Prevalence and co-infection of *Toxoplasma gondii* and *Neospora caninum* in *Apodemus sylvaticus* in an area relatively free of cats

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SUMMARY

The protozoan parasite *Toxoplasma gondii* is prevalent worldwide and can infect a remarkably wide range of hosts despite felids being the only definitive host. As cats play a major role in transmission to secondary mammalian hosts, the interaction between cats and these hosts should be a major factor determining final prevalence in the secondary host. This study investigates the prevalence of *T. gondii* in a natural population of *Apodemus sylvaticus* collected from an area with low cat density (<2·5 cats/km^2). A surprisingly high prevalence of 40·78% (95% CI: 34·07%–47·79%) was observed despite this. A comparable level of prevalence was observed in a previously published study using the same approaches where a prevalence of 59% (95% CI: 50·13%–67·87%) was observed in a natural population of *Mus domesticus* from an area with high cat density (>500 cats/km^2). Detection of infected foetuses from pregnant dams in both populations suggests that congenital transmission may enable persistence of infection in the absence of cats. The prevalences of the related parasite, *Neospora caninum* were found to be low in both populations (*A. sylvaticus*: 3·39% (95% CI: 0·12%–6·66%); *M. domesticus*: 3·08% (95% CI: 0·11%–6·05%)). These results suggest that cat density may have a lower than expected effect on final prevalence in these ecosystems.

Key words: *Toxoplasma gondii*, *Neospora caninum*, PCR, *Apodemus sylvaticus*, *Mus domesticus*, vertical transmission.

INTRODUCTION

The protozoan parasite *Toxoplasma gondii* is prevalent worldwide, infects a wide range of hosts and is generally found in high prevalence (Tenter *et al*. 2000). Amongst the *Apicomplexa* it is distinguished for its ability to readily infect almost any mammal, as well as birds, despite felids being the only definitive host (Dubey *et al*. 1970; Dubey, 2009*a*,*b*). Rodents, and in particular mice, are considered to be an important intermediate host as they are a potential prey species for cats (Tenter *et al*. 2000). They may play an important role in the completion of the life cycle of *T. gondii* by providing a source of infected meat for the cat which leads to subsequent infection of more secondary hosts. Despite this apparently important role, a number of serological studies have shown that *T. gondii* has a relatively low prevalence in mice (Franti *et al*. 1976; Jackson *et al*. 1986; Webster, 1994; Dubey *et al*. 1995; Smith and Frenkel, 1995) although some PCR-based studies of *T. gondii* in *Mus domesticus*, and other species of mice, have shown higher prevalences (Marshall *et al*. 2004; Zhang *et al*. 2004). Few studies have been conducted to date on the woodmouse, *Apodemus sylvaticus*. Prevalences of *T. gondii* of 14·3% have been reported, using PCR, in one study (Kijlstra *et al*. 2008) and seroprevalences of 1·49% and 7·4% were found in *Apodemus agrarius* in Korea (Jeon and Yong, 2000) and the Czech Republic (Hejlicek and Literak, 1998). The assumption is that these natural populations of wild mammals are infected through interaction with cats.

Infected cats release large quantities of oocysts (10^7–10^8 per cat) that are environmentally persistent and previous reports suggested that younger cats shed more frequently than older or previously exposed cats (Dubey, 1995). However, recent large-scale studies (Schares *et al*. 2008; Herrmann *et al*. 2010) have demonstrated frequent shedding of oocysts by older cats. It is estimated that only a small proportion (1–2%) of domestic cats may be shedding oocysts at

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any one time (Dubey and Beattie, 1988; Dubey and Jones, 2008) but this could be as low as 0·25% (Herrmann *et al*. 2010). Overall, these data imply that a higher prevalence of *T. gondii* would be found in intermediate hosts in an area with large cat populations.

T. gondii is transmitted via 3 routes; the ingestion of oocysts from the environment, ingestion of tissue cysts by the intermediate host, and congenitally through transplacental transmission from mother to foetus. The contribution of each of these routes to the overall prevalence of the *T. gondii* is currently a subject of debate (Hide *et al*. 2007, 2009; Innes *et al*. 2009). There is considerable evidence now that the parasite might frequently engage in transmission cycles that bypass cats (Su *et al*. 2003; Dubey *et al*. 2005) but the relative importance of different transmission routes is still unclear. A number of studies have suggested that vertical transmission may be an important transmission route in natural populations of mammals such as sheep (Duncanson *et al*. 2001; Williams *et al*. 2005; Morley *et al*. 2005, 2008) and perhaps humans (Hide *et al*. 2007, 2009). Although congenital transmission of *T. gondii* has been studied in mice under laboratory conditions, few studies have looked at this phenomenon in naturally infected wild populations.

