

West Greenland harbour porpoises assayed for antibodies against *Toxoplasma gondii*: false positives with the direct agglutination method

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ABSTRACT: We assayed blood/tissue fluid samples from 20 harbour porpoises *Phocoena phocoena* from western Greenland coastal waters for antibodies against the protozoan parasite *Toxoplasma gondii* by the direct agglutination test (DAT). Nine individuals (45%) were interpreted to be seropositive at 1:40 dilution and 4 (20%) were seropositive up to 1:160. Samples from these individuals were assayed by an enzyme-linked immunosorbent assay (ELISA), and tissue samples of the DAT-positive animals were tested by a nested polymerase chain reaction (nPCR). Results from both methods were negative, suggesting the absence of infection in the tested animals. After chloroform clean-up, all were negative when re-assayed by DAT. We concluded that infection with *T. gondii* was absent in all 20 animals, despite the initially positive DAT results, and that the false positives resulted from non-specific adherence to tachyzoites in the DAT assay which could be removed by the chloroform clean-up method. Our results suggest that detecting antibodies against *T. gondii* using the DAT or the modified agglutination technique, particularly on samples from Arctic marine animals which often are rich in lipids, may lead to false positive results. For such samples, the use of ELISA or PCR on available tissue samples may be advocated as confirmatory tests in order to avoid false positives and overestimating seroprevalence.

KEY WORDS: *Toxoplasma gondii* · *Phocoena phocoena* · Greenland · Seroprevalence · DAT · ELISA · PCR

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INTRODUCTION

Toxoplasma gondii is a globally distributed intracellular coccidian protozoan parasite capable of infecting virtually all warm-blooded animals, including humans and birds (Schmidt & Roberts 2005). Infection with *T. gondii* is mainly acquired by ingestion of meat containing infective tissue cysts or food

and drink contaminated with oocysts. The parasite also infects transplacentally, and congenital toxoplasmosis is a serious disease, often resulting in abortions, foetal damage or death in humans and other endothermic species (Dubey & Beattie 1988, Petersen & Liesenfeld 2007). Animals of the family Felidae only (domestic and wild cats) are known as definitive hosts in which the sexual phase with production of

oocysts takes place, while in hosts other than cats (intermediate hosts) the sexual life cycle is never completed (Hutchison et al. 1969, Dubey et al. 1970). From the first reports on *T. gondii* in marine mammals (in captive California sea lions *Zalophus californianus*: Ratcliffe & Worth 1951, Migaki et al. 1977; and northern fur seal *Callorhinus ursinus*: Holshuh et al. 1985), 33 species of marine mammals (representing 8 families) have been reported to be infected by *T. gondii*, mostly detected by serological methods (e.g. Gajadhar et al. 2004, Fujii et al. 2007, Oksanen et al. 2009, Di Guardo et al. 2011). Furthermore, numerous reports have presented findings related to *T. gondii* in several Arctic animal species (see Table S1 in the Supplement at www.int-res.com/articles/suppl/d108p181_supp.pdf).

The harbour porpoise *Phocoena phocoena* has a circumpolar distribution and can be found in the Barents Sea up to 80° N (Bjørge & Øien 1995, Teilmann & Dietz 1998, Teilmann et al. 2008, Acquarone et al. 2010). Along the east coast of Greenland, the distribution ranges from Ammassalik to south of Kap Farvel, while on the west coast the species is found mainly in the central part and up to Upernavik (Teilmann & Dietz 1998). The West Greenland population is believed to be relatively isolated from other populations and is presumed to have limited contact with the neighbouring populations in Canada and Iceland. Studies even suggest that this population is genetically different from other sub-populations in the northern Atlantic (Andersen et al. 2001, Lockyer et al. 2003). Although the harbour porpoise is a fairly common species in coastal regions where humans live, presence of antibodies against *Toxoplasma gondii* in harbour porpoises have to our knowledge been reported only twice, from the Mediterranean (Cabezón et al. 2004) and British waters (Forman et al. 2009). In this study, we tested 20 harbour porpoises from the west coast of Greenland for *T. gondii* infection by 2 serological methods and PCR on tissue samples.

