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Occurrence of zoonotic parasites in free-ranging dolphins and sea turtles in the Gulf of Taranto (Northern Ionian Sea, Central-eastern Mediterranean Sea)

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***Abstract* – The occurrence of enteric protozoan parasites *Giardia duodenalis* and *Cryptosporidium* sp. was molecularly investigated in free ranging species of striped (*Stenella coeruleoalba)* and Risso's dolphins (*Grampus griseus*) and loggerhead (*Caretta caretta*) and green (*Chelonia mydas*) sea turtles occurring in the Gulf of Taranto (Mediterranean Sea). Out of forty-two examined faecal samples, 2 samples of striped dolphins were found positive to *G. duodenalis* and 3 of loggerhead sea turtles were found positive to *Cryptosporidium*. Zoonotic *G. duodenalis* assemblage A and *Cryptosporidium parvum* were identified. This is the first report in which the presence of these pathogens have been investigated in free ranging species in this area and the first report of *C. parvum* in loggerhead sea turtles. These results extend the known host range of these water and foodborne parasites and confirm the widespread of zoonotic assemblages/species in the marine environment and their inhabitants probably as a results of an increasing in anthropogenic activities.**

I. INTRODUCTION

Globally, populations of cetaceans and sea turtles have drastically decreased in recent decades, largely due to anthropogenic activities [1]. In fact, according to the International Union for Conservation of the Nature (IUCN) Red List, at least a quarter of the world's cetacean and sea turtle species are classified as Endangered (EN), although the situation may be worse for some species. Their ecological role in the marine food web, global distribution in both coastal and off shore waters, long lifespans as well as prey diversity and migratory pattern [2, 3, 4] makes cetaceans and sea turtles more susceptible to a variety of human-induced risks, including chemical pollutants, emerging and zoonotic pathogens and infectious diseases [5]. Otherwise, often the same bio-ecological traits respond in measurable and interpretable pathway for natural and anthropogenic changes [6, 7, 8]. Consequently, the conservation assessment of their population can be used to indirectly measure marine ecosystem quality and investigate both magnitude and severity of anthropogenic impacts [9, 10]. Moreover, since these species share the coastal environment with humans and feed on overlapped marine resources, they may also serve as effective sentinels even for public health status [11].

Giardia duodenalis and *Cryptosporidium* sp. are emerging water- and foodborne enteric zoonotic pathogens [12], able to infect humans and a wide range of animals, including domestic and wild [13]. *G. duodenalis* cysts and *Cryptosporidium* oocysts may be released into the terrestrial and marine environment through human/animals excreta [13]. Cetaceans and sea turtles may become infected either via contamination of coastal waters by sewage, run-off and agricultural and medical waste or by consumption of infected prey such as fish and shellfish, resulting in increased infections and mortality in some populations

[14, 15]. Out of eight Assemblages (A-H) recognized for *G. duodenalis*, the zoonotic assemblages A and B infect both humans and animals, while the others do not infect humans and are mainly host specific [16]. At least thirty species have been recognized in the *Cryptosporidium* genus, with *C. parvum* that exhibits the highest zoonotic potential [12].

The presence of *G. duodenalis* and *Cryptosporidium* sp. cysts/oocysts has been already reported in the faecal samples of bowhead whales (*Balaena mysticetus)* and right whales (*Eubalaena glacialis*) from North Atlantic Sea [17] and *Cryptosporidium* oocysts in some samples of harbor porpoises *(Phocoena phocoena)* from Baltic Seas [18]**.** Moreover**,** zoonotic *G. duodenalis* assemblages and *Cryptosporidium* species have been reported in several species of dead cetaceans such as minke whales *(Balaenoptera acutorostrata)* (*G. duodenalis* Assemblage A)*,* common bottlenose dolphins (*Tursiops truncatus*) *(C. parvum),* striped *(Stenella coeruleoalba)* and short-beaked common dolphins (*Delphinus delphis*) *(G. duodenalis* Assemblage A and *C. parvum),* stranded along the European Atlantic Coast [19, 20].

Within the Mediterranean Sea, the only available studies regard the presence of *G. duodenalis* and *Cryptosporidium* sp. cysts/oocysts in free-ranging bottlenose dolphins (*Tursiops aduncus*) and sperm whales (*Physeter macrocephalus*) inhabiting waters of the Red Sea at Hurghada, Egypt [21] and Balearic Archipelago, Spain [22].