In a laboratory study, Owen and Trees (1998) looked at chronically infected house mice (*Mus musculus*) and wood mice (*A. sylvaticus*) using PCR. Vertical transmission was demonstrated in 82·7% of all pups and in 95% of all litters of *A. sylvaticus* showing that it is extremely efficient in this species and probably endures for the life of the breeding female. Another earlier study by Beverley (1959) looked at congenital transmission of toxoplasmosis through successive generations of mice. Beverley (1959) reported that 23 of the infected litters were born of mothers, themselves congenitally infected, and in one instance a congenitally infected mother produced congenitally infected offspring even in her fourth litter. In a field study, PCR testing of *T. gondii* in foetuses, collected from a natural population of wild *M. domesticus*, demonstrated that congenital transmission was occurring in 75% of mice and rose to 100% when only PCR positive mothers were considered (Marshall *et al*. 2004). However, most studies conducted using serological techniques have suggested lower rates of congenital transmission in rodents (Dubey *et al*. 1995) although this may be due to sensitivity of the serological tests which often show that congenitally infected mice can test seronegative while harbouring parasites (Beverley, 1959; Jacobs, 1964; Dubey *et al*. 1997; Owen and Trees, 1998; Araujo *et al*. 2010).

The related parasite, *Neospora caninum*, undergoes frequent vertical transmission (Cole *et al*. 1995; Williams *et al*. 2009) but little is known about its prevalence in natural populations of rodents nor the degree of co-infection with *T. gondii*. Studies using PCR detection of *Neospora* and *Toxoplasma* have demonstrated evidence of co-infection in sheep, mice, rats (Hughes *et al*. 2006) and rabbits (Hughes *et al*. 2008) but no significant association between the two parasites.

In this paper we report the results of a study that investigates the prevalence of *T. gondii* and *N. caninum* in a wild population of *A. sylvaticus*. At the location of the sampling site there are few cats present locally – less than 2·5 cats per km^2 (based on the data of Hughes *et al*. 2008). This offers the opportunity to compare directly, using an identical methodology, the prevalence of *T. gondii* in rodents in an area relatively free of cats with a previously published study on an area where there were more than 500 cats per km^2 (based on the data of Murphy *et al*. 2008). The objectives of this study are to investigate the prevalence and co-infection of *T. gondii* and *N. caninum* in *A. sylvaticus* and to compare the prevalence of *T. gondii* in rodents in areas with high and low cat densities.

MATERIALS AND METHODS

The woodmice (*A. sylvaticus*) were sampled, using Longworth Traps, from the surrounding area of Malham Tarn Field Centre, North Yorkshire. Mice weighing less than 14 g were considered juveniles (Higgs and Nowell, 2000). They were euthanased, measured, weighed, sexed and the brains dissected out, using sterile technique, and transferred into sterile tubes containing 400 μl of lysis buffer (100 mM sodium chloride, 25 mM EDTA, 0·5% SDS, 20 mM Tris pH 8·0) and stored at −20 °C. Instruments were sterilized between dissections and cross-contamination of samples was prevented at all stages by the use of disposable sterile equipment (e.g. tubes, tips), careful handling, labelling and sample processing during DNA extraction.

DNA was extracted using a phenol/chloroform procedure as previously described (Terry *et al*. 2001). Uninfected and *T. gondii* (RH Strain)-infected mouse tissue (J.E. Smith, University of Leeds) were used as DNA-processing controls. The extracted DNA was tested to ensure viability for PCR using generic primers to mammalian α-tubulin as previously described (Duncanson *et al*. 2001; Morley *et al*. 2005). The presence of *T. gondii* was detected using PCR amplification of the Surface Antigen Gene 1 (SAG1) gene and resolved by gel electrophoresis as previously described (Williams *et al*. 2005). Precautions were taken to avoid contamination during nested PCR reactions as follows: negative controls (sterile distilled water) were included as the first and last sample of any set of PCR reactions. Positive controls used were DNA extracted from *T. gondii* reference strains (typically RH) and a cloned

Table 1. Summary of the SAG 1 PCR detection of *Toxoplasma gondii* and *Neospora caninum* in *A. sylvaticus* and *M. domesticus*

(Data from *Mus domesticus* were obtained from previously published studies (Marshall *et al.* 2004; Murphy *et al.* 2008.)

