), and only 7% were calves (n = 2). The sex of these calves was not determined, whereas 57% of the remaining samples were males (n = 17), and 37% were females (n = 11).

All of the strandings were from single events. Most of the responded cetaceans came from Luzon Island, where a relatively higher number of strandings were reported during the duration of the study (see Figure 1). There were more live (n = 18) than dead stranders (n = 12). Among live stranders, the majority of cetaceans (n = 16) either died while being responded to or re-stranded dead after they were released back into the wild. Only two were released and did not re-strand.

Swab samples, blood, sera, and feces were appropriately collected from cetaceans based on animal disposition, physical preservation, and sampling conditions. Depending on the type of biological material(s) obtained, the stranders were screened for target pathogens: nine individuals (30% of all cetacean samples) with swab samples for the presence of bacteria; 15 individuals (50% of all cetacean samples) and 8 individuals (27% of all cetacean samples) with blood and serum samples, respectively, for the presence of *T. gondii*; and 10 individuals (33% of all cetacean samples) with fecal samples for the presence of *Giardia* spp. (see Table 2).

Bacteria in Stranded Cetaceans

Twenty-five bacteria isolates were obtained from the following cetacean species: two short-finned pilot whales, one dwarf sperm whale, three pygmy sperm whales, one spinner dolphin, and two rough-toothed dolphins. Phenotypic and genotypic methods of isolate identification yielded 12 consensus genera: *Acinetobacter, Aeromonas, Burkholderia, Enterococcus, Moraxella, Proteus, Providencia, Rhizobium, Serratia, Sphingomonas, Staphylococcus,* and *Vibrio*. However, there were differences in species-level identifications between the two methods used. In particular, 23 (92%) out of 25 isolates subjected to phenotypic methods were successfully identified to the species, while 19 (90%) out of 21 isolates (with available nucleic acid samples) processed for 16S rDNA sequencing had highest matches with similar sequences in the Genbank database at the species level. Species-level agreements between phenotypic and genotypic identifications were established for the following isolates: *Burkholderia cepacia, Staphylococcus epidermidis*, *Serratia marcescens*, *Proteus mirabilis*, and *Providencia stuartii*.

Toxoplasma *in Stranded Cetaceans*

The following cetaceans were screened for the presence of *T. gondii*: three short-finned pilot whales, three pygmy sperm whales, one beaked whale, three pantropical spotted dolphins, two spinner dolphins, two rough-toothed dolphins, and one Indo-Pacific bottlenose dolphin. All these cetaceans had qualified blood samples for the amplification of the target parasite's B1 gene by nested PCR, while only eight had serum samples available for Toxocell LAT. Two individuals were PCR-positive, while five were serologically positive for the protozoan parasite.

Giardia *in Stranded Cetaceans*

Giardia was not detected in the following cetaceans: three short-finned pilot whales, three pygmy sperm whales, one spinner dolphin, two rough-toothed dolphins, and one beaked whale.