MATERIAL AND METHODS

Animals and samples

The study included 20 harbour porpoises (Table 1) caught in September 2009 for scientific purposes in the vicinity of Maniitsoq (65° 25' N, 25° 54' W) in western Greenland. The animals were shot at sea by Inuit hunters, brought to the Greenland Institute of Natural Resources in Nuuk and stored (−20°C) until

Table 1. *Phocoena phocoena*. Morphometric information of the 20 sampled harbour porpoises and results of the direct agglutination tests (Run 1 = initial screening of samples, Run 2 = retesting following clean-up) on blood and/or tissue fluid samples. Ratios show the highest dilutions at which the samples were interpreted as positive

ID	Sex	Age (yr)	Weight (kg)	Length (cm)	Run 1	Run 2
5041	F	4	72.7	156	Neg	Neg
5045	F	7	51.1	136	Neg	Neg
5046	F	3	49.3	138	Neg	Neg
5055	M	2	40.3	124	Neg	Neg
5056	F	<1	25.8	105	Neg	Neg
5057	M	<1	27.9	113	1:40	Neg
5059	F	6	63.8	152	1:160	Neg
5060	F	3	46.7	135	1:80	Neg
5061	M	6	58.6	147	1:80	Neg
5062	M	4	46.2	134	1:160	Neg
5063	F	10	66.0	151	Neg	Neg
5069	F	4	68.4	157	1:160	Neg
5072	F	>10	60.8	142	1:80	Neg
5076	M	>9	60.4	150	1:40	Neg
5078	M	2	38.8	130	1:160	Neg
5082	F	6	60.1	153	Neg	Neg
5083	F	1	34.1	120	Neg	Neg
5084	F	5	53.0	139	Neg	Neg
5086	F	6	58.1	146	Neg	Neg
5087	M	10	43.1	128	Neg	Neg

necropsy in January 2010. The animals were examined for external lesions, and morphometric measurements were taken (Table 1). Samples of blood and tissue fluid were collected from severed blood vessels at wound sites, or from the abdominal cavity, along with samples of tonsil, tongue, masseter muscle, diaphragm and brain, and stored at −20°C until they were processed. From each individual, up to 4 teeth were extracted from the lower jaw, and age was determined by counting dentine growth layers of the least damaged tooth (for details see Lockyer et al. 2003).

Direct agglutination test (DAT)

Blood/tissue fluid samples were thawed, centrifuged (1000 × *g*, 10 min) and assayed for antibodies against *Toxoplasma gondii* using the Toxo-Screen DA kit (DAT) (BioMerieux). The procedure was based on the manufacturer's instructions and samples that showed agglutination at a dilution of 1:40 were interpreted as positive. Blood samples from 2 arctic foxes *Vulpes lagopus* from Svalbard, one of which was previously diagnosed with *T. gondii* infection by polymerase chain reaction (PCR) performed

on brain tissue samples, and with a high level of antibodies against *T. gondii* (DAT positive at a dilution >1:1280; Prestrud et al. 2008a,b), and the other classified negative by DAT, were used as controls. Samples interpreted as positive or suspicious were assayed further in serial 2-fold dilutions ending at 1:320.

Enzyme-linked immunosorbent assay (ELISA)

An ELISA for antibodies against *Toxoplasma gondii* was performed using the ID Screen® Toxoplasmosis Indirect Multiple-Species ELISA kit (IDvet Innovative Diagnostics). The procedure was based on antibody detection by enzyme-conjugated chimeric Protein A/Protein G, as recently described (Schaefer et al. 2012). Substrate colour development was stopped and read as the optical density at 450 nm (OD₄₅₀) after 15 min. The arctic fox positive and negative control sera were run in separate wells. Samples and controls were run in duplicate wells.

