Veterinary Parasitology 230 (2016) 25–32

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Toxoplasma gondii in stranded marine mammals from the North Sea and Eastern Atlantic Ocean: Findings and diagnostic difficulties

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ARTICLE INFO

Article history:
Received 16 June 2016
Received in revised form 19 October 2016
Accepted 22 October 2016

Keywords:
Toxoplasma gondii
Marine mammals
Seroprevalence
PCR
MAT
IFA
ELISA

ABSTRACT

The occurrence of the zoonotic protozoan parasite *Toxoplasma gondii* in marine mammals remains a poorly understood phenomenon. In this study, samples from 589 marine mammal species and 34 European otters *(Lutra lutra)*, stranded on the coasts of Scotland, Belgium, France, The Netherlands and Germany, were tested for the presence of *T. gondii*. Brain samples were analysed by polymerase chain reaction (PCR) for detection of parasite DNA. Blood and muscle fluid samples were tested for specific antibodies using a modified agglutination test (MAT), a commercial multi-species enzyme-linked immunosorbent assay (ELISA) and an immunofluorescence assay (IFA). Out of 193 animals tested by PCR, only two harbour porpoise (*Phocoena phocoena*) cerebrum samples, obtained from animals stranded on the Dutch coast, tested positive. The serological results showed a wide variation depending on the test used. Using a cut-off value of 1/40 dilution in MAT, 141 out of 292 animals (41%) were positive. Using IFA, 30 out of 244 tested samples (12%) were positive at a 1/50 dilution. The commercial ELISA yielded 7% positives with a cut-off of the sample-to-positive (S/P) ratio $\geq$ 50; and 12% when the cut-off was set at S/P ratio $\geq$ 20. The high number of positives in MAT may be an overestimation due to the high degree of haemolysis of the samples and/or the presence of lipids. The ELISA results could be an underestimation due to the use of a multispecies conjugate. Our results confirm the presence of *T. gondii* in marine mammals in The Netherlands and show exposure to the parasite in both the North Sea and the Eastern Atlantic Ocean. We also highlight the limitations of the tests used to diagnose *T. gondii* in stranded marine mammals.

1. Introduction

Toxoplasma gondii is a globally distributed zoonotic parasite. It is assumed that all warm-blooded animals, including humans, can act as intermediate hosts, while only felids act as the final hosts (Tenter et al., 2000). A primary infection in felids allows *T. gondii* to complete its sexual cycle, resulting in the faecal shedding of millions of oocysts into the environment (Dubey, 2010). These

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http://dx.doi.org/10.1016/j.vetpar.2016.10.021
0304-4017/

oocysts are able to reach coastal seawaters and several studies have demonstrated the presence of *T. gondii* in marine mammal species including pinnipeds, cetaceans, sirenians, sea otters *(Enhydra lutris)* and polar bears *(Ursus maritimus)* (Cole et al., 2000; Dubey, 2010; Mathews et al., 2012; Oksanen et al., 2009). Different transmission routes such as coastal run-off and mechanical vectors have been hypothesized, but the exact mode of transmission remains unknown (Arkush et al., 2003; Lindsay et al., 2001; Massie et al., 2010). Immunosuppressive factors, such as polychlorbiphenyls and Morbillivirus infection could make marine mammals more susceptible to disease induced by *T. gondii* infection (Mazzariol et al., 2012). Besides being a pathogenic agent capable of affecting marine mammal health, *T. gondii* acts as an indicator of marine pollution (Jessup et al., 2004), with marine mammals being excellent sentinel species (Conrad et al., 2005; de Moura et al., 2014; Jessup et al., 2004; Moore, 2008; Stewart et al., 2008).

Various serological tests are available to detect the presence of *T. gondii* antibodies in humans and animals. The Sabin-Feldman dye test is considered the gold standard test, but is time consuming and makes use of live parasites. Although not validated for marine mammals, the modified agglutination test (MAT) may be an appealing alternative method, because of its speed and ease of use, but also because it does not require species-specific secondary antibodies (Desmonts and Remington, 1980). This is particularly useful when testing wildlife, such as marine mammals (Cabezón et al., 2011; Santos et al., 2011; Jensen et al., 2012). Recently, however, Blanchet et al. (2014) questioned the reliability of MAT in the serological diagnosis of *T. gondii* infection in harbour porpoises *(Phocoena phocoena)*. This was based on a comparison of MAT on untreated serum samples with enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR), which suggested that MAT may give false positive results. Adherence of components to tachyzoites other than specific antibodies, such as samples rich in particulate matter and lipids, may be responsible for these false positive reactions. A chloroform 'clean-up' of serum samples may prevent this problem (Blanchet et al., 2014). With the exception of harbour porpoises (*Phocoena phocoena*), however, this method has not been evaluated for other marine mammal species.