	Toxoplasma gondii			*Neospora caninum*		
	PCR positive	Total tested	Prevalence% (95% CI)	PCR positive	Total tested	Prevalence% (95% CI)
Apodemus sylvaticus	84	206	40·78 (34·1–47·8)	4	118	3·39 (0·12–6·66)
Mus domesticus	118	200	59 (50·1–67·9)	4	130	3·08 (0·11–6·05)

SAG1 gene product which has been verified by DNA sequencing. Preparation of PCR reactions at different stages (e.g. addition of DNA and transfer of second-round products) was conducted in different rooms or in separate clean hoods. Due to unknown ratios of parasite to host DNA, all samples were tested 3 times at 1 μl, 2 μl and 1 μl 1:5 dilution of sample DNA. Further validation of the reliability of the SAG1 PCR system has been described by us previously (Williams *et al.* 2005).

A nested PCR was used to detect the SAG3. This method has been modified from the method described by Grigg *et al.* (2001). Amplification was carried out in a final volume of 50 μl containing 5 μl of 10× HT PCR buffer (HT Biotechnologies) (100 mM Tris HCl (pH 9·0), 15 mM $MgCl_2$, 500 mM KCl, 1% TritonX-100, 0·1% (w/v) stabilizer), 0·5 μl of dNTP mix (100 mM), 2·5 μl of (10 pM/μl) forward primer F_{ext} (5′CAACTCTCACCATTCCACCC3′) and reverse primer R_{ext} (5′GCGCGTTGTTAGACAAGACA3′) and 2·5 units Biotaq polymerase (Bioline). DNA-free water made the final volume to 50 μl. Due to unknown ratios of parasite to host DNA, all samples were tested 3 times at 1 μl, 2 μl and 1 μl 1:5 dilution of sample DNA. Amplification was carried out using a Stratagene Robocycler as follows: an initial denaturation step of 5 min at 94 °C, was followed by 35 cycles of PCR performed for 40 sec at 94 °C, 40 sec at 60 °C and 60 sec at 72 °C, with a final extension step of 10 min at 72 °C. Second-round PCR was carried out using the same reaction and cycling conditions as the first round with the exception of the primers which were F_{int} (5′TCTTGTCGGGTGTTCACTCA3′) and R_{int} (5′CACAAGGAGACCGAGAAGGA3′). A volume of 2 μl of first-round product was added to act as a template. Amplification products (10 μl) were visualized by agarose gel electrophoresis on a 2% agarose gel containing Gelred or ethidium bromide. Positive PCR reactions were further analysed by restriction enzyme digestion with each of the enzymes Nci1 and AlwN1, 8·5 μl of PCR product, 1 μl of buffer 4 (NEB) and 0·5 μl of enzyme. These were incubated at 37 °C for a minimum of 4 h. Products were visualized by gel electrophoresis on a 2·5% agarose gel. *N. caninum* was detected by PCR as previously described (Hughes *et al.* 2006, 2008).

To confirm the sequence of PCR products, bands were excised from the agarose gel and cleaned using Geneclean® before sequencing. Fragments of DNA sequence from SAG1 and SAG3 PCR amplifications were sent to Lark Technologies for bidirectional sequencing using the primers DS 38 and DS 39 (SAG1) and Fint and Rint (SAG3). *N. caninum* fragments were sequenced as previously described (Hughes *et al.* 2006, 2008). Sequence data were compared to previously published sequences using CLUSTAL W software available on the European Bioinformatics Institute website (www.ebi.ac.uk/clustalw/).

Statistical analysis of data was carried out using Chi-Squared tests (Microsoft, EXCEL) or 2 × 2 contingency tables using Fishers Exact Test (Graphpad Software: http://www.graphpad.com/).

RESULTS

DNA was extracted from brain tissue taken from 206 *A. sylvaticus* and DNA integrity checked using PCR amplification of the *Apodemus* tubulin gene. SAG1 PCR was used to detect the presence of *T. gondii* DNA in the DNA extracted from brain tissue. Positive amplifications in 84 of the 206 samples demonstrated the prevalence of *Toxoplasma* infection to be 40·78% (95% CI: 34·07%–47·79%) (Table 1). To confirm that the correct DNA sequence was being amplified, DNA sequencing was carried out on a sample of PCR amplified product from a PCR-positive *A. sylvaticus* (Accession no. JF412000). There was a 100% match to published SAG1 sequences.