Blood/tissue fluid sample clean-up

Samples were subjected to a chloroform/centrifugation clean-up method (Castro et al. 2000). Briefly, a 1 ml sample in an Eppendorf (polypropylene) tube was mixed thoroughly (by vortexing) with 1 ml chloroform and centrifuged (1000 × *g*, 30 min). The upper phase was pipetted off and centrifuged (10 000 × *g*, 30 min) and assayed by DAT (1:40 and 1:80 dilution). Same samples that were only centrifuged were run in control wells. The arctic fox control samples were treated and assayed in the same manner.

PCR

DNA was extracted from 25 mg of tissue samples (tonsil, tongue, masseter muscle, diaphragm, brain). The samples were first homogenized (pellet pestle device; Kontes, Fisher Thermo Scientific) in phosphate-buffered saline (PBS, pH 7.2), and DNA was extracted using the QIAamp DNA mini kit (Qiagen). The samples were assayed by a nested PCR (nPCR) amplifying a *Toxoplasma gondii*-specific 300-copy 529 bp element (Homan et al. 2000). Samples of brain from arctic foxes previously identified as infected or non-infected with *T. gondii* (Prestrud et al. 2008a,b) were used as positive and negative controls, respectively.

RESULTS

Twenty animals (13 females and 7 males) ranging from 1 to >10 yr old, were sampled (Table 1). The mean (±SD) age was 4.8 ± 3.7 yr for the males and 5 ± 2.9 yr for the females, with weights of 45 ± 11.4 kg and 54 ± 13.4 kg and lengths of 132.3 ± 12.9 cm and 140 ± 14.9 cm, respectively.

Harbour porpoise blood/tissue fluid samples were assayed by DAT, and 9 of the 20 individuals (45%) were interpreted positive or suspicious (4 females, 5 males) at 1:40 dilution. Of these, 3 remained positive at 1:80 dilution and 4 at 1:160 dilution (Table 1). Following the clean-up of the samples (porpoises) and controls (arctic foxes) by the chloroform method, all porpoises were negative at all dilutions tested, whereas the controls remained positive or negative as before. Assaying of the porpoise samples after they had only been centrifuged gave the same results as before centrifugation. The porpoise samples, when assayed by ELISA, were all negative both before and after chloroform clean-up. PCR of tissue samples (tonsil, tongue, masseter muscle, diaphragm, brain) from the 9 individuals interpreted as positive by the first DAT run yielded negative results.

DISCUSSION

Blood/tissue fluid samples collected from opened blood vessels/body cavities of the porpoise cadavers were used in this study. Such samples (mixture of blood/tissue fluid) are often the only samples available, particularly in wildlife studies, and are considered a good alternative to serum samples for serological surveys (Tryland et al. 2006, Simon et al. 2011, Glor et al. 2013). However, such samples are often opaque and rich in particulate matter, especially if the carcass has been stored over a long time or frozen. In addition, marine mammal samples, including fresh serum or plasma, are often rich in lipids (Nelson 1971). The principle of the DAT-assay is that *Toxoplasma gondii* tachyzoites, when not bound to specific IgG antibodies, freely roll to the bottom of the U-shaped test well to form a tiny blob (red in the kit used here) interpreted as a negative test result. In contrast, tachyzoites bound by antibodies (positive test result) are cross-linked via antibody 'bridges' and form a mat more or less covering the bottom of the well (positive test result). If substances present in the sample non-specifically adhere to tachyzoites, their free rolling may be prevented, resulting in false positives. This scenario is likely to be the case here,