The aim of this study was to assess the prevalence of *T. gondii* in various marine mammal species stranded on Scottish, Belgian, French, Dutch and German coastlines of the North Sea and the Atlantic Ocean around Scotland. As the reliability of MAT in marine mammal samples has been questioned (Blanchet et al., 2014), it was decided to perform additional testing to evaluate and compare various serological detection methods.

2. Materials and methods

2.1. Samples

From 589 free-ranging stranded marine mammals necropsied at different institutes in The Netherlands, Belgium, France, Germany and Scotland (Table 1), a selection of samples including cerebrum and cerebellum, serum and/or muscle fluid was obtained. Additionally, samples from 34 free-ranging European otters *(Lutra lutra)* living along the North Sea coast in Scotland were also analysed. Convenience-based sampling from these different institutes resulted in a collection of samples from a time period of 1992–2013 that covered 17 different marine mammal species. An overview of the numbers and species of collected and tested animals is given in Table 2. Brain samples (n = 193) were tested by PCR. Positive PCR samples were further analysed by histopathology and immunohistochemistry. All serum (n = 292) and muscle fluid (n = 193) samples were analysed with MAT and a large part of the collected serum samples was further analysed with an immunofluorescence assay (IFA) (n = 244) and a multispecies ELISA (n = 241) (Table 3). Due to the relatively large volumes required by MAT and IFA, it was not possible to test all samples with all three tests.

2.2. PCR

2.2.1. DNA extraction

DNA from the brain samples was isolated with the QIAmp DNA Mini Kit (Qiagen, Venlo, The Netherlands) as previously described by De Craeye et al. (2011). Briefly, 400 mg of each brain sample was mixed with 20 μl of proteinase K and 400 μl of a first lysis buffer and vortexed thoroughly prior to overnight incubation at 56 °C. The next day, 400 μl of a second lysis buffer was added and the mixture was homogenized and further incubated at 70 °C for 20 min. After cooling on ice, 600 μl of a chloroform/isoamyl alcohol (24/1) mixture was added and each sample was vortexed and then centrifuged at 22,000 × *g* (4 °C) for 30 min. The supernatant was transferred into a new tube and the DNA was precipitated by adding 400 μl of 95% ethanol cooled at −20 °C. From this point onwards, the manufacturer's protocol was followed. The mixture was transferred to a spin-column and washed once with 500 μl of a first washing buffer and once with 500 μl of a second washing buffer. The purified DNA was then eluted from the column using 200 μl of an elution buffer. The samples were stored at −20 °C until further testing.

2.2.2. Real-time PCR, histopathology and immunohistochemistry

The isolated DNA samples were tested by duplex real-time PCR (qPCR) as described by De Craeye et al. (2011). The 529-basepair repeat element was used as a target (Homan et al., 2000). Table 4 gives an overview of the primers and probes used and their final concentration per reaction. DNA quality and PCR inhibition were assessed using cellular r18S as a target (Table 4). Samples were run and analysed on a BioRad CFX 96 thermocycler/detection system (BioRad, Hercules, CA, USA). In each run, a positive extraction control sample consisting of DNA extracted from a brain sample from a mouse experimentally infected with the 1984-IPB-GAN *T. gondii* strain was included as well as one sample containing only phosphate buffered saline (PBS) as a negative control. Positive results from PCR were evaluated using histopathology and immunochemical stains for detecting *T. gondii* tachyzoites. Neutral-buffered 10% formalin fixed samples were routinely processed in paraffin for light microscopy using haematoxylin-eosin and immunohistochemical staining. For immunohistochemical staining, an avidin-biotin complex method using polyclonal antibodies against *T. gondii* (1:100) was applied (Key, 2009).

2.3. Serological examination

2.3.1. Modified agglutination test (MAT)

The sera were tested with a commercial MAT kit (Toxo-Screen DA® kit, Biomérieux, Marcy-l'Etoile, France) according to manufacturer's instructions. Briefly, samples were diluted in PBS and 25 μl of each dilution was applied in a round bottom microtiter plate with 25 μl 2-mercaptoethanol at 0.2 mol/l. To this, 50 μl of a 1/5 dilution of whole formolized tachyzoites in bovine albumin borate saline (supplied in the kit) buffer was added. The plate was mixed for 1 min on a plate mixer at 300 revolutions per minute and incubated overnight at room temperature. The negative and positive controls were both supplied by the manufacturer and consisted of goat serum. The positive control was calibrated against the World Health Organization standard, a positive standard serum calibrated to international units. Agglutination in a 1/40 dilution was considered to be a positive result according to the manual of the diagnostic kit and earlier studies on marine mammal sera (Jensen et al., 2010, 2012; Oksanen et al., 1998). According to the manufac-

Table 1
Institutes from which samples were collected.