Given the widespread of these emerging pathogens and zoonotic assemblages/species commonly associated with humans in marine ecosystem, the aim of this study is to provide information on *G. duodenalis* and *Cryptosporidium* sp. occurrence in free-ranging individuals of striped dolphin, Risso's dolphin (*Grampus griseus*), loggerhead turtle (*Caretta caretta*) and green turtle (*Chelonia midas*) occurring in the Gulf of Taranto (Northern Ionian Sea, Central-Eastern Mediterranean Sea), where these species are exposed to elevated levels of anthropogenic pressure [23, 24]. The role of these emerging and zoonotic pathogens has been also evaluated.

II. MATERIAL AND METHODS

A. Study area

The Gulf of Taranto (Northern Ionian Sea-Central Mediterranean Sea) extends on about 14000 km^2 from Santa Maria di Leuca to Punta Alice and it is characterized by terraces descending toward a system of submarine canyons that identify the Taranto Valley [25]. This singular morphology involves a complex distribution of water masses with a mixing of surface and dense bottom waters and the occurrence of upwelling currents with high seasonal variability [26].

In the basin, different cetacean and sea turtle species coexist with several anthropogenic pressures, such as fishery, industrial discharges, marine traffic and navy exercise areas [23, 27]. In particular, the striped dolphin is the most frequent and abundant species, followed by the common bottlenose dolphin, the Risso's dolphin and the sperm whale [28, 29, 30, 31]. Concerning the sea turtles, the most frequent species in the basin is the loggerhead sea turtle, followed by the occasionally green and leatherback sea turtle (*Dermochelys coriacea*) [32, 33].

B. Sampling

From August 2018 to August 2019, faecal samples of free-ranging striped and Risso's dolphins were collected during vessel-based surveys aimed to monitoring the cetacean populations carried out in the study area. A total of 17 individual faecal samples were collected, of which 11 samples from striped and 6 from Risso's dolphins. Whenever individual dolphin defecated, floating faces were collected at the water surface by using a fine nylon mesh net, changed between each sample as reported in [34]. During the same sampling period, 25 sea turtles founded stranded along the Ionian Sea coast but still alive, were hospitalized at two Sea Turtle Rescue Centres alongside the Northern Ionian Sea. Out of 25 sea turtles, 23 were loggerhead and 2 green turtle. Once reaching the Rescue Center, each turtle was subjected to the first clinical examination according to the Ministerial Guidelines of the Italian Institute for Environmental Protection and Research, ISPRA [35] and if needed, they received, rehydration with fluidic therapy and vitamin administration. Successively, they were kept in an individual sterilized basin with salt water and faecal sample was collected, as soon as possible, after the first spontaneous faecal voiding from each turtle [36].

Both faecal samples of dolphins and sea turtles were collected in a sterile tubes individually labelled for identification, refrigerated at 5°C, and delivered to the laboratory analysis within 24h for the analysis.

C. DNA extraction

Genomic DNA was isolated from individual faecal sample by using the Qiagen Stool kit (Qiagen, Germany), according to the manufacturer's instructions. DNA samples were eluted in 50 µl of H_2O, quantified by using a Qubit 2.0 fluorimeter and stored at –20 °C, pending molecular analysis. The individual genomic DNA samples contained approximately from 0.2 to 100 ng μL^{-1}.

D. Giardia duodenalis and Cryptosporidium sp. PCR

For the genetic characterization of *G. duodenalis* and *Cryptosporidium* sp., part of the TPI gene (~530 bp) and of GP60 gene (~358 bp), were amplified following the nested-PCR protocol as described in [37].

All the PCRs were carried out in 25 µL, including 10 µL of Ready Mix REDTaq (Sigma, St. Louis, MO) and 100 pmol of each primer. Approximately 50-100 ng of genomic DNA was incorporated into each reaction and a negative control sample (no-template) and a known positive control sample were included in each PCR run.

