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Development and evaluation of a modified agglutination test for diagnosis of *Toxoplasma* infection using tachyzoites cultivated in cell culture

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ABSTRACT

Toxoplasma gondii is a zoonotic protozoan parasite that can cause significant disease in most warm-blooded animals, including humans. Surveillance testing of human and animal populations is essential to estimate disease prevalence, assess food safety risks and establish control programmes. Serological tests are the most practical methods to detect the prevalence of infection in a broad range of host populations. The modified agglutination test (MAT) is a sensitive serological method to detect *Toxoplasma* IgG antibodies in livestock and wild animals. An in-house MAT was developed using tachyzoites produced by *in vitro* cultivation instead of traditional propagation in murine peritoneal cavity. The assay was evaluated using samples of serum and/or meat juice from pigs and cats experimentally infected with *T. gondii*. Samples were also tested by a commercially available MAT kit. Comparative analysis of test results from serum and meat juice samples showed excellent agreement between the in-house MAT and the commercial MAT. Serum and/or blood samples from naturally infected cats, sheep, and 20 wildlife host species were also tested by the in-house MAT, with overall results comparable to those obtained using the commercial MAT kit. Therefore, this new MAT is an efficient and convenient method for testing a variety of terrestrial and aquatic domestic or wild host species for *T. gondii*.

1. Introduction

Toxoplasma gondii is an important coccidian parasite that has a complex reproductive cycle involving two hosts. Members of the family Felidae are the only known definitive hosts, whereas humans and a wide range of domestic and wild animals are intermediate hosts for this parasite (Dubey, 2010). Infection with *T. gondii* can cause abortion or neurological symptoms in their intermediate hosts (Gilbert et al., 2000; Sushrut and Davis, 2012). Transmission occurs *via* consumption of meat or other animal tissues containing tissue cysts of this parasite, or of water or other materials contaminated with sporulated *Toxoplasma* oocysts.

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Several serological tests have been developed to detect antibodies to *T. gondii* in humans and animals for the detection of infection (Dubey et al., 1995). The modified agglutination test (MAT) is a sensitive and specific method for the detection of *T. gondii* in a wide range of host species, and is simple, rapid and of relatively low cost compared to other serological tests (Dubey, 1997; Hill et al., 2006; Dubey, 2010). However, the routine use of MAT for the diagnosis of *T. gondii* infection can be challenging because of the lack of readily available commercial kits and the tachyzoite-derived antigen. Typically, tachyzoites used for MAT antigen are generated by intraperitoneal propagation in mice (Desmonts and Remington, 1980). To reduce the use of live animals, antigen required for the assay in this study was prepared from tachyzoites grown in cell culture. Performance of the test was assessed using samples of serum, whole blood or meat juice from experimentally infected pigs and cats, and naturally infected cats, sheep and 20 species of wild animals.

2. Materials and methods

2.1. Samples

Samples of serum, whole blood (EDTA) and meat juice were assigned to 1 of 3 groups, based on origin of the samples (Table 1).

2.1.1. Experimentally-infected domestic animals group

A total of 74 serum and 39 meat juice samples previously generated from 13 pigs experimentally inoculated with *T. gondii* were used (Forbes et al., 2012). The *Toxoplasma-free* pigs had been inoculated with 1×10^2, 3×10^2, 5×10^2 or 1×10^3 sporulated oocysts, and three uninoculated pigs served as controls (Table 1). Serum samples were collected prior to inoculation and serially post-inoculation. Post-mortem samples of meat juice from each carcass were obtained from diaphragm, heart and sternomastoideaus muscle. An additional 36 serum samples (provided by Dr. Dolores Hill, USDA, Beltsville, Maryland, USA) were collected from nine pigs each experimentally infected with 1000 oocysts of *T. gondii per os* (Hill et al., 2006). Samples were collected prior to inoculation and at 11, 31 and 66 days post-inoculation (DPI). Thirty serum samples obtained from cats experimentally infected with *T. gondii via* various routes of inoculations and described in an earlier study were also used. The samples were collected from 11 SPF cats at pre-inoculation and 32 weeks post-inoculation. These cats were inoculated orally with mouse brain tissues containing tissue cysts of *T. gondii* (Al-Adhami and Gajadhar, 2014). An additional eight serum samples obtained from a previous study in which four SPF cats were fed brain or muscle tissues of seals experimentally infected with *T. gondii*. Samples were collected at pre- and post-inoculation (Gajadhar et al., 2004).