Name	Location	Location of the stranded animals
Utrecht University	Faculty of Veterinary Medicine, Dept. Pathobiology, Utrecht, The Netherlands	Dutch coastline – North Sea and Wadden Sea
Institute for Marine Resources and Ecosystem Studies (IMARES)	Texel, The Netherlands	Dutch coastline – North Sea and Wadden Sea
Institute for Terrestrial and Aquatic Wildlife Research (ITAW)	University of Veterinary Medicine Hannover, Büsum, Germany	German coastline – North Sea and Wadden Sea
Royal Belgian Institute of Natural Sciences	Ostend, Belgium	Belgian coastline – North Sea
University of Liège	Faculty of Veterinary Medicine, University of Liège, Belgium	Belgian, Dutch and northern French coastline – North Sea
Scottish Marine mammal Stranding Scheme (SMASS)	Scotland's Rural College, Inverness, Scotland	Scottish coastline – Atlantic Ocean and North Sea

Table 2
Number and species of animals tested for *Toxoplasma gondii* from the different locations.

	Serum	Serum + Brain	Brain	Brain + Muscle	Muscle	Total
Belgian coastline–North Sea						
Fin whale (*Balaenoptera physalus*)					1	**1**
Grey seal (*Halichoerus grypus*)					2	**2**
Harbour porpoise (*Phocoena phocoena*)			20	1	48	**69**
Harbour seal (*Phoca vitulina*)			8		1	**9**
Hooded seal (*Cystophora cristata*)			1			**1**
White-beaked dolphin (*Lagenorhynchus albirostris*)			1			**1**
Northern French coastline – North Sea						
Fin whale *(Balaenoptera physalus)*			1		1	**2**
Grey seal (*Halichoerus grypus*)			1			**1**
Harbour porpoise (*Phocoena phocoena*)			3		12	**15**
Harbour seal (*Phoca vitulina*)			2		3	**5**
Striped dolphin (*Stenella coeruleoalba*)			1			**1**
Dutch coastline –North Sea and Wadden Sea						
Harbour porpoise *(Phocoena phocoena)*	16	15	68	5	81	**185**
Harbour seal *(Phoca vitulina)*			28			**28**
German coastline –North Sea and Wadden Sea						
Harbour porpoise *(Phocoena phocoena)*			2	19		**21**
Harbour seal *(Phoca vitulina)*			2	19		**21**
Scottish coastline – Atlantic Ocean						
Atlantic white-sided dolphin *(Lagenorhynchus acutus)*	7					**7**
Bottlenose dolphin *(Tursiops truncatus)*	4					**4**
Fin whale *(Balaenoptera physalus)*	1					**1**
Grey seal *(Halichoerus grypus)*	5					**5**
Harbour porpoise *(Phocoena phocoena)*	47					**47**
Harbour seal *(Phoca vitulina)*	6					**6**
Killer whale *(Orcinus orca)*	2					**2**
Long-finned pilot whale *(Globicephala melas)*	6					**6**
Minke whale *(Balaenoptera acutorostrata)*	2					**2**
Risso's dolphin *(Grampus griseus)*	4					**4**
Short-beaked common dolphin *(Delphinus delphis)*	9					**9**
Sowerby's beaked whale *(Mesoplodon bidens)*	2					**2**
Sperm whale *(Physeter macrocephalus)*	1					**1**
Striped dolphin *(Stenella coeruleoalba)*	5					**5**
White-beaked dolphin *(Lagenorhynchus albirostris)*	4					**4**
Scottish coastline – North Sea						
Atlantic white-sided dolphin *(Lagenorhynchus acutus)*	11					**11**
Bottlenose dolphin *(Tursiops truncatus)*	2					**2**
Grey seal *(Halichoerus grypus)*	12					**12**
Harbour porpoise *(Phocoena phocoena)*	56					**56**
Harbour seal *(Phoca vitulina)*	14					**14**
Killer whale *(Orcinus orca)*	1					**1**
Long-finned pilot whale *(Globicephala melas)*	5					**5**
Minke whale *(Balaenoptera acutorostrata)*	3					**3**
Risso's dolphin *(Grampus griseus)*	4					**4**
Sei whale *(Balaenoptera borealis)*	1					**1**
Short-beaked common dolphin *(Delphinus delphis)*	4					**4**
Sowerby's beaked whale *(Mesoplodon bidens)*	2					**2**
Sperm whale *(Physeter macrocephalus)*	1					**1**
Striped dolphin *(Stenella coeruleoalba)*	4					**4**
White-beaked dolphin *(Lagenorhynchus albirostris)*	2					**2**
European otter *(Lutra lutra)*	34					**34**
Total	**277**	**15**	**138**	**44**	**149**	**623**

Table 3
Results by species of serum samples tested for *Toxoplasma gondii* with modified agglutination test (MAT), enzyme-linked immunosorbent assay (ELISA) with a sample-to-positive ratio (S/P) cut-off at 20 and 50, and immunofluorescent assay (IFA).

