Detection of *Toxoplasma gondii* infection in dolphins stranded along the Tuscan coast, Italy

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**SUMMARY**

The rate of *Toxoplasma gondii* infection was surveyed in 8 bottlenose dolphins (*Tursiops truncatus*) and 6 striped dolphins (*Stenella coeruleoalba*) stranded along the coast of Tuscany. Specimens of brain (n=13), liver (n=8), muscle (n=2), and whole blood (n=4) were examined by a nested polymerase chain reaction (PCR) targeting the B1 gene of *T. gondii* in different tissues (n=10) or by a modified agglutination test in combination with nested PCR (n=4). Overall, *Toxoplasma* infection was detected in 13 of 14 (93%) dolphins (in 10 by nested-PCR, in 2 by MAT, and in 1 by both). These results suggest that the level of *T. gondii* infection among dolphins in the marine area of Tuscany is high.

**Keywords:** *Toxoplasma gondii*, dolphins, Italy.

**RÉSUMÉ**

Détection de *Toxoplasma gondii* chez les dauphins échoués sur la côte de la Toscane, Italie

La présence de *Toxoplasma gondii* a été étudiée chez 8 grands dauphins (*Tursiops truncatus*) et 6 dauphins bleus et blancs (*Stenella coeruleoalba*), échoués sur la côte de la Toscane. Des échantillons de cerveau (n = 13), foie (n = 8), muscle (n = 2), et sang entier (n = 4) ont été examinés par réaction de polymérisation en chaîne (PCR) nichée le gène B1 de *T. gondii* dans les différents tissus (n=10), ou par un test d’agglutination modifié en combinaison avec la PCR nichée (n = 4). En général, l’infection par *Toxoplasma* a été détecté chez 13 des 14 (93%) dauphins (10 par PCR nichée, 2 par MAT, et 1 par les deux). Ces résultats suggèrent que le niveau d’infection par *T. gondii* chez les espèces de mammifères dans la zone marine de la Toscane est élevé.

**Mots clés :** *Toxoplasma gondii*, dauphins, Italie.

**Introduction**

The protozoan parasite *Toxoplasma gondii* infects a wide range of warm-blooded hosts, including birds, domestic and wild mammals, and humans. Hosts, including the definitive host (felids), can acquire *T. gondii* by ingesting either food or drink contaminated with sporulated oocysts or tissues of infected animals, or by transplacental transmission.

Recently, *T. gondii* infection in marine mammals has drawn attention because toxoplasmosis can cause mortality in certain marine mammals which have been suggested as sentinels of contamination of marine waters [4, 16].

Cases of *Toxoplasma* infection were detected in cetaceans stranded on the coasts of Italy [6-8]. However, data about the rates of infection among mammal species inhabiting the marine area of Tuscany are limited and date back to several years ago [6, 7]. Here, we report *T. gondii* infection in dolphins stranded on the Tuscan coast during 2006-2008.

**Materials and Methods**

**STUDY AREA**

Tuscany (geographical coordinates: 43° 24’ 36” N, 11° 0’ 0” E) is a region of central Italy. Its 397 km of continental coastline are bathed by the Ligurian Sea (43° 29’ 54” N, 9° 2’ 30” E) in the north-central portion and by the Tyrrenian Sea (39° 31’ 22” N, 13° 21’ 12” E) in the southern portion. The marine area of Tuscany includes the Tuscan Archipelago National Park which is the largest marine park in Europe. The park was founded in 1966, covers 56,766 hectares of sea and 17,887 hectares of land including seven major islands, smaller islands, islets and reefs located between Tuscany and Corsica. Moreover, the Tuscan Archipelago National Park is part of the Cetacean Sanctuary. This is a special marine protected area in the north-western Mediterranean Sea, was founded in 1999, and covers about 90,000 km² of international waters including three countries: France (Côte d’Azur and Corsica), Principality of Monaco, and Italy (Liguria, Tuscany, and northern Sardinia).
COLLECTION OF SPECIMENS

Fourteen dolphins (8 bottlenose dolphins, *Tursiops truncates*, 6 striped dolphins, *Stenella coeruleoalba*), were found stranded along the coast of Tuscany during 2006–2008. Species, condition of carcass, gender and length of each animal are given in Table 1. Unfortunately, in two cases the gender could not be determined due to high decomposition of these carcasses. Collection and transport of all carcasses were authorized and supervised by the Centro Studi Cetacei (Milan, Italy). Specimens of brain (n=14), liver (n=8), muscle (n=2), and whole blood (n=4) were collected, refrigerated at +4°C, and transported as soon as possible to the laboratory. Upon arrival, samples of brain, liver and muscle were stored at -20°C pending molecular assay while blood samples were immediately examined. The infection rate of *T. gondii* was surveyed using a nested polymerase chain reaction (PCR) targeted to *T. gondii* B1 gene in the different tissues (n=10), or a modified agglutination test in combination with nested PCR (n=4) according to the availability of samples.