2.1.2. Naturally-infected domestic animals group

A total of 134 serum samples were obtained from pet cats in France (provided by Drs. Pascal Boireau and Radu Blaga), and 20 serum samples were collected from outdoor-raised sheep in Saskatoon, Canada (provided by Dr. Gita Malik-Dahiya).

2.1.3. Naturally-infected wildlife animals group

A total of 244 serum and 424 whole blood samples were collected from 20 wildlife species between 2008 and 2010 at various locations in the northern and arctic regions in Canada as part of an International Polar Year project (Fig 1, Table 1).

2.2. Serological tests

Serum and whole blood samples from all 3 groups were tested for antibodies to *T. gondii* using the commercial MAT kit (Toxo-Screen DA, Biomerieux, France) as the gold standard, and the in-house MAT. Kit controls and test samples were tested at 1:40 and 1:40,000 dilutions as per the kit manufacturer's instructions.

The in-house MAT was developed using *Toxoplasma* antigen produced from *in vitro* cultured tachyzoites. To produce the antigen, cryopreserved tachyzoites obtained from VEG, type III strain of *T. gondii* were propagated in tissue culture using MDBK cells and appropriate culture medium (Minimum Essential Medium-MEM-GIBCO-USA) supplemented with foetal bovine serum (FBS) and antibiotics (Lindsay et al., 1991). *Toxoplasma* tachyzoites were grown in conventional cell culture flasks (T75) and Falcon® cell culture multi-flasks (Corning-USA) to improve tachyzoites yield and productivity. Tachyzoites were harvested from the culture medium by washing in phosphate buffer saline (PBS), followed by filtration through PD-10-Sephadex column (GE-Healthcare-USA) to remove cells and other particulate materials. The viability of the purified tachyzoites was assessed by trypan blue dye exclusion assay (Elsheikha et al., 2006). Purified tachyzoites were fixed in 6% formaldehyde solution and stored at 4 °C overnight. After fixation, the formalin suspension was centrifuged, washed 3× in sterile filtered PBS and resuspended in alkaline borate buffer (pH 8.7) containing 0.4% bovine serum albumin (BSA/borate buffer) and 0.2% sodium azide to a final concentration of approximately 2×10^8/ml (antigen stock suspension), and stored at 4 °C until used. The test method described by Dubey (2010) was followed with modifications as described below. Agglutination was performed in round-bottom 96-well plates (Corning-USA). Test serum and blood samples were titrated at serial 2-fold dilutions from 1:25 to 1:200. Negative (pre-inoculation and uninoculated controls) and positive (terminal bleed) sera from various species experimentally infected with *T. gondii* were chosen as negative and positive controls, respectively. The positive control was required to have a minimum titre of 1:200. Meat juice samples were diluted at 1:10 and 1:25 prior to testing. Serum, whole blood, and meat juice samples were diluted with sterile filtered PBS. The antigen mixture for each plate was prepared by mixing 200 μl of formalin-fixed tachyzoites,

Table 1
Source of serum, blood and meat juice samples used for in-house MAT and comparative results from commercial MAT kit for the detection of IgG antibodies to *Toxoplasma gondii*.