	MAT		ELISA (S/P ≥ 20)		ELISA (S/P ≥ 50)		IFA	
	No. *tested*	No. *positive (%)*	No.*tested*	No. *positive (%)*	No. *tested*	No. *positive (%)*	No. *tested*	No. *positive (%)*
Dutch coastline– North and Wadden Sea	**31**	**4 (13)**						
Harbour porpoise *(Phocoena phocoena)*	31	4 (13)						
Scottish coastline – Atlantic Ocean	**105**	**54 (51)**	**98**	**7 (7.1)**	**98**	**3 (3.1)**	**100**	**7 (7.0)**
Atlantic white-sided dolphin *(Lagenorhynchus acutus)*	7	7 (100)	7	0 (0)	7	0 (0)	7	0 (0)
Bottlenose dolphin *(Tursiops truncatus)*	4	3 (75)	4	0 (0)	4	0 (0)	4	0 (0)
Fin whale *(Balaenoptera physalus)*	1	0 (0)					1	0 (0)
Grey seal *(Halichoerus grypus)*	5	2 (40)	4	0 (0)	4	0 (0)	4	0 (0)
Harbour porpoise *(Phocoena phocoena)*	47	22 (47)	43	0 (0)	43	0 (0)	44	**1** (2)
Harbour seal *(Phoca vitulina)*	6	0 (0)	6	0 (0)	6	0 (0)	6	0 (0)
Killer whale *(Orcinus orca)*	2	1 (50)	2	0 (0)	2	0 (0)	2	0 (0)
Long-finned pilot whale *(Globicephala melas)*	6	5 (83)	5	**3** (60)	5	**2** (40)	5	**3** (60)
Minke whale *(Balaenoptera acutorostrata)*	2	0 (0)	2	0 (0)	2	0 (0)	2	0 (0)
Risso's dolphin *(Grampus griseus)*	4	2 (50)	4	**1** (25)	4	0 (0)	4	**1** (25)
Short-beaked common dolphin *(Delphinus delphis)*	9	5 (56)	9	**1** (11)	9	0 (0)	9	**1** (11)
Sowerby's beaked whale *(Mesoplodon bidens)*	2	1 (50)	2	0 (0)	2	0 (0)	2	0 (0)
Sperm whale *(Physeter macrocephalus)*	1	1 (100)	1	0 (0)	1	0 (0)	1	0 (0)
Striped dolphin *(Stenella coeruleoalba)*	5	2 (40)	5	0 (0)	5	0 (0)	5	0 (0)
White-beaked dolphin *(Lagenorhynchus albirostris)*	4	3 (75)	4	**2** (50)	4	**1** (25)	4	**1** (25)
Scottish coastline – North Sea	**156**	**63 (40)**	**143**	**21 (15)**	**143**	**13 (9.1)**	**144**	**23 (16)**
Atlantic white-sided dolphin *(Lagenorhynchus acutus)*	11	4 (36)	11	0 (0)	11	0 (0)	11	0 (0)
Bottlenose dolphin *(Tursiops truncatus)*	2	0 (0)	1	0 (0)	1	0 (0)	1	0 (0)
Grey seal *(Halichoerus grypus)*	12	3 (25)	9	**1** (11)	9	0 (0)	9	0 (0)
Harbour porpoise *(Phocoena phocoena)*	56	21 (38)	53	0 (0)	53	0 (0)	54	**1** (2)
Harbour seal *(Phoca vitulina)*	14	4 (29)	11	**2** (18)	11	**1** (9)	11	**2** (18)
Killer whale *(Orcinus orca)*	1	1 (100)	1	0 (0)	1	0 (0)	1	0 (0)
Long-finned pilot whale *(Globicephala melas)*	5	3 (60)	5	**1** (20)	5	**1** (20)	5	**1** (20)
Minke whale *(Balaenoptera acutorostrata)*	3	1 (33)	3	0 (0)	3	0 (0)	3	0 (0)
Risso's dolphin *(Grampus griseus)*	4	2 (50)	3	**1** (33)	3	0 (0)	3	**1** (33)
Sei whale *(Balaenoptera borealis)*	1	1 (100)	1	0 (0)	1	0 (0)	1	0 (0)
Short-beaked common dolphin *(Delphinus delphis)*	4	3 (75)	4	**1** (25)	4	0 (0)	4	**1** (25)
Sowerby's beaked whale *(Mesoplodon bidens)*	2	1 (50)	2	0 (0)	2	0 (0)	2	0 (0)
Sperm whale *(Physeter macrocephalus)*	1	0 (0)	1	0 (0)	1	0 (0)	1	0 (0)
Striped dolphin *(Stenella coeruleoalba)*	4	2 (50)	4	0 (0)	4	0 (0)	4	0 (0)
White-beaked dolphin *(Lagenorhynchus albirostris)*	2	1 (50)	2	**1** (50)	2	0 (0)	2	0 (0)
European otter *(Lutra lutra)*	34	16 (47)	32	**14** (44)	32	**11** (34)	32	**17** (53)
Total	**292**	**121 (41)**	**241**	**28 (12)**	**241**	**16 (6.6)**	**244**	**30 (12)**

Table 4
Primers and probes used in the real-time PCR.