A subset of 111 mice (42·3% PCR positive), which had an associated comprehensive data set on parameters such as sex, weight and length, was used to investigate the relationship with *Toxoplasma* infection. To determine whether there was a relationship of infection with sex of the mice, 54 females and 57 males were found to have 23 (42·6%) and 24 (42·1%) PCR-positive mice respectively. No significant difference between *T. gondii* infection and sex was

Table 2. Frequency of infection of *Apodemus sylvaticus* with *Neospora caninum* and *Toxoplasma gondii*

	N. caninum positive	*N. caninum* negative
T. gondii positive	1	55
T. gondii negative	3	59
Total	4	114

Table 3. Frequency of infection of *Mus domesticus* with *Neospora caninum* and *Toxoplasma gondii*

	N. caninum positive	*N. caninum* negative
T. gondii positive	3	66
T. gondii negative	1	60
Total	4	126

found ($\chi^2 = 0{\cdot}003$, D.F. = 1, $P = 0{\cdot}957$). As studies of *Toxoplasma* infection often show the presence of an age-dependent prevalence (i.e. higher prevalences are found in older cohorts), we tested this using these data. A total of 97 adults and 14 juveniles (those under 14 g) were observed in this cohort of 111 of which 44 (45·3%) and 5 (35·7%) were PCR positive for *Toxoplasma* respectively. No significant difference between infection of adults and juveniles was found ($\chi^2 = 0{\cdot}153$, D.F. = 1, $P = 0{\cdot}6953$) showing that no age-dependent prevalence increase was evident in this study.

For this study, 2 pregnant *A. sylvaticus* dams were collected. One was found to be PCR positive for *T. gondii* and SAG1 PCR detected parasite DNA in the brain tissue of 2 out of the 6 foetuses within this pregnant dam.

To gain an insight into the diversity of *T. gondii* strains within the *A. sylvaticus* population, as many as possible of the SAG1-positive animals were tested for RFLP with the SAG3-PCR method (Grigg *et al.* 2001) to determine strain type. Considerable difficulty was experienced in successfully amplifying sufficient quantities of the SAG3 product from the DNA of these tissue samples. SAG3 genotypes were determined for 22 of the PCR-positive mice. Of the 22 mice, 12 were of SAG3 Type I, 9 of Type II and 1 Type III.

PCR was used to investigate the prevalence of *N. caninum* in this population of *A. sylvaticus*. Of the *A. sylvaticus* there were 118 tested for *N. caninum* of which only 4 were positive, giving a prevalence of 3·39% (95% CI: 0·12–6·66%) (Table 1).

The question of co-infection between *T. gondii* and *N. caninum* has not been explored in *A. sylvaticus* to our knowledge although it has been investigated using a smaller sample of *M. domesticus* in a previous study (Hughes *et al.* 2006). Tables 2 and 3, respectively, summarize the infection data for *A. sylvaticus* and the previously published *M. domesticus* data (Hughes *et al.* 2006) augmented with additional samples new to this study. Co-infection was observed in both populations but no significant association was found between *T. gondii* and *N. caninum* infection in either *A. sylvaticus* ($P = 0{\cdot}621$) or *M. domesticus* ($P = 0{\cdot}622$).

DISCUSSION

In this study a sympatric collection of *A. sylvaticus* was tested for the presence of *T. gondii* and *N. caninum* with prevalences of 40·78% (95%CI: 34·1%–47·8%) and 3·39% (95%CI: 0·12%–6·66%) respectively being measured. To our knowledge only one other PCR study has been conducted on the prevalence of *N. caninum* in *A. sylvaticus* – a prevalence of 1·8% was seen in a sample set of 55 animals collected in Italy (Ferroglio *et al.* 2007). This low prevalence was not significantly different from that seen with our larger collection ($\chi^2 = 0{\cdot}412$, D.F. = 1, $P = 0{\cdot}52$). A comparison of *N. caninum* prevalences in this population of *A. sylvaticus* and an *M. domesticus* population collected from an urban area within Manchester, UK, showed a non-significantly different ($\chi^2 = 0{\cdot}046$, D.F. = 1, $P = 0{\cdot}83$) prevalence of 3·08% (95%CI: 0·11%–6·05%) using identical collection and detection systems. It is unclear how this parasite is transmitted in both of these rodent species. The dog is known to be the definitive host (McAllister *et al.* 1998) and vertical transmission is considered to be frequent in *N. caninum* (Williams *et al.* 2009). Co-infection was observed but no significant association was seen between infection with *N. caninum* and *T. gondii* in either the *Apodemus* or *Mus* hosts. This is consistent with PCR-based studies on other mammalian species (Hughes *et al.* 2006, 2008).