NESTED PCR

DNA from tissue samples and tachyzoites were extracted in guanidine lysis buffer precipitated with isopropanol and dissolved in deionised water [20]. DNA pellets were resuspended in TE buffer (10 mM Tris–HCl [pH 7.5], 1 mM EDTA) and concentration assessed by UV absorbance at 260 nm. The DNA was diluted to 10 ng/ml with water and stored at -20°C.

Two pairs of oligonucleotide primers directed against the B1 gene of *T. gondii* were used to perform a nested PCR as described by Jones et al. (2000). Briefly, the following primers were used:

- Outer primer (sense strand) 5’-GGAACTGCATCCGGTT-CATGAG-3’ (position: 694–714)
- Outer primer (sense strand) 5’-TCTTTAAAGCGTTC-GGAACTGCATCCGTT-CATGAG-3’ (position: 853–831)
- Inner primer (sense strand) 5’-TGCATAGGTTGCAGT-GGTGGTC-3’ (position: 887–868)
- Inner primer (sense strand) 5’-TGATACGTTGTGCACT-CTGAG-3’ (position: 757–776)
- Inner primer (sense strand) 5’-GGCGACCAATCTGC-GAATACACC-3’ (position: 833–811)

Purified *T. gondii* DNA was used as a template. First-Round PCR contained 10 mM Tris–HCl, pH 8.3 (at 25°C), 50 mM KCl, 2 mM MgCl2, 0.1 mM each primer, 0.1 mM each dNTP, and 1 U Taq DNA polymerase. Reactions were cycled 40 times using a denaturation step of 93°C for 10 seconds, followed by annealing at 62.5°C for 10 seconds and extension at 72°C for 15 seconds. Negative control samples from first-round amplification and an additional second-round negative control of sterile water were included in the nested reactions.

Sensitivity controls of PCR test were achieved using several dilutions (from 0.69 to 6900 cells) of *T. gondii* tachyzoites RH strain from acetic fluid of mice. The cells were quantified using a modified Fuchs Rosenthal CSF counting chamber.

Unless otherwise stated, all chemicals used were purchased from Sigma (St. Louis, USA) and were of the highest grade available.

Ten micro-litre B1 amplification products were visualized under UV illumination after electrophoresis on 1.2% TBE/agarose gels and staining with ethidium bromide. A molecular weight marker was included in each run (DNA Molecular Weight marker XIV, Roche Applied Science, Mannheim, Germany). Samples were scored as positive when a PCR product of 96 bp was detected.

PCR products were purified using High Pure PCR Product Purification Kit (Roche Applied Science). The sequencing reactions were performed in a total volume of 10 μl containing 0.5 μl of premix from the ABI prism BigDye Terminator v3.0 ready-reaction cycle-sequencing kit (Applied Biosystems), 10 pmol of sequencing primer SSU-hact-519r, and 2 μl of the purified PCR product. The sequencing products were analyzed on an ABI 3730XL capillary sequencer. An alignment B1 gene sequences available in GenBank was performed using ClustalW, as implemented in BioEdit Version 7.0.1 [12]. DNA sequences were compared with target sequences and found to be identical in all cases [2].

SEROLOGICAL ANALYSIS

Whole blood specimens were centrifuged at 1500 rpm (151 x g) for 10 min. Supernatants were collected and filtrated using sterile 0.2-μm micro-filters (Nalgene®, PBI, Milan, Italy) to remove larger particles. Sera were screened for *T. gondii* IgG and IgM antibodies using a MAT (Toxo-Screen DA, Biomérieux, France), according to the manufacturer’s instructions. Samples were two-fold diluted starting from 1:10 dilution.

DESCRIPTIVE STATISTICAL ANALYSIS

Dolphins were considered as *Toxoplasma* positive when one of the tissue samples examined by nested-PCR, or whole blood examined by MAT, or both gave a positive result. Infection rate was determined as number of positive animals/number of examined animals x 100.
Results

A single amplicon with a predicted size of 96 bp was amplified at the end of the nested amplification (Figure 1). This reaction was able to detect 0.69 cells of *T. gondii* DNA after analysis on ethidium bromide-stained TBE/agarose gels. The nested reaction did not yield product from the negative controls. The nested PCR products were cycles sequenced, which confirmed that the amplified products were identical with the published sequence of the *T. gondii* B1 gene. Comparison of the sequencing products obtained with *T. gondii* sequences available in the GenBank™ database revealed 99% identity with the *T. gondii* B1 gene (Genbank accession number: AF179871).