	Target	Name	Sequence 5′ → 3′	5′ Modification[a]	3′ Modification[b]	Concentration[c]
Primers	*T. gondii*	T2	CGGAGAGGGAGAAGATGTT			400 nM
		T3	GCCATCACCACGAGGAAA			400 nM
	Cellular r18S	VF1	GATTAAGTCCCTGCCCTTT			200 nM
		VR1	CACACCGCCCGTCGCTACTACC			200 nM
Probes	*T. gondii*	TP	CTTGGCTGCTTTTCCTGGAGGG	FAM 488	BHQ1	66 nM
	Cellular r18S	VP	CACACCGCCCGTCGCTACTACC	Texas Red 547	BHQ2	66 nM

[a] Fluorophore used as reporter.
[b] Quencher molecule.
[c] Final concentration per reaction.

turer's instructions, samples were also tested at a 1/4000 dilution to monitor for a potential prozone effect at a 1/40 dilution.

2.3.2. Enzyme-linked immunosorbent assay (ELISA)

Two hundred and forty-one sera of the 292 collected sera were tested for the presence of anti-*T. gondii* IgG with the ID Screen Toxoplasmosis Indirect Multi-species ELISA kit (ID.vet, Montpellier, France). Samples were tested and analysed according to the manufacturer's instructions. Briefly, each sample was diluted 1/10 in the supplied dilution buffer and 100 μl was applied in the antigen-coated 96 well plate. After 45 min of incubation at room temperature, the plate was washed 3 times with the supplied washing buffer. 100 μl of the multispecies conjugate diluted 1/10 in the supplied buffer was then applied in each well. The plate was incubated for another 30 min and washed 3 times. The colorimetric reaction was done with the supplied 3, 3′, 5, 5′-tetramethylbenzidine and stopped after 15 min with the supplied stop solution. For each sample the sample-to-positive (S/P) ratio was calculated using the optical density (OD) as $(OD_{sample} - OD_{negativecontrol})/(OD_{positivecontrol} - OD_{negativecontrol})$, with the positive and negative controls supplied with the kit. According to the manufacturer's instructions, a sample was considered positive if the S/P ratio was ≥50.

2.3.3. Immunofluorescent assay (IFA)

Two hundred and forty-four of the 292 collected serum samples were tested for the presence of anti-*T. gondii* IgG using IFA. The IFA was performed using commercially available antigen coated slides (Toxo-Spot IF, Biomerieux, Capronne, France). The sera were diluted 1/50 in PBS and 50 μl of each serum dilution was applied on

Table 5
Conjugates used in the immunofluorescence assay.

Fluorescein isothiocyanate labelled conjugate[a]	Manufacturer	Used for
goat anti-bovine IgG (H + L)	KPL (Gaithersburg, Maryland, USA)	Dolphins, porpoises, whales[b]
goat anti-dog IgG (H + L)	KPL (Gaithersburg, Maryland, USA)	Seals[c]
goat anti-ferret IgG (H + L)	KPL (Gaithersburg, Maryland, USA)	Otter[c]

[a] (H + L) conjugate against IgG heavy and light chains.
[b] Nollens et al. (2008).
[c] Miller et al. (2002).

the antigen coated slides. The slides were incubated at 37 °C in a saturated moist atmosphere for 30 min. The slides were then washed once with PBS and once with distilled water. After drying, 30 μl of the fluorescein isothiocyanate labelled conjugate was applied in a 1/25 dilution in PBS with Evans Blue as counter dye. The following conjugates were used: anti-bovine IgG to test cetacean sera (Jaber et al., 2003), anti-ferret IgG to test otter sera (Miller et al., 2002) and anti-dog IgG to test seal sera (Carlson-Bremer et al., 2015). Table 5 lists the conjugates used per tested animal species. After washing and drying, the slides were read with a fluorescence microscope (Nikon eclipse Ni-U). Samples that had tested positive and negative in ELISA were used as positive and negative controls for each type of conjugate.

2.3.4. Muscle fluid

Muscle fluid samples were tested using MAT. Muscle fluid was collected by squeezing the defrosted muscle samples above a 15 ml centrifuge tube. Firstly, 47 samples from harbour porpoises (*Phocoena phocoena*) from The Netherlands were tested at the following dilutions: 1/4, 1/200 and 1/400. Since no positivity was found at the 1/200 and 1/400 dilution, the remaining 146 muscle fluid samples were tested at a 1/2, 1/10 and 1/64 dilution to better estimate the antibody titres. Doubtful results were retested for confirmation. Forbes et al. (2012) showed that a 1/10 dilution of muscle fluid samples from both experimentally infected and negative pigs identified all positive cases. Therefore, samples in this survey that showed agglutination at 1/10 dilution were considered positive.

