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Research paper

Detection of *Toxoplasma gondii* in three common bottlenose dolphins (*Tursiops truncatus*); A first description from the Eastern Mediterranean Sea

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ABSTRACT

Toxoplasma gondii has been described in several marine mammals around the world including numerous species of cetaceans, yet infection and transmission mechanisms in the marine environment are not clearly defined. The Israel Marine Mammal Research and Assistance Center has been collating a database of all marine mammal stranding events along the country's national coastlines since 1993. In this study, we describe the molecular detection and characterisation of *T. gondii* in three common bottlenose dolphins (*Tursiops truncatus*) including one case of coinfection with herpesvirus. The animals were found stranded on the Mediterranean coast of Israel in May and November 2013. In one of the three cases, the dolphin was found alive and admitted to intensive care. To our knowledge, this is the first report of *T. gondii* infection of marine mammals in the Eastern Mediterranean Sea. As this parasite acts as an indicator for marine pollution and marine mammal health, we believe these findings add important information regarding the state of the environment in this region.

1. Introduction

Toxoplasma gondii is a coccidian protozoan that infects a wide range of homoeothermic organisms including humans (Dubey and Beattie, 1988). It is spread through ingestion of oocysts shed in the faecal matter of its definitive host, the cat, as well as through transplacental transfer or consumption of infected tissue (Frenkel et al., 1970). The oocysts are able to reach coastal waters and may be sufficiently resistant to survive in the sea (Dubey et al., 2003; van de Velde et al., 2016); the presence of *T. gondii* has been described in numerous marine mammals around the world, including cetaceans, pinnipeds, sirenians, sea otters and polar bears (Herder et al., 2015). However, the mechanisms by which these animals are infected remain uncertain as most marine mammals derive their nutritional requirements from cold-blooded organisms or are exclusively herbivorous; in dolphins, this is particularly intriguing as they drink little or no water, thus transmission through the faecal-oral route is ostensibly unlikely (Dubey et al., 2003). Several biotic vectors have been suggested capable of conveying oocysts from the nearshore to offshore environment. However, their affirmative uptake by phoretic agents, such as sessile or migratory filter feeders, has only been confirmed under experiment conditions (e.g., Lindsay et al., 2001; Massie et al., 2010). The epidemiology of *T. gondii* infection of offshore species has also been linked to waterborne transmission through ship runoff waters, when hygienic conditions are poor and rodents, cats, or contaminated soil are present onboard (Van Bressem et al., 2009; Di Guardo et al., 2010). The publication of clinical cases may improve our understanding of *T. gondii* infection in marine mammal species. Yet, monitoring of cetacean health poses great difficulties and is largely reliant on postmortem examinations, which often fail to inform about the primary cause of death. It is therefore imperative to scrutinise each

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necropsy case of a deceased marine mammal. The Israel Marine Mammal Research and Assistance Center (IMMRAC) has been collating a database of all stranding events along the country's Red Sea and Mediterranean coasts since 1993 (n = 348), including 21 cases of live-beached cetaceans (Morick, personal communication). Reported in this paper is the detection of *T. gondii* in three common bottlenose dolphins (*Tursiops truncatus*), including one case of coinfection with herpesvirus. All animals were found stranded on the Mediterranean coast of Israel in May and November 2013. In one of the three cases, the dolphin was found alive and admitted to intensive care. Nevertheless, the animal died after five days of attempted rehabilitation. Full necropsies were performed on all specimens as well as ancillary examinations. To our knowledge, this is the first report of *T. gondii* infection of marine mammals in the Eastern Mediterranean Sea.

2. Materials and methods

2.1. Necropsy

Necropsies were conducted as described by Kuiken and García-Hartmann (1993). Samples of internal organ tissues, including brain, spleen, liver, lymph node and lung were collected and stored for further DNA extraction as well as histology and bacteriology.

2.2. Histology

During necropsy, tissue samples of 500 mg were submerged in 10% neutral-buffered formalin. The specimens were embedded in paraffin (Paraplast Plus; Diapath S.r.l., Belgamo, Italy), cut by microtome (Reichert-Jung 2050) into serial 5 µm sections, stained with hematoxylin and eosin (H&E) and examined under light microscopy (Leica DMRB). Images were acquired by a Nikon digital light system.

2.3. Immunohistochemistry

Indirect immunohistochemistry was performed on lung, adrenal gland and brain tissues from each examined dolphin. Following deparaffinization and peroxidase blocking, sections were covered with a rabbit polyclonal anti-*T. gondii* primary antibody (dilution 1:100) (Novus Biologicals, CO, USA) and incubated. A One-Step Polymer anti-Mouse/Rabbit/Rat secondary antibody (ready to use) (ZytoChem Plus HRP; Zytomed Systems, Berlin, Germany) was applied, rinsed with a substrate-chromogen solution (Zytomed Systems, Berlin, Germany) and counter-stained with Meyer's hematoxylin.