Source animals for samples			Inoculum[b]	Type of sample	No. of samples tested	Results of serological tests		Kappa value
Animal groups	Species[a]	No. of animals				No. tested positive/total No. of samples (% positive)		
						In-house MAT	Toxo-Screen-DA Kit MAT	
Experimental infection-domestic[a]	Pig (*Sus scrofa domesticus*)	3	0	Serum	14	4/14 (28.57)	4/14 (28.57)	1.0
		2	1×10^2	Serum	14	9/14 (64.29)	9/14 (64.29)	1.0
		2	3×10^2	Serum	14	10/14 (71.42)	10/14 (71.42)	1.0
		3	5×10^2	Serum	16	10/16 (62.5)	10/16 (62.5)	1.0
		3	1×10^3	Serum	16	10/16 (62.5)	10/16 (62.5)	1.0
	Pig (*Sus scrofa domesticus*)	3	0	Meat Juice	9	3/9 (33.33)	3/9 (33.33)	1.0
		2	1×10^2	Meat Juice	6	6/6 (100)	6/6 (100)	1.0
		2	3×10^2	Meat Juice	6	6/6 (100)	6/6 (100)	1.0
		3	5×10^2	Meat Juice	9	9/9 (100)	9/9 (100)	1.0
		3	1×10^3	Meat Juice	9	9/9 (100)	9/9 (100)	1.0
	Pig (*Sus scrofa domesticus*)	9	1×10^3	Serum	36	27/36 (75)	9/9 (100)[c]	1.0
	Cat (*Felis catus*)	11	Mouse brain	Serum	22	11/22 (50)	11/22 (50)	1.0
	Cat (*Felis catus*)	4	Seal brain or tissue	Serum	8	4/8 (50)	4/8 (50)	1.0
Natural infection-domestic	Cat (*Felis catus*)	134	NA	Serum	134	51/134 (38.05)	49/134 (36.06)	0.841
	Sheep (*Ovis aries*)	20	NA	Serum	20	0/20 (0)	0/20 (0)	1.0
Natural infection-wildlife	Bison (*Bison bison*)	31	NA	Serum	31	0/31 (0)	0/31 (0)	1.0
	Muskox (*Ovibos moschatus*)	144	NA	Serum	144	2/144 (1.38)	2/144 (1.38)	1.0
	Muskox (*Ovibos moschatus*)	1	NA	Blood	1	1/1 (100)	1/1 (100)	1.0
	Caribou (*Rangifer tarandus*)	69	NA	Serum	69	8/69 (11.59)	7/69 (11.29)	0.925
	Caribou (*Rangifer tarandus*)	28	NA	Blood	28	3/28 (10.71)	2/28 (7.14)	0.781
	Red fox (*Vulpes vulpes*)	11	NA	Blood	11	3/11 (27.27)	4/11 (36.36)	0.792
	Wolf (*Canis lupus*) Wolf	37	NA	Blood	37	7/37 (18.91)	16/37 (43.24)	0.469
	Lynx (*Lynx canadensis*) Lynx	5	NA	Blood	5	2/5 (40.0)	2/5 (40)	1.0
	Arctic Hare (*Lepus arcticus*)	2	NA	Blood	2	0/2 (0)	0/2 (0)	1.0
	Snow shoe Hare (*Lepus americanus*)	8	NA	Blood	8	0/8 (0)	0/8 (0)	1.0
	Polar bear (*Ursus maritimus*)	3	NA	Blood	3	0/3 (0)	0/3 (0)	1.0
	Black bear (*Ursus americanus*)	5	NA	Blood	5	3/5 (60.0)	3/5 (60.0)	1.0
	Grizzle bear (*Ursus arctos horribillis*)	2	NA	Blood	2	0/2 (0)	0/2 (0)	1.0
	Ringed seal (*Pusa hispida*)	108	NA	Blood	108	16/108 (14.81)	14/108 (12.96)	0.845
	Harp seal (*Pagophilus groenlandicus*)	1	NA	Blood	1	0/1 (0)	0/1 (0)	1.0
	Walrus (*Odobenus rosmarus*)	34	NA	Blood	34	5/34 (14.74)	9/34 (26.47)	0.648
	Whale/Beluga (*Delphinapterus leucas*)	46	NA	Blood	46	10/46 (21.73)	10/46 (21.73)	1.0
	Bowhead whale (*Balaena mysticetus*)	2	NA	Blood	2	1/2 (50.0)	1/2 (50.0)	1.0
	Rock ptarmigan (*Lagopus muta*)	25	NA	Blood	25	1/25 (4.0)	1/25 (4.0)	1.0
	Willow ptarmigan (*Lagopus lagopus*)	24	NA	Blood	24	0/24 (0)	0/24 (0)	1.0
	Canadian goose (*Branta canadensis*)	71	NA	Blood	71	2/71 (2.81)	2/71 (2.81)	1.0
	Snow goose (*Chen caerulescens*)	11	NA	Blood	11	0/11 (0)	0/11 (0)	1.0
Total tested					1001			

[a] Serology results of 13 pigs and 15 cats have been previously reported (Forbes et al., 2012; Al-Adhami and Gajadhar, 2014)
[b] Number of oocysts/or type of infected tissue
[c] Data for samples collected at different DPI was not available except for those obtained at the time of euthanasia (Hill et al., 2006).