3. Results

3.1. PCR

The positive and negative controls and the negative results of the non-template controls confirmed proper testing conditions without contamination during extraction.

Two out of 193 brain samples were positive for *T. gondii* DNA: a cerebrum sample from a young male harbour porpoise (*Phocoena phocoena*) stranded in 2010 near Katwijk, The Netherlands (Ct value of 34.4) and a cerebrum sample from an adult female harbour porpoise (*Phocoena phocoena*) stranded in 2010 near Noordwijk, The Netherlands (Ct value of cycle 40.4). The cerebellum samples from both porpoises tested negative. Further histopathological and immunohistochemical examinations of brain tissues of both animals could not confirm the presence of *T. gondii*. Other tissues of these animals were not available for further serological testing.

3.2. Serology—serum samples

Table 3 summarizes the results of the serological assays per animal species. Marine mammals collected from the Dutch coastline, the Scottish coastline along the Atlantic Ocean and the Scottish coastline along the North Sea showed a seroprevalence of 13%, 51% and 39%, respectively in MAT. Sixteen of the 34 (47%) European otter *(Lutra lutra)* samples from the Scottish coastline along the North Sea tested positive. From the 261 sera collected from Scotland, 244 and 241 sera were retested using IFA and ELISA, respectively. In IFA, 13 out of 212 marine mammal samples (6.1%) tested positive at a 1/50 dilution and 17 of the 32 (53%) European otter *(Lutra lutra)* samples tested positive. The commercial ELISA yielded a seroprevalence of 2% in marine mammal samples when using a S/P ratio cut-off of 50, and 7% when lowering the cut-off to 20. ELISA identified 11 (34%) and 14 (44%) of the 32 European otter *(Lutra lutra)* samples per cut-off respectively.

The one harbour porpoise (*Phocoena phocoena*) from the Atlantic Ocean, which tested positive in IFA, also tested positive in MAT. The one harbour porpoise (*Phocoena phocoena*) from the North Sea, which tested positive in IFA, tested negative in MAT. The one grey seal (*Halichoerus grypus*) from the North Sea, which tested positive in ELISA with a cut-off value of 20, tested positive in MAT. Table 6 shows the agreement between results in testing long-finned pilot whale (*Globicephala melas*), Risso's dolphin (*Grampus griseus*), short-beaked common dolphin (*Delphinus delphis*) and white-beaked dolphin (*Lagenorhynchus albirostris*) samples. Agreement was defined as a similar result in all three diagnostic methods. Of the 209 marine mammals tested with all three serological techniques, 4 (1.9%) marine mammals (1 harbour seal *(Phoca vitulina)*, 2 long-finned pilot whales (*Globicephala melas*) and 1 white-beaked dolphin (*Lagenorhynchus albirostris*)) tested positive on all tests and 104 marine mammals (50%) tested negative on all tests, leaving 101 marine mammals (48%) with inconsistent results across tests. Nine out of 32 (28%) European otter *(Lutra lutra)* samples tested positive on all three tests.

3.3. Serology—muscle fluid samples

Out of the first batch of 47 muscle samples from The Netherlands, 21 (43%) tested positive in a 1/4 dilution in MAT. No positivity was found at 1/200 and 1/400 dilutions. Further testing was done using lowered dilutions, i.e., 1/2, 1/10, 1/64. Out of all 146 animals tested with these latter dilutions, only 12 samples (8.2%) showed agglutination in a 1/2 dilution (data not shown). All these samples were negative using the cut-off value of 1/10.

4. Discussion

In this study, 589 marine mammals of 17 different species and 34 European otters *(Lutra lutra)* were examined for the presence of *T. gondii,* using various detection methods. Previous studies on *T. gondii* in marine mammals showed that this parasite is able to reach and infect marine mammals (Cabezón et al., 2011; Rengifo-Herrera et al., 2012). Infection with *T. gondii* in marine mammals may result in clinical disease (Carlson-Bremer et al., 2015; Mikaelian et al., 2000; Van Bressem et al., 2009). This has been clearly demonstrated in California sea otters (*Euhydra lutris nereis*) (Miller et al., 2004), Hector's dolphins *(Cephalorhynchus hectori)* (Roe et al., 2013) and harbour porpoises (*Phocoena phocoena*) (Herder et al., 2015). *T. gondii* clinical disease has also been recorded with co-infections of *Brucella ceti,* and *Listeria monocytogenes* in striped dolphins (*Stenella*

Table 6
Agreement between results in serological testing of long-finned pilot whale, Risso's dolphin, short-beaked common dolphin and white-beaked dolphin samples.