2.4. Toxoplasma and neospora polymerase chain reactions (PCR)

For detection of Toxoplasma, tissues of 200 mg underwent extraction using the QIAgen DNeasy Tissue Extraction kit (CA, USA). The manufacturer's instructions were followed with two modifications: Proteinase K incubation was carried out at 60 °C for 1 h instead of 10 min, and elution of DNA from spin columns was performed twice instead of once. The final elution volume was 200 µL per sample. The DNA samples obtained by extraction were stored at −20 °C until examined. *T. gondii*-specific primers TOX4 (5′-CGCTGCAGGGAGGAAGACGAAAGTTG-3′) and TOX5 (5′-CGCTGCAGACACAGTGCATCTGGATT-3′) were used from the 5′ and 3′ ends of the 529 bp repeated sequence (Homan et al., 2000), respectively. The PCR mixtures contained 0.2 mM of each primer, 100 mM dNTP (Fermentas Inc., ML, USA), 60 mM Tris−HCl (pH 9.0), 15 mM (NH_4) $2SO_4$, 2 mM $MgCl_2$ and 1U Biotaq (Bioline, MA, USA) per reaction. Amplification was performed on a PTC-150 Minicycler thermocycler (MJ Research Inc., MA, USA) with initial denaturation for 7 min at 94 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 60 °C, 1 min at 72 °C and a final 10 min incubation at 72 °C. PCR reactions were carried out in 50 µL mixtures containing 10 µL of sample extract. PCR amplification products were run on 1.1% ethidium bromide agarose gels alongside a 100 bp size marker (Biolabs, MA, USA) and a positive control containing the purified DNA equivalent of 5 *T. gondii* tachyzoites. A negative control containing no DNA material was also included by adding equivalent volumes of ddw only. Detection of *N. caninum* DNA was performed by nested PCR as previously described (Fish et al., 2007), using two sets of primers located on gene Nc5 and based on the DNA sequence of *N. caninum* (GenBank accession number X84238). The PCR reaction contained 1 µL of genomic DNA. Each primer's concentration was 0.4 µM and 1 × ReddyMix (Thermo Fisher Scientific Inc., Hemel Hampstead, UK), in a final volume of 25 µL. Samples from *N. caninum* isolate, NcIs491 and negative (no DNA) were added to each reaction as positive and negative controls, respectively. Products were visualised on 1.5% ethidium bromide-stained agarose gels. Positive *T. gondii* PCR products were purified using a PCR Purification kit (Qiagen, Germantown, USA) and sequenced (Hy Laboratories, Rehovot, Israel).

2.5. Distemper and herpesvirus real-time PCR

For the detection of distemper virus, total RNA was extracted using the Nucleo Spin RNA II Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. The PCR was performed according to Rubio-Guerri et al. (2013) and Centelleghe et al. (2016). Total DNA was extracted from the sampled tissues, using the High Pure Template Preparation Mix according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). A pan-herpesvirus nested PCR targeting the DNA polymerase gene was performed (Vandevanter et al., 1996). The PCR product was purified using a PCR Purification kit (Qiagen, Germantown, USA) and sequenced.

3. Results

3.1. Case #1

On May 2, 2013 at 22:00 h, a dead female dolphin was found stranded on Ga'ash beach, Israel (Fig. 1). The decomposition state of the carcass was classed as 'fresh' (condition code 2; CC2), as defined by Kuiken and García-Hartmann (1993). The specimen was transported for necropsy which was carried out on the morning of 4 May. Identified as *T. truncatus*, the dolphin measured 233 cm in length and weighed 120 kg. Lacerations and teeth marks were observed on the external surface of the body. Blubber sampled from a mid-ventral incision was 2 cm thick. The animal was estimated to have been 5 years of age upon death. Undigested fish were present in the oesophagus and first stomach chamber, but no food residues were found further down the digestive tracts and no pathological signs were observed in the stomach. Additional lesions included multifocal abscess in the lungs, 1–2 cm in diameter at each site, as well as splenomegaly (Fig. 2). Histopathological examination of internal organ tissues revealed initial autolysis and mild pleuropneumonia. *T. gondii* was not detected by means of immunohistochemistry. PCR was performed on lung, spleen, liver and brain tissues. The latter tested positive for *T. gondii* (GenBank accession number MG003442). No pathological findings were observed in this tissue. All other tissues were negative for *T. gondii*, *N. caninum*, distemper and herpesvirus.

3.2. Case #2

On May 10, 2013, another dead bottlenose dolphin was found drifting off the coast of Mikhmoret (Fig. 1) and towed into a nearby boat anchorage. The CC3 carcass was kept cool until necropsy which was carried out on the evening of May 12. Measuring a total length of 211 cm and weighing 93 kg, the male dolphin was estimated to have been two years of age. Mid-ventral blubber was 1.5 cm thick. No fish were found in the digestive system. Foam was observed in the tracheae and the lungs had warts and disseminated abscess, ranging up to 10 cm