Fig 1. Map of Canada with stars indicating capture areas for wildlife species tested for *Toxoplasma gondii* in the study.

2.5 ml of BSA/borate buffer, 35 µl of 2-mercaptoethanol, and 50 µl of Evans blue dye solution (2 mg/ml water). Equal volumes (25 µl) of freshly prepared antigen mixture and serum, blood or meat juice dilutions were added into each well and mixed gently by repeated pipetting action. The plate was covered with a sealing tape, incubated at room temperature and results read after 24 h. A blue button at the bottom of the well indicated a negative result. A clear bottom of the well indicated positive result. Results were interpreted as positive, negative, or inconclusive. However, all inconclusive results were considered negative in this study.

2.3. Statistical analysis

The degree of agreement between the results of the commercial MAT and in-house MAT was estimated by calculating the kappa value. Kappa (k) values of ≥0.81, 0.61–0.80, 0.40–0.60, and ≤0.40 were considered to represent excellent, substantial, moderate to good, and slight to poor agreement, respectively (Viera and Garret, 2005). The receiver operating characteristic curve (ROC) was constructed to evaluate the performance of the in-house MAT in relation to the commercial MAT (as the gold standard). Statistical analyses were performed using SPSS 18.0 for windows (SPSS Inc., IBM-USA).

3. Results

The serum samples from pigs experimentally infected with *T. gondii* were negative at 0 and 7 DPI by the in-house MAT and the commercial MAT kit which was used as the gold standard. By 11–14 DPI, all inoculated pigs were positive by both tests at a 1:25 serum dilution and remained positive for the duration of the respective experiments until euthanasia (35–66 DPI). All samples of meat juice collected from each of the three muscle sites of experimentally-infected pigs also tested positive at 1:10 and 1:25

dilutions by both tests. Samples of all negative controls used for this study by both methods yielded negative results. Similar results were obtained with serum samples collected from the experimentally infected cats. For these animals, all samples collected at 0 DPI tested negative, whereas those collected post-inoculation at euthanasia tested positive. No significant difference was detected between positive and negative results when comparing MAT results for all tested serum and meat juice samples collected from experimentally infected pigs and cats obtained by either test, using Pearson Chi-square test ($P < 0.05$). The relative specificity and sensitivity of the in-house test were both 100%, compared to the commercial MAT kit. Thus, excellent agreement ($k = 1.0$) was observed between results from samples of serum and/or meat juice from experimentally infected pigs and cats tested by in-house MAT or commercial MAT kit. The results of this study are summarized in Table 1. ROC curve analysis, using the MAT kit result as the gold standard, showed that the in-house MAT performed similarly with area under the curve of 1.0 (Fig. 2).

For samples collected from a variety of naturally infected animals, test results for serum and/or whole blood samples obtained from two domestic (cat and sheep) and 20 wildlife species and tested by in-house MAT (1:50 dilution) and commercial MAT kit (1:40 dilution) showed comparable results (Table 1). Samples obtained from domestic cats showed a relatively high rate of positive results by both in-house MAT and commercial MAT (38.05% and 36.06%, respectively) with excellent agreement between the two tests ($k = 0.841$). All samples from sheep tested negative by both tests, indicating no exposure to *T. gondii*. A total of 668 serum and/or whole blood samples collected from 20 diverse species of wildlife animals in the northern and arctic regions of Canada were tested. Results of all wildlife species, except wolf, indicated substantial to excellent agreement between the two test methods.