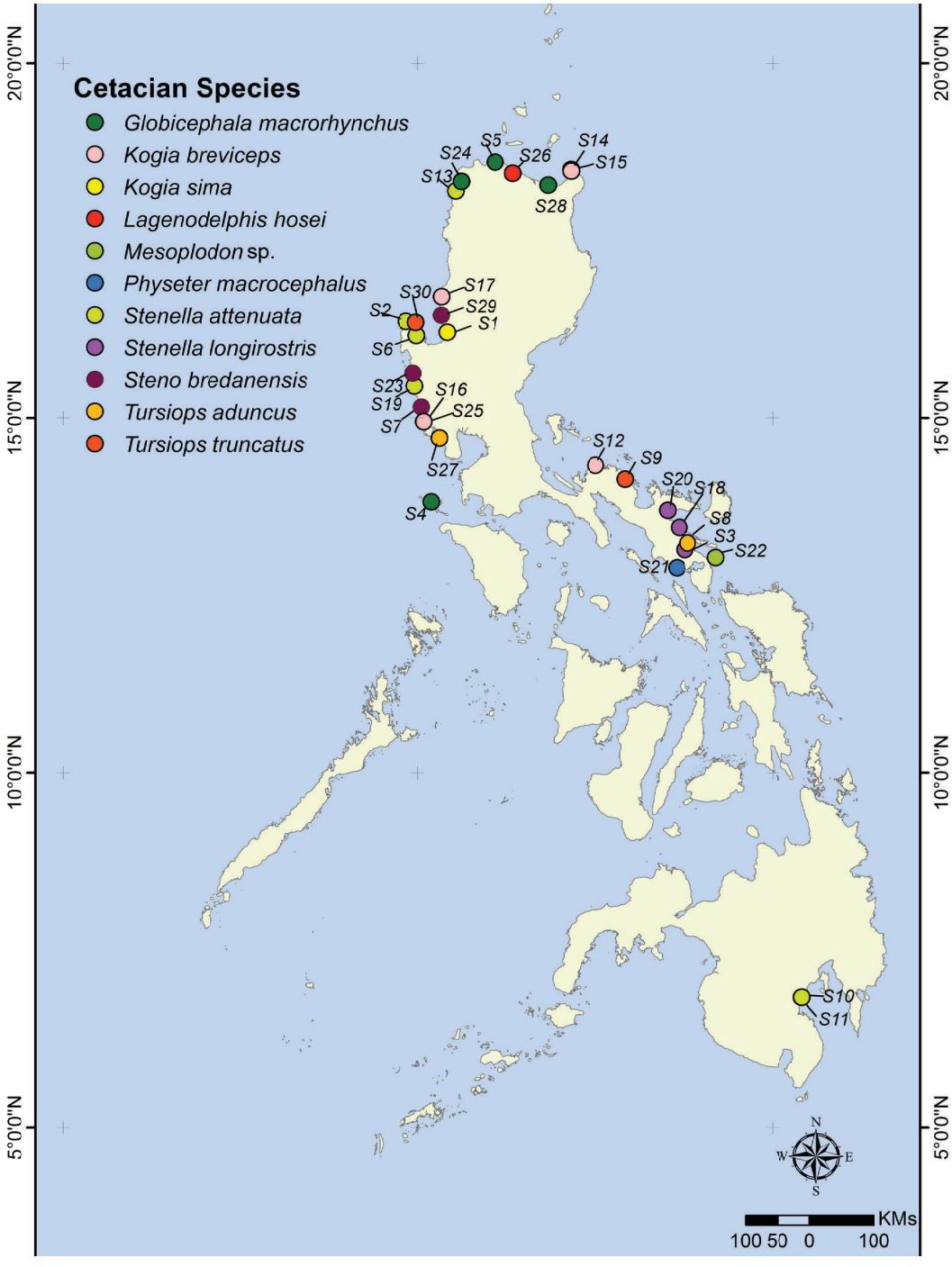

Figure 1. Stranding sites of responded cetaceans (samples S1 to S30) in the Philippines from January 2012 through March 2013

Table 2. Occurrence of target microorganisms in stranded cetaceans sampled in the Philippines from January 2012 through March 2013

Strander code and number	Cetacean species	Identification of isolated bacteria			Detection of protozoa[b]
		Phenotypic method (API/VITEK 2 % probability)	Genotypic method (16S rDNA highest sequence match in GenBank based on % bp/bp similarity)	Consensus taxon	
S1	*Kogia sima* Dwarf sperm whale	*Moraxella*[a] (82.2%)			
		Ochrobactrum[a] (96.9%)			
		Vibrio[a] (87.9%)			
		Moraxella sp. (82.2%)	*Moraxella lincolnii* (HF558363) (83%)	*Moraxella*	
S2	*Stenella attenuata* Pantropical spotted dolphin				
S3	*Stenella longirostris* Spinner dolphin				
S4	*Globicephala macrorhynchus* Short-finned pilot whale	*Aeromonas sobria* (98.7%)	*Aeromonas salmonicida* subsp. *achromogenes* (AY910844) (86%)	*Aeromonas*	TPCR negative GPCR negative
		Aeromonas hydrophila[a] (83.8%)			
		Vibrio parahaemolyticus (96.2%)	*Vibrio harveyi* (FJ227111) (96%)	*Vibrio*	
		Burkholderia cepacia (99.4%)	*Burkholderia cepacia* (EU734821) (97%)	*Burkholderia cepacia*	