		Animal no.	MAT	ELISA (S/P ≥ 20)	ELISA (S/P ≥ 50)	IFA	Agreement
Long-finned pilot whale *(Globicephala melas)*	Atlantic Ocean	**1**	N	N	N	N	Yes
		2	P	P	P	P	Yes
		3	P	P	N	P	No
		4	P	–	–	–	–
		5	P	P	P	P	Yes
		6	P	N	N	N	No
	North Sea	**1**	N	N	N	N	Yes
		2	P	N	N	N	No
		3	N	N	N	N	Yes
		4	P	N	N	N	No
		5	P	P	P	P	Yes
Risso's dolphin *(Grampus griseus)*	Atlantic Ocean	**1**	N	N	N	N	Yes
		2	N	N	N	N	Yes
		3	P	P	N	P	No
		4	P	N	N	N	No
	North Sea	**1**	N	–	–	–	–
		2	N	N	N	N	Yes
		3	P	P	N	P	No
		4	P	N	N	N	No
Short-beaked common dolphin *(Delphinus delphis)*	Atlantic Ocean	**1**	P	N	N	N	No
		2	P	N	N	N	No
		3	P	N	N	N	No
		4	P	N	N	N	No
		5	P	N	N	N	No
		6	N	P	N	P	No
		7	N	N	N	N	Yes
		8	N	N	N	N	Yes
		9	N	N	N	N	Yes
	North Sea	**1**	P	P	N	P	No
		2	P	N	N	N	No
		3	P	N	N	N	No
		4	N	N	N	N	Yes
White-beaked dolphin *(Lagenorhynchus albirostris)*	Atlantic Ocean	**1**	P	P	P	P	Yes
		2	P	N	N	N	No
		3	P	N	N	N	No
		4	N	N	N	N	Yes
	North Sea	**1**	P	P	N	N	No
		2	N	N	N	N	Yes

MAT = modified agglutination test; ELISA = enzyme-linked immunosorbent assay; S/P = sample-to-positive ratio; IFA = immunofluorescent assay; P = positive, N = negative, – = not tested.

coeruleoalba) (Di Guardo et al., 2010; Grattarola et al., 2016), and with morbillivirus in fin whales (*Balaenoptera physalus*) (Mazzariol et al., 2012). Additionally, research on *T. gondii* in marine mammals is relevant as *T. gondii* acts as an indicator for marine pollution and marine mammals are sentinel species for the health of the marine ecosystems they live in (de Moura et al., 2014; Gibson et al., 2011; Moore, 2008).

Sampling of wildlife is challenging and comes with unknown factors and biases (Nusser et al., 2008). Convenience-based sampling is not a random selection of animals and therefore does not represent the complete wildlife population. In this study only stranded animals were tested. Seals are more coastal species in comparison with most cetaceans and could therefore be overrepresented. The sampled population was largely dominated by harbour porpoises (*Phocoena phocoena*), as this is the most abundant sea mammal species in the investigated areas. The convenience-based sampling further explains the differences in sampling methods used across animals. Indeed, the set of samples per animal varied across institutes and further depended on factors such as the preservation state of the animal carcass – e.g., fewer samples were taken from decomposed animals. As our study covered samples taken in a time span of 21 years, not all necropsied animals were sampled specifically for *Toxoplasma* research; brain and muscle samples were therefore not available from all animals.

The diagnostic techniques used in this study are not validated for marine mammals by the manufacturers and therefore their diagnostic test characteristics for marine mammal samples are not exactly known. For detecting *T. gondii* in wild animals, brain is demonstrated to be a useful sampling tissue (De Craeye et al., 2011). Out of 193 animals tested by PCR, two harbour porpoise (*Phocoena phocoena*) cerebrum samples proved positive for *T. gondii* DNA. The cerebellum samples of the same two animals tested negative in PCR, which might be due to the heterogeneous distribution of *T. gondii* in the host's organs or to a low intensity of infection. Also the sample size of 400 mg for DNA extraction is low. Using PCR on marine mammal samples to detect *T. gondii* DNA has not identified many positive animals in other studies either (Dubey et al., 2009; Omata et al., 2006). Furthermore, a large discrepancy between the PCR and serologic results was found, similar to other studies on *T. gondii* prevalence in other hosts (De Craeye et al., 2011; Wang et al., 2011; Prestrud et al., 2007). This could be due to difficulties in determining serological parameters such as sensitivity and specificity.

Blanchet et al. (2014) tested tissue fluid and blood (thawed and centrifuged) with MAT and ELISA. In the current study, no chloroform clean-up was applied on the serum samples. In accordance to the findings of Blanchet et al. (2014), the results obtained by IFA and ELISA indeed gave a lower number of positive animals compared to the MAT results. MAT might be useful as a first screening method, but the results must be interpreted with caution.