4. Discussion

In the present study, an in-house MAT using tachyzoites antigen of *T. gondii* cultivated in tissue culture instead of the peritoneal cavity of mice was developed and its performance compared with that of a commercial MAT kit (Toxo-Screen DA, Biomerieux, France) as the gold standard. The commercial MAT test kit is widely accepted as a reliable test for screening animals for *Toxoplasma* infection regardless of host species. Commercial MAT kits for *T. gondii* are only intermittently available and there is difficulty in obtaining the antigen to perform the test which is usually prepared in the peritoneal cavity of mice. Whole tachyzoites grown in cell culture are available commercially (Kerafast-USA), but there are no published data on the validity of these cultured derived tachyzoites. Currently, we are aware of only a single commercial supplier of MAT kit (*Toxoplasma* Microwell Kit-New Life Diagnostics-USA). The in-house MAT using cultured tachyzoites was shown to be effective and specific in detecting anti-*Toxoplasma* IgG antibodies in serum, blood, and meat juice samples from experimentally and naturally infected animals. Also, culture conditions were improved using cell culture multi-flasks instead of conventional cell culture flasks to scale-up tachyzoites productivity and yield. Under these culture conditions, the yield of harvested tachyzoites increased approximately 10 fold from 1.6×10^9/ml (using conventional cell culture flasks) to 1.2×10^{10}/ml when multi-flasks were used. Therefore, a significant increase in tachyzoite production was achieved with improvement in cell culture conditions for large batch reagents.

The modified agglutination test (MAT) developed by Fulton and Turk (1959) and improved by others (Desmonts and Remington, 1980; Dubey and Desmonts, 1987; and Dubey, 2010) was designed for screening serum and blood samples from

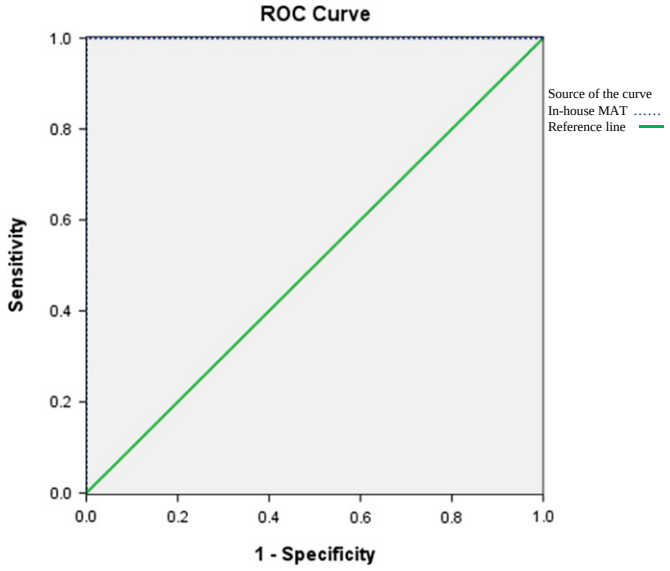

Fig 2. Receiver operating characteristic calculations for commercial MAT kit (gold standard) in relation to in-house MAT using serum samples from pigs experimentally infected with *Toxoplasma gondii*.

multiple host species for *T. gondii* infection. The present in-house MAT was adapted from the direct agglutination test described by Dubey (2010) with modifications involving changes in the preparation of antigen and incubation period of test plate. The *T. gondii* antigen used in the in-house MAT was prepared from tachyzoites harvested from MDBK cells grown in tissue culture. To preserve the parasite integrity and reduce cell culture debris, the tachyzoites were drawn through a sephadex column instead of using needles and millipore filters to break up and separate intact cells. Dubey (2010) suggested using 2×10^4/μl as the final concentration of tachyzoites for the MAT, whereas in the in-house MAT, the antigen concentration was optimized to 3×10^4/μl to detect positive or negative reactions. The negative results (blue button) were not clearly detected if the antigen concentration was too low, and appear to be inconclusive rather than true negative when the antigen concentration was too high. The incubation temperature affects the pattern of agglutination and sensitivity of the plate. Results from the in-house MAT could be read after incubating the test plate at room temperature (23 ± 5 °C) overnight, which facilitated the performance of the test without the requirement of incubation equipment. However, previous studies recommended overnight incubation at 32 °C or 37 °C, respectively Desmonts and Remington (1980) and Dubey (2010). Adjustment of test conditions was required to optimize test performance when using *in vitro* cultured antigen. Similar observations were reported when using *Neospora caninum* tachyzoites harvested from Vero cells grown in tissue culture to develop a MAT for detecting infection in various animal species (Packham et al., 1998).