E. Sequencing

PCR products were run on 1.2% agarose gel, and positive samples were purified with exonuclease I (EXO I) and thermosensitive alkaline phosphatase (FAST AP) (Fermentas, Whaltham, MA, U.S.A.) enzymes, in accordance with the manufacturer's instructions. PCR fragments obtained were directly sequenced in both directions using the ABI PRIMS BygDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) with the same primers as the respective PCR reactions, in accordance with the manufacturer's instructions. The sequences obtained were determined using an ABI PRISM 3130 Genetic Analyser (Applied Biosystems), chromatograms were inspected by eye using FinchTV (https://digitalworldbiology.com/FinchTV) and primer regions plus bad-quality regions were removed. Once the sequences had been cleaned up, to investigate the assemblages and species, each sequence was compared with the *G. duodenalis* and *Cryptosporidium* sp. homologous nucleotide sequences available in GenBank database using the BLAST program (Basic Local Alignment SearchTool; https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Subsequently the sequences were aligned using the CLUSTALW implementation of BIOEDIT, version 7.0.5 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

III. RESULTS

Overall, out of forty-two DNA samples subjected to molecular analysis, 5 (11.9%) were found positive to *G. duodenalis* or *Cryptosporidium* sp. In particular, 2 samples of striped dolphin were positive to *G. duodenalis* and 3 samples of loggerhead sea turtle were positive to *Cryptosporidium* sp. Risso's dolphin and green sea turtle samples were found negative to at least one or two protozoan parasites. After sequencing, *G. duodenalis* Assemblage A and *C. parvum* were characterized.

IV. DISCUSSION

This is the first study in which the presence of *G. duodenalis* and *Cryptosporidium* sp. has been molecularly investigated in free-ranging species of striped and Risso's dolphins as well as in loggerhead and green sea turtles occurring in the Gulf of Taranto, Northern Ionian Sea Central-eastern Mediterranean Sea. Zoonotic *G. duodenalis* assemblage A have been characterized in two individuals of striped dolphins whereas *C. parvum* was found in three of loggerhead sea turtles.

Currently, published data about *G. duodenalis* and *Cryptosporidium* infections in marine animals are limited and they are mainly referred to stranded and dead cetaceans [17, 18, 19, 20]. Few studies investigated the prevalence of *G. duodenalis* and *Cryptosporidium* sp. infections in free-ranging bottlenose dolphins and sperm whales within the Mediterranean Sea [21, 22].

In our study, two individuals of striped dolphins were positive to *G. duodenalis*. Moreover, zoonotic Assemblage A was reported. The finding of a zoonotic assemblage is in line of what reported in the study carried out from [19] in European Atlantic coast, although investigated in dead cetaceans, highlight as anthropogenic activities can be a source of contamination for the marine environment. Contrarily of what reported in the same study [19], striped dolphin samples were founded negative to *Cryptosporidium* sp. Moreover, Risso's dolphin samples were founded negative to both the parasites. Variations in parasites composition and prevalence might be related to several factors such as dietary differences, the parasite life cycle, the availability of hosts necessary to complete the life cycle, the interactions between parasite species, the host immune response, and the host population density [38]. Moreover, parasites can also spread in different way in marine animals, particularly when they act together with ecological, biological, and anthropogenic factors [39].

In this work, *C. parvum* has been molecularly characterized in three individuals of loggerhead sea turtles while the same sample were found negative to *G. duodenalis*. The green turtles were negative to both the parasites. Parasites prevalence difference may be related to the different diet for the two sea turtle species. Several studies have investigated the presence of *C. parvum* in different species of mussels (*Mytilus galloprovincialis)* [37] and a diet based on mussels can be hypothesized for the loggerhead sea turtles.

To date, no data about the presence of *Giardia* and/or *Cryptosporidium* cysts/oocysts in any sea turtles are available. Oocysts of *Cryptosporidium* and zoonotic species (*C. parvum*) have been reported in several species of terrestrial turtles [40]. Therefore, to the best of our knowledge, this is the first report of *C. parvum*

in *C. caretta* and these results extend the known host range of this zoonotic protozoan. The possible role as sea turtles as reservoir of these protozoan need to be further investigated.

The results obtained in the present study confirm the widespread of zoonotic assemblages/species of these protozoan parasites in the marine environment and their inhabitants probably as a result of an increasing in anthropogenic activities. Indeed, different factors have been suggested to influence the prevalence of protozoans in aquatic animals, including proximity to human sewage or agricultural outflow [41].

In the future, zoonotic pathogens prevalence and origin routes establishment, are needed in order to implement management actions and preserve their habitats and, hence, to protect animal and public health.

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