In the current study, using MAT, 51% and 39% of the sera from marine mammals stranded on Scottish coastlines along the Atlantic

Ocean and the North Sea, respectively, were found positive compared to 13% of the sera from animals stranded on the Dutch coastline. Extrapolating these differences in seroprevalence should be done with caution, because the sampling was biased and not representative for the whole population. Nevertheless, the difference in seroprevalence between the animals found on Dutch and Scottish coastlines is striking and deserves further investigation.

While MAT was designed for testing serum samples, the Toxo-Screen DA® test kit was reported to give reliable results when used on swine muscle fluid (Forbes et al., 2012). MAT has proven its usefulness as a diagnostic method in experimentally infected seals (Gajadhar et al., 2004). In our study, no positive results were obtained from the muscle fluid samples at a 1/10 cut-off value as described by Forbes et al. (2012). The muscle fluid samples showed various degrees of degradation, which may have interfered with the reading of MAT.

Across the three serological methods used in this study, more samples were found positive in MAT than in ELISA and IFA. A major difference between these tests is the use of a multispecies conjugate in ELISA and species specific conjugates in IFA. Finding appropriate specific conjugates for all the tested species of sea mammals is not only challenging (as for most of them they are not commercially available), it is also rather expensive and impractical. Therefore, for the IFA we relied on previously published cross-reactivity between species (Miller et al., 2002; Nollens et al., 2008) (Table 5). Another way to approach this problem is using a multispecies conjugate consisting of protein G, which non-specifically binds to the Fc portion of IgG antibodies. The commercial multispecies ELISA used in this study relies on such a protein G conjugate for detecting the adsorbed IgG's. However, it is also possible that the conjugates used in IFA and the commercial ELISA are not optimal, leading to possible false negatives. The fact that we obtained similar results with IFA and ELISA when lowering the S/P cut-off ratio of the latter to $\geq$20, could be explained by protein G having less affinity for marine mammal antibodies. Indeed, differences in binding affinities between protein G and immunoglobulins have already been described for various other animal species (Phillips, 2015).

Although it remains poorly understood how marine mammals acquire *T. gondii* infection, it has been shown that certain prey species, such as anchovies (*Engraulidae* family), sardines (*Clupeidae* family) and bivalves, are able to concentrate viable *T. gondii* oocysts in their digestive tract (Massie et al., 2010). As these prey species are also consumed by humans, *T. gondii* infection in marine mammals could be an indirect indicator of the risk these species pose for causing *T. gondii* infection in humans. Otters are hypothesized to be an interesting link in the transmission of *T. gondii* from land to marine environments and were therefore included in this study (Conrad et al., 2005). A higher percentage of samples testing positive on all three tests was found in otters (28%) compared to marine mammals (2%), supporting this hypothesis. The presence of *T. gondii* oocysts in the marine environment could be an indicator of marine pollution in general (Meireles et al., 2004; Stewart et al., 2008). However, for marine mammals to be truly used as sentinels for both the zoonotic and ecological implications of *T. gondii* infection, further research is needed to overcome the current diagnostic difficulties.

5. Conclusions

This study has confirmed the presence of *T. gondii* DNA and antibodies in marine mammals living in the North Sea (and Eastern Atlantic Ocean), to our knowledge for the first time. The comparison between the different detection methods highlighted the diagnostic difficulties when testing marine mammal samples for this parasite. To gain more insights in the significance and potential threat of *T. gondii* in marine mammals, the serological techniques for detecting *T. gondii* antibodies in marine mammals should be further optimized. Despite these diagnostic difficulties, the difference in seroprevalence between animals found on the Scottish and Dutch coastlines should be further investigated.

Acknowledgments

We would like to thank Zehra Yildirimer, Nabila El Moussaoui, Mariel ten Doeschate, Kornelia Schmidt, Miriam Hillmann, Jurgen Willems and Laurie Flament for their kind cooperation during this project. Samples gained from Utrecht University were possible through a grant from the Dutch Ministry of Economic Affairs (140000353). We are greatly indebted to all volunteers spending their time and effort to collect the stranded animals. Without their dedication, this project would not have been possible. The marine mammals washed ashore in Belgium were collected by the Royal Belgian Institute of Natural Sciences (RBINS). We would like to thank and acknowledge all members of the French stranding networks for their continuous effort in collecting stranding data. We specifically would like to thank the following nature protection associations of Northern France: OCEAMM (Observatoire pour la Conservation et l'Etude des Animaux et Milieux Marins), LPA (Animal Protection League) and CMNF (Coordination Mammalogique du Nord de la France) and the Observatoire PELAGIS in charge of the national stranding network. The Observatoire PELAGIS is funded by the ministry in charge of the environment, and by the Communauté d'Agglomération de la Ville de La Rochelle, with support from the Région Poitou-Charentes and the European Union.

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