Test results of the in-house MAT using pig serum and meat juice samples obtained from 13 pigs experimentally infected with *T. gondii* and controls all showed excellent performance agreement between the in-house MAT and the previously reported results of commercial MAT and ELISA (Forbes et al., 2012; Al-Adhami and Gajadhar, 2014). Similar results were obtained in this study with serum samples collected from experimentally infected pigs from USDA. Although data for samples collected at different DPI were not available except for those obtained at the time of euthanasia (Hill et al., 2006), the in-house MAT test results indicated that seroconversion in these pigs started at 11 DPI and remained positive for the duration of the experiment until euthanasia (66 DPI). Results obtained with serum samples collected from domestic cats and sheep also suggest that the in-house MAT is a useful tool when applied to routinely collected serum samples to screen for the presence of specific anti-*T. gondii* antibodies in domestic animals.

Comparison of wildlife serum or whole blood results obtained at a 1:50 dilution by the in-house MAT with those of the commercial MAT kit at 1:40 dilution demonstrated excellent agreement between the two tests for the animal species tested (k values ranged from 0.845–1.0), except for caribou ($k = 0.781$), red fox ($k = 0.792$), wolf ($k = 0.469$) and walrus ($k = 0.648$) blood (Table 1). Blood samples from wolf showed moderate to good agreement ($k = 0.469$), which could possibly be due, as previously reported, to the low sensitivity of the MAT for the detection of *T. gondii* antibodies in canine sera (Macri et al., 2009). However, this was not the case for the results obtained for foxes ($k = 0.792$). In contrast, data for serum samples collected from wildlife animals including wolves and foxes and tested by MAT indicated high sensitivity of the test as confirmed by bioassay in mice (Dubey et al., 2011; Dubey et al., 2013). These differences between test results for serum or blood samples obtained from naturally infected animals including canines may reflect diversity in antibody profiles that may be related to the duration of infection and/or differences in individual or species-specific immunological responses. Demonstrating seroconversion after a second sampling from the same individual, collected a few weeks after the first, will continue to provide the best diagnostic evidence of exposure/infection, but this is obviously impractical for wildlife sampling.

Human populations in northern and arctic regions of Canada are at higher risk of *Toxoplasma* infection due to cultural habits favouring handling and consuming raw wild game meat (Elmore et al., 2012). In this study, some of the tested wildlife species such as bison, muskox, caribou, and black bear may represent an important source of traditional 'country' food for the indigenous peoples (Forbes et al., 2009). Thus, our results indicate that meat from these sources could pose a risk for *T. gondii* infection when consumed raw or not cooked to a minimum internal temperature of 70 °C (Dubey, 2010). The in-house MAT described in this study is a simple, rapid, inexpensive and multi-host species tool for use in food production and surveillance programmes for estimating and managing the risk of *T. gondii* infection in the food supply web.

Conflicts of interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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References

Al-Adhami, B.H., Gajadhar, A.A., 2014. A new multi-host species indirect ELISA using protein A/G conjugate for detection of anti-*Toxoplasma gondii* IgG antibodies with comparison to ELISA-IgG, agglutination assay and Western blot. Vet. Parasitol. 200, 66–73.

Desmonts, G., Remington, J.S., 1980. Direct agglutination test for diagnosis of *Toxoplasma* infection: method for increasing sensitivity and specificity. J. Clin. Microbiol. 11, 562–568.

Dubey, J.P., 1997. Validation of the specificity of the modified agglutination test for toxoplasmosis in pigs. Vet. Parasitol. 71, 307–310.

Dubey, J.P., 2010. Toxoplasmosis of Animals and Humans. second ed. CRC Press, Boca Raton, London, NY.

Dubey, J.P., Desmonts, G., 1987. Serological responses of equids fed *Toxoplasma gondii* oocysts. Equine Vet. J. 19, 337–339.

Dubey, J.P., Thulliez, P., Weigel, R.M., Andrews, D.C., Lind, P., Powell, E.C., 1995. Sensitivity and specificity of various serologic tests for detection of *Toxoplasma gondii* infection in naturally infected sows. Am. J. Vet. Res. 56, 1030–1036.