as (1) all of the porpoise blood/tissue fluid samples were classified as negative when assayed by ELISA, both before and after the chloroform-method clean-up, (2) all were classified as negative by DAT after the clean-up procedure, and (3) all tissue samples tested negative for *T. gondii* by PCR. The DAT and the modified agglutination test (MAT), in principle the same method (IgG-induced agglutination of formalin-fixed tachyzoites after destruction of IgM), remains the most commonly used method among the many serological methods for detection of anti-*T. gondii* antibodies found in the literature, and cut-off values as low as 1:20 have been used (Dubey et al. 1995; and see references in Table S1 in the Supplement). Therefore, the comparison of seroprevalence between different species, regions and time periods reported in different studies is difficult. If false positives occur due to adherence of components to tachyzoites other than specific antibodies, which can be a problem with samples rich in particulate matter and lipids in a test-sample dilution of 1:40, as demonstrated here, there is good reason to believe that the problem will be even more pronounced if test samples are less diluted. If there is doubt with respect to false positives and sample quality (presence of particulate matter and lipids), our results suggest that an ELISA can be run on agglutination test positive samples (serial testing, which will increase the specificity of the detection). Lastly, PCR could be performed on ELISA-positive samples to provide a diagnosis of certainty.

In the present study, all 20 harbour porpoises from western Greenland coastal waters were seronegative for antibodies against *Toxoplasma gondii*. The limited number of samples does not allow inference as to whether the harbour porpoise population is exposed to *T. gondii*. The transmission routes by which marine animals can become infected are largely unknown but likely involve a marine pathway (Jensen et al. 2010, Massie et al. 2010). Oocysts remain viable in molluscs, in the gills of migratory fish and in seawater (Arkush et al. 2003, Lindsay et al. 2003, 2004, Massie et al. 2010, Shapiro et al. 2012), and waterborne transmission is likely (Dubey 2004, Winiacka-Krusnell et al. 2009, Jones & Dubey 2010), particularly in the Arctic (Jensen et al. 2010, Simon et al. 2011, 2013). Contamination by oocysts from terrestrial ecosystems has been associated with toxoplasmosis outbreaks in sea otters (Miller et al. 2002) and beluga whales (Mikaelian et al. 2000). Harbour porpoises are believed to primarily inhabit coastal waters and continental shelves from temperate to Arctic regions (Lockyer & Kinze 2003, Marubini et al.

2009, Acquarone et al. 2010) and are therefore exposed to coastal freshwater runoff potentially contaminated by oocysts. Surprisingly, despite their proximity to potential sources of infections, only 2 cases of seropositivity in harbour porpoises have been documented so far, one from the UK (Forman et al. 2009) and the other from the Spanish Mediterranean (Cabezón et al. 2004). This could of course reflect the general lack of systematic screening in harbour porpoise populations.

Harbour porpoises are hunted in Greenland without regulation (Teilmann & Dietz 1998), and the meat, skin and intestines, highly valued by the Inuit, are sometimes consumed raw. Inuit from Nunavik in Canada show a 60% seroprevalence for antibodies against *Toxoplasma gondii*, mostly associated with consumption of raw caribou and pinniped meat, as well as with activities linked to skinning of animals (Forbes et al. 2009). We suggest that *T. gondii* exposure should be routinely tested in marine mammals especially in the context of a changing Arctic to provide an assessment of the potential risk from this infection (both as a zoonosis and an epidemic), establish a baseline for future studies (Burek et al. 2008, Prestrud et al. 2010, Heide-Jørgensen et al. 2011, Hueffer et al. 2013) and to provide indications on the contamination path.

In summary, to rule out false positivity, samples that have tested positive by MAT/DAT should be either tested after clean-up or by ELISA (serial testing). The isolation of *Toxoplasma gondii* or detection of its DNA is the only diagnosis of certainty. Therefore, PCR could be performed on tissue samples before making any definitive conclusions on the exposure to *T. gondii* in a given 'at risk' population.

Acknowledgements. We thank J. Sethsen, A. Geisler, F. O. Christensen, K. Lehnert and H. Seibel for their invaluable help during the necropsies. The sampling and necropsies of the porpoises was funded by the Greenland Institute of Natural Resources.

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*Editorial responsibility: Michael Moore,
Woods Hole, Massachusetts, USA*

*Submitted: July 1, 2013; Accepted: December 19, 2013
Proofs received from author(s): February 17, 2014*