S5	*Globicephala macrorhynchus* Short-finned pilot whale	*Aeromonas hydrophila* (99.7%)	*Aeromonas* sp. (HM161724) (81%)	*Aeromonas*	TPCR negative GPCR negative
		Enterococcus faecium (91%)	*Enterococcus casseliflavus* (AF367977) (100%)	*Enterococcus*	
S6	*Stenella attenuata* Pantropical spotted dolphin				
S7	*Steno bredanensis* Rough-toothed dolphin	*Rhizobium radiobacter* (90%)	*Rhizobium* sp. (FM173817) (93%)	*Rhizobium*	TPCR negative TLAT negative GPCR negative
S8	*Tursiops aduncus* Indo-Pacific bottlenose dolphin				
S9	*Tursiops truncatus* Common bottlenose dolphin				
S10	*Stenella attenuata* Pantropical spotted dolphin				TPCR negative
S11	*Stenella attenuata* Pantropical spotted dolphin				TPCR negative
S12	*Kogia breviceps* Pygmy sperm whale				
S13	*Stenella attenuata* Pantropical spotted dolphin				GPCR negative
S14	*Kogia breviceps* Pygmy sperm whale	*Sphingomonas paucimobilis* (88%)	*Sphingomonas melonis* (JF343163) (88%)	*Sphingomonas*	TPCR negative TLAT positive GPCR negative
		Staphylococcus epidermidis (99%)	*Staphylococcus epidermidis* (KC443110) (99%)	*Staphylococcus epidermidis*	
		Serratia marcescens (91%)	*Serratia marcescens* (CP003959) (98%)	*Serratia marcescens*	
		Vibrio alginolyticus (95%)	*Vibrio harveyi* (FJ227111) (98%)	*Vibrio*	

S15	*Kogia breviceps* Pygmy sperm whale				GPCR negative
S16	*Kogia breviceps* Pygmy sperm whale	*Vibrio alginolyticus* (96%)	*Vibrio harveyi* (FJ227111) (98%)	*Vibrio*	TPCR negative TLAT positive GPCR negative
		Vibrio alginolyticus (99%)	*Vibrio harveyi* (FJ227111) (98%)	*Vibrio*	
S17	*Kogia breviceps* Pygmy sperm whale	*Proteus mirabilis* (99%)	*Proteus mirabilis* (NR074898) (100%)	*Proteus mirabilis*	TPCR positive TLAT positive
		Acinetobacter haemolyticus (96%)	*Acinetobacter baumannii* (JF513192) (98%)	*Acinetobacter*	
		Acinetobacter haemolyticus (96%)	*Acinetobacter baumanni* (JF513192) (95%)	*Acinetobacter*	
S18	*Stenella longirostris* Spinner dolphin				TPCR negative
S19	*Stenella attenuata* Pantropical spotted dolphin	*Vibrio alginolyticus* (90%)	*Vibrio harveyi* (FJ227111) (97%)	*Vibrio*	TPCR negative TLAT positive
		Vibrio alginolyticus (91%)	*Vibrio harveyi* (FJ227111) (97%)	*Vibrio*	
S20	*Stenella longirostris* Spinner dolphin				TPCR negative
S21	*Physeter macrocephalus* Giant sperm whale				
S22	*Mesoplodon* sp. Beaked whale				TPCR negative TLAT positive GPCR negative

S23	*Steno bredanensis* Rough-toothed dolphin	*Providencia stuartii* (99%) *Providencia stuartii* (97%)	*Providencia stuartii* (CP003488) (98%) *Providencia stuartii* (CP003488) (97%)	*Providencia stuartii* *Providencia stuartii*	TPCR negative TLAT negative GPCR negative
S24	*Globicephala macrorhynchus* Short-finned pilot whale				TPCR negative TLAT negative GPCR negative
S25	*Kogia breviceps* Pygmy sperm whale				
S26	*Lagenodelphis hosei* Fraser's dolphin				
S27	*Tursiops aduncus* Indo-Pacific bottlenose dolphin				TPCR positive
S28	*Globicephala macrorhynchus* Short-finned pilot whale				
S29	*Steno bredanensis* Rough-toothed dolphin				
S30	*Tursiops truncatus* Common bottlenose dolphin				

[a] No genotypic identification

[b] TPCR (PCR screening for *Toxoplasma gondii*); TLAT (LAT screening for *T. gondii*); and GPCR (PCR screening for *Giardia*)

These were the stranders with fecal material qualified for screening.

Discussion

Screening of sampled cetacean stranders for bacteria revealed that a comparatively high proportion (28%) of isolates were comprised of species of *Vibrio*. *Vibrio* spp. appear to be the most commonly isolated bacteria in cetaceans (Buck et al., 2006; Morris et al., 2011). Although *Vibrio* spp. are natural inhabitants of the marine environment, many of its species are known to associate with animals and are pathogenic (Hervio-Heath et al., 2002; Panicker et al., 2004; Thompson et al., 2007). Some species (e.g., *V. cholerae, V. vulnificus, V. parahaemolyticus, V. mimicus,* and *V. fluvialis*) were reported as serious human pathogens (Vora et al., 2005; Saha et al., 2006; Izumiya et al., 2011). In the Philippines, studies on *Vibrio* involved isolations of pathogenic and non-pathogenic strains from shrimps and scleractinian corals (Monsalud et al., 2003; Arboleda & Reichardt, 2008; Caipang & Aguana, 2011).