Dubey, J.P., Velmurugan, G.V., Rajendran, C., Yabsley, M.J., Thomas, N.J., Beckmen, K.B., Sinnett, D., Ruid, D., Hart, J., Fair, P.A., McFee, W.E., Shearn-Bochsler, V., Kwok, O.C.H., Ferreira, L.R., Choudhary, S., Faria, E.B., Zhou, H., Felix, T.A., Su, C., 2011. Genetic characterisation of *Toxoplasma gondii* in wildlife from North America revealed widespread and high prevalence of the fourth clonal type. Int. J. Parasitol. 41, 1139–1147.

Dubey, J.P., Choudhary, S., Ferreira, L.R., Kwok, O.C.H., Butler, E., Carstensen, M., Yu, L., Su, C., 2013. Isolation and RFLP genotyping of *Toxoplasma gondii* from the gray wolf (*Canis lupus*). Vet. Parasitol. 197, 685–690.

Elmore, S.A., Jenkins, E.J., Huyvaert, K.P., Polley, L., Jeffrey Root, J., Moore, C.J., 2012. *Toxoplasma gondii* in circumpolar people and wildlife (review). Vector Borne Zoonotic Dis. 12, 1–9.

Elsheikha, H.M., Rosenthal, B.M., Murphy, A.J., Dunmas, D.B., Neelis, D.D., Mansfield, L.S., 2006. Generally applicable methods to purify intracellular coccidia from cell culture and to quantify purification efficacy using quantitative PCR. Vet. Parasitol. 135, 223–234.

Forbes, L.B., Measures, L., Gajadhar, A., 2009. Infectivity of traditional (country) foods prepared with meat from experimentally infected seals. J. Food Prot. 72, 1756–1760.

Forbes, L.B., Parker, S., Gajadhar, A.A., 2012. Performance of commercial ELISA and agglutination test kits for the detection of *Toxoplasma gondii* antibodies in serum and muscle fluid of swine infected with 100, 300, 500 or 1000 oocysts. Vet. Parasitol. 190, 362–367.

Fulton, J.D., Turk, J.L., 1959. Direct agglutination test for *Toxoplasma gondii*. Lancet 274, 1068–1069.

Gajadhar, A.A., Measures, L., Forbes, L.B., Kapel, C., Dubey, J.P., 2004. Experimental *Toxoplasma gondii* infection in grey seals (*Halichoerus grypus*). J. Parasitol. 90, 255–259.

Gilbert, R., Cook, A., Dunn, D., 2000. Sources of *Toxoplasma* infection in pregnant women: a European multicenter case–control study. Br. Med. J. 312, 142–147.

Hill, D.E., Chirukandoth, S., Dubey, J.P., Lunney, J.K., Gamble, H.R., 2006. Comparison of detection methods of *T. gondii* in naturally and experimentally infected swine. Vet. Parasitol. 141, 9–17.

Lindsay, D.S., Dubey, J.P., Blagburn, B.L., Toivio-Kinnucan, M., 1991. Examination of tissue cyst formation by *Toxoplasma gondii* in cell cultures using bradyzoites, tachyzoites, and sporozoites. J. Parasitol. 77, 126–132.

Macri, G., Sala, M., Linder, A.A., Pettirossi, N., Scrapulla, M., 2009. Comparison of indirect fluorescent antibody test and modified agglutination test for detecting *Toxoplasma gondii* immunoglobulin G antibodies in dog and cat. Parasitol. Res. 105, 35–40.

Packham, A.E., Sverlow, K.W., Conrad, P.A., Loomis, E.F., Rowe, J.D., Anderson, M.L., Marsh, A.E., Cray, C., Barr, B.C., 1998. A modified agglutination test for *Neopspora caninum*: development, optimization, and comparison to the indirect fluorescent-antibody test and enzyme-linked immunosorbent assay. Clin. Diagn. Lab. Immunol. 5, 467–473.

Sushrut, K., Davis, P.H., 2012. *Toxoplasma* on the brain: understanding host–pathogen interactions in chronic CNS infection. J. Parasitol. Res. 2012, 1–10.

Viera, A.J., Garret, J.M., 2005. Understanding interobserver agreement: the kappa statistics. Fam. Med. 37, 360–363.