Results of nested-PCR and serological tests are shown in Table 1. Following nested-PCR amplification, 9 of 14 (64%) specimens from brain and 4 of 8 (50%) specimens from liver were positive for parasite DNA. The two specimens from muscle failed to show the presence of *Toxoplasma* DNA. In 2 cases brain positivity and liver positivity were associated, whereas in 4 and 2 cases only brain positivity or liver positivity were detected, respectively. Combining results of nested-PCR amplification from brain and liver, 11 of 14 (79%) dolphins were harbouring *T. gondii* DNA in their tissues.

Antibodies to *T. gondii* (MAT titre 1:20) were found in 3 of 4 whole blood samples. Serological positivity was associated with brain negativity in 2 cases, with brain positivity and liver negativity in another case. The serologically negative blood sample was from a dolphin harbouring *T. gondii* DNA in brain. Combining molecular and serological results, total infection rate raised to 13 of 14 (93%) dolphins examined.

Discussion

The present results confirm those of previous investigations in which *Toxoplasma* was detected in 4 of 25 and 8 of 37 cetaceans stranded along the Italian peninsula, including the Tuscan coast [6, 7]. Thus *T. gondii* is still circulating actively in the marine area of Tuscany. A few studies have recently investigated the rates of *Toxoplasma* infection in cetaceans inhabiting the Mediterranean Sea. Antibodies to *T. gondii* were found in 11 of 58 dolphins stranded along the Spanish Mediterranean coasts, using the MAT [3]. DI GUARDO et al. [8] tested sera from 8 striped dolphins stranded on the

![Figure 1: Electrophoresis of final nested-PCR products for *T. gondii* DNA from brains of stranded dolphins. PCR products (10 µl) were resolved on 1.2% agarose/TBE gel and visualized after ethidium bromide staining under UV illumination. Lines 1 and 20: 100 bp DNA ladder. Lines 2 and 19: empty. Line 3: positive control (*T. gondii* tachyzoites genomic DNA, 0.69 cells). Line 18: negative control. Lines 4, 5, 7, 10, and 12: negative samples. Lines 6, 8, 9, 11, and 13 to 17: positive samples.](image-url)
Ligurian coast by an immunofluorescence antibody test. Antibodies to *T. gondii* were found in 4 of these dolphins with titres ranging from 1:80 to 1:320. In our study, *Toxoplasma* infection was detected overall in 13 of 14 (93%) dolphins stranded on the Tuscan coast by nested PCR assay, or MAT, or both. This suggests that the level of *T. gondii* infection among mammal species in the marine area of Tuscany appear to be comparable to or higher than those reported elsewhere [3, 8, 11, 19]. Viable *T. gondii* has been isolated from dolphins from North and Central America [9, 10].

Felids are the only known hosts that excrete environmentally resistant oocysts of *T. gondii*. Cats can contaminate the environment with as many as 94 to 4,671 oocysts/m² [5]. Many owners dispose of their cat faeces by flushing them down the toilet [11]. A positive correlation between the presence of coastal storm runoff water and the presence of antibodies to *T. gondii* in sea otters was found [16]. Thus it is possible that *T. gondii* oocysts enter the marine environment via runoff contaminated by cat excrements [17]. Oocysts have been shown to sporulate in seawater and remain orally infectious to mice for up to 24 months after storage in seawater at +4°C [14]. Sporulated oocysts can be concentrated by bivalve molluscs and retain their infectivity in oysters (*Crassostrea virginica*) or mussels (*Mytilus galloprovincialis*) for up to 3-85 days under experimental conditions [1, 13]. Thus infected filter feeders may serve as a source of *T. gondii* for marine mammals and possibly humans [17]. However, their epidemiological role in the marine transmission pathway of the parasite is unknown. Similarly, the transmission pathway by which dolphins are being infected still remains unresolved. It is known that toxoplasmosis can occur by ingesting either food or drink contaminated with sporulated oocysts or tissues of infected warm-blooded animals. However, dolphins drink little or no water and feed on fish, squid, or other cold-blooded sea animals. In addition, experimental studies on the role of fishes as hypothetical source of *T. gondii* have yielded conflicting results [15, 18].

In conclusion, the present results document *T. gondii* infection in dolphins in Tuscany and warrant further studies on contamination of marine life with *T. gondii*.

References