Other isolated bacteria species are either previously known or emerging agents of primary or nosocomial infections in humans and other animals: *Burkholderia cepacia*; *Ochrobactrum anthropi*; *Serratia marcescens*; *Proteus mirabilis*; *Providencia stuartii*; *Staphylococcus epidermidis*; and members of the genus *Sphingomonas*, *Acinetobacter*, and *Aeromonas* (Daily et al., 1981; Parke & Sherman, 2001; Salles et al., 2002; Tumbarello et al., 2004; Guillou, 2005; Teyssier et al., 2005; Grimont & Grimont, 2006; Fernandez-Delgado et al., 2007; Ryan & Adley, 2010; Abulreesh, 2011). It was previously recognized that bacterial pathogens are routinely recovered even from healthy captive marine mammals (Dunn et al., 2001). However, more isolates are being found in stranders than in free-living or captive cetaceans, implying the probability of finding more opportunistic bacteria in stranded animals which are usually debilitated or physically compromised (Buck et al., 1991; Chan et al., 2001; Rose et al., 2009). Among stranded marine mammal populations, bacterial infections were found to comprise a considerable proportion of infectious mortality, accounting for 31% probable cause of death in bottlenose dolphins from the coastal region of South Carolina (McFee & Lipscomb, 2009), 14% of dead strandings along the Oregon Coast (Stroud & Roffe, 1979), and in 29% of mortality cases classified as human-induced types (i.e., fishery-related and traumatic injury) in Hong Kong (Parsons & Jefferson, 2000).

The non-concurrence between genotypic and phenotypic species-level identifications of most isolates (71%) may have resulted from the limitations of the methods used. The study did not aim to compare the taxonomic discriminations of the methods used; rather, 16S rDNA sequencing was done to complement the phenotypic identification technique. This is due to the efficiency of commercial systems used being limited by the need to perform additional tests to definitively identify strains at the species level (Adderson et al., 2008). The identified bacteria isolates are data relevant to the medical management of cetaceans that locally strand in the Philippines. Many stranding events in the country are being responded to by trained members of stranding networks (e.g., PMMSN) or by a concerned government agency (i.e., BFAR), and some of these cases necessitate cetacean rehabilitation involving antibiotic treatments. Thus, sensitivity tests should be part of any monitoring work on cetacean health. As suggested earlier (Bogomolni et al., 2008; Rose et al., 2009; Wallace et al., 2013), the exposure of marine mammals to antibiotic resistances must be considered given the ability of these animals not only to serve as vectors of resistant bacteria but also to indicate the geographical extent of bacterial resistance development.

As for *Giardia* detection, the negative results parallel the findings of Rengifo-Herrera et al. (2011) in seals sampled from the Antarctic as well as that of Fayer et al. (2008) in bottlenose dolphins sampled from South Carolina and Florida. It should be considered that the stranded cetaceans screened in this study only represent their counterparts in the wild, and so there would always be the possibility of *Giardia* occurrence in their populations.

As for *Toxoplasma*, the amplification of *T. gondii* B1 gene from the blood samples of Indo-Pacific bottlenose dolphin and pygmy sperm whale suggests acute infection of these animals. However, the presence of antibodies directed against *T. gondii* cannot conclusively establish whether the other serologically positive cetaceans are suffering from chronic or acute infection though it does confirm their exposure to the parasite. This is because body tissues (e.g., skeletal and smooth muscles of the brain, liver, etc.) that may be harbouring different stages of the parasite (i.e., latent bradyzoites in tissue cysts or active tachyzoites and bradyzoites) were not examined histopathologically or otherwise. Aside from the serologic and molecular evidence, other pathological findings clinically associated with toxoplasmosis in marine mammals were not available in all samples due to limitations in stranding response. Other documented cases of toxoplasmosis in the country involved terrestrial mammals such as rats (Salibay & Claveria, 2006) and cats (Advincula et al., 2010).

As it is in other marine mammals, the occurrence of *T. gondii* in cetaceans tested in the present study (i.e., kogiids, spinner dolphin, bottlenose dolphin, and ziphiid) is intriguing given that the route for postnatal infection includes the ingestion of either (1) oocysts from contaminated food and water or (2) latent bradyzoite-infected tissue by carnivorism (Dubey et al., 2003, 2008). One interesting case is that of infected Antillean manatees (*T. manatus manatus*) in Puerto Rico; these animals are exclusively herbivorous and, thus, ingestion of infected meat or animal tissue is unlikely (Bossart et al., 2012). In the southern sea otter (*E. lutris nereis*) California population that experienced significant mortality due to toxoplasmic meningoencephalitis (Conrad et al., 2005), a source of infection was hypothesized to be through the predation of filter-feeding marine bivalve shellfish (*Mytilus galloprovincialis*) found to assimilate and concentrate infective oocysts from contaminated marine water (Arkush et al., 2003). Dietary information on pygmy sperm whales supports cephalopods (most commonly members of the families Cranchiidae, Enoploteuthidae, Histioteuthidae, Lycoteuthidae, and Ommastrephidae) as the staple diet as well as consumption of deep-sea shrimps and, rarely, mesopelagic fishes (Bloodworth & Odell, 2008). On the other hand, mesopelagic fishes, particularly myctophids (mainly *Ceratoscopelus warmingi*, *Diaphus* spp., and *Myctophum asperum*) were found to be the consistent portion of the spinner dolphins' diet inhabiting the Sulu Sea, Philippines (Dolar et al., 2003). Likewise, cephalopods and fishes were found to comprise the diets of beaked whales (MacLeod et al., 2003) as well as Indo-Pacific bottlenose dolphins (Amir et al., 2005). Such cold-blooded preys are not hosts to *T. gondii*, and it is not known whether sporozoite excysts if they do ingest oocysts (Jones & Dubey, 2010).

The presence of *T. gondii* in some stranded cetaceans in the Philippines offers the possibility of toxoplasmosis in their free-ranging populations. This is especially important in the case of kogiids for which not much is known about owing to their cryptic and solitary behavior, difficulty in taxonomic identification, and generally deepwater distribution (Bloodworth & Odell, 2008). It is important to note that among the five serologically positive samples, three are pygmy sperm whales (kogiids) that separately beached in the northern part of the country (i.e., North Luzon) within 6 d. Similarly, information regarding beaked whales is said to be so sparse that even the most basic aspects of their biology are poorly known for some species (MacLeod et al., 2006). A 12-y marine mammal stranding database (1998-2009) in the Philippines documented only two single strandings of pygmy sperm whales and three single strandings of beaked whales involving two Blainville's beaked whale (*Mesoplodon densirostris*) (Aragones et al., 2010) and one Longman's beaked whale (*Indopacetus pacificus*) (Acebes et al., 2005).

The high proportion of live responded cetacean stranders herein (i.e., 60%) is consistent with the reported percentage (i.e., 65%) for marine mammal strandings in the Philippines by Aragones et al. (2010). In their paper, the authors hypothesized three possible reasons for the relatively high number of live stranding events in the country: (1) acoustic trauma, (2) gear entanglement, and (3) biotoxins coupled in their prey items. While physical damages from fishing gears were observed in some of the sampled cetaceans herein, it is also possible that infections or diseases contribute to live stranding frequencies given the determined occurrences of the target pathogens. This deserves further investigation in the future.

Until this study, there has been no information on the presence of the detected microorganisms in cetacean species found in the Philippines. This study provides the first report on bacteria isolated in local cetaceans. Likewise, this is the first effort to document *T. gondii* occurrence from a marine environment and among marine mammals in the country. The current findings provide clues to the health status of free-ranging cetacean populations and underscore the potential of stranded representatives to serve as sentinels for indicating the conditions of their habitats. As the detected microorganisms include known and emerging pathogens of humans and other terrestrial animals, such may have implications on potential contamination of marine habitats by land-based disease agents or the unrecognized existence of these agents in marine environments. Knowledge on the kinds of pathogens found in cetaceans may be treated as feedback information on how anthropogenic activities (e.g., discharge of untreated effluents to oceans) are affecting the ecology of these species. Likewise, it presents the health risks available to humans who share the same water resource and interact with these animals through responding to stranding events, doing research work, or involvement in other activities. As the proponents of the "one health" concept suggest, the health status of marine mammals provides one of the links between ocean and human health. Therefore, it is high time to consider the health conditions of the diverse cetacean assemblage in the Philippines. As an offshoot of this study, a local stranding event response protocol involving organized specimen collection and improved necropsy work for investigating emerging diseases in stranded cetaceans is in the planning stages.

Acknowledgments

We thank the Philippine Marine Mammal Stranding Network (PMMSN), the Bureau of Fisheries and Aquatic Resources (BFAR) Regional Offices, Ocean Adventure, and Bryan Clark Hernandez for assistance provided. This study was funded by the Philippine Council for Health Research and Development–Department of Science and Technology (PCHRD-DOST) through the Accelerated Science and Technology Human Resource Development Program (ASTHRDP) scholarship grant for MCMO.

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