In the case of *T. gondii*, the cat clearly has a key role in the life cycle, raising the question as to how the prevalence of infection in rodents may differ in areas with high and low cat densities. The *Apodemus* in this study were collected from an area with an estimated cat density of less than 2·5 cats per km^2 (based on re-calculation of data from Hughes *et al.* 2008). In other, previously published studies (Marshall *et al.* 2004; Murphy *et al.* 2008) exactly the same techniques and approaches were used to measure the prevalence of *T. gondii* in an urban population of *M. domesticus* from an area of considerably higher cat density than the sampling site for *A. sylvaticus*. Details of the locations of cats within the study site were collated (Murphy *et al.* 2008) and a cat density of more than 500 cats per km^2 was calculated (based on the data

presented by Murphy *et al*. 2008). Two hundred *M*. *domesticus* collected from this urban population were found to have a prevalence of 59% using SAG1 PCR of brain tissue (Marshall *et al*. 2004). Compared with the 40·78% found in the *A*. *sylvaticus* samples, there was a higher prevalence (59%) in the *M*. *domesticus* samples (Table 1) suggesting that the presence of cats clearly contributes to the prevalence of *T*. *gondii*. However, despite the 200 times higher density of cats in the *M*. *domesticus* sample site, only a further 19% (95%CI: 11·2%–26·8%) increase in prevalence was seen. Clearly, therefore, high levels of infection in the *Apodemus* samples are being maintained in the relative absence of cats. We appreciate that other factors, such as the difference in species studied here (for example, in behaviour), may also influence the different prevalences although we found no evidence in the literature that might suggest differences in virulence towards *Apodemus* or *Mus*.

One possible transmission route that could maintain these relatively high prevalences is congenital transmission. Previous studies on the *M*. *domesticus* population showed the occurrence of high frequencies of congenital transmission (Marshall *et al*. 2004). In this study, 2 pregnant females (1 PCR positive) were found within the population of *A*. *sylvaticus*. Although only a very small sample size, the PCR-positive foetuses in one of them shows that congenital transmission is occurring in this *A*. *sylvaticus* population. This offers a possible explanation of how transmission might be occurring in the relative absence of cats. This hypothesis is further supported by the lack of an age prevalence effect when comparing the juvenile *Apodemus* with adult mice – a phenomenon which would be expected to be seen if the *Apodemus* acquired infection by ingestion of oocysts or carnivory.

Parasite multilocus genotyping might be one way of investigating whether frequent congenital transmission might be occurring. High degrees of similarity between genotypes might indicate the level of clonality expected to be associated with vertical transmission. In our studies, considerable difficulty was experienced in successfully amplifying sufficient quantities of the SAG3 product to directly genotype from these tissue samples, although all 3 main clonal genotypes were seen. The difficulty was probably due to a low parasite to host DNA ratio. The consequences of this difficulty were that we were unable to attempt multilocus genotyping which is now considered the norm for *T*. *gondii* genotyping. The range of SAG3 genotypes present in this *Apodemus* population may argue against congenital transmission occurring frequently.

The prevalence of *T*. *gondii* infection in both the *Apodemus* and *Mus* populations is high. This is in contrast to many serological studies that have reported relatively low *Toxoplasma* prevalence in mice (Franti *et al*. 1976; Jackson *et al*. 1986; Webster, 1994; Dubey *et al*. 1995; Hejlicek *et al*. 1997; Hejlicek and Literak, 1998). Few studies have been conducted to date on the woodmouse, *A*. *sylvaticus*. Prevalences of *T*. *gondii* of 14·3% have been reported, using real-time PCR, in one study (Kijlstra *et al*. 2008) and seroprevalences of 1·49% and 7·4% found in *A*. *agrarius* in Korea (Jeon and Yong, 2000) and the Czech republic (Hejlicek and Literak, 1998). A study of prevalence of *T*. *gondii* in another rodent species, the Deer Mouse, (*Peromyscus maniculatus*) reported a prevalence rate of 26% (Dabritz *et al*. 2008). Interestingly, recent research has shown that high levels of congenital transmission can be detected following experimental infection of this latter species (Rejmanek *et al*. 2010).

The reasons behind the variability in prevalences of *T*. *gondii* in small rodents is unclear and especially the explanation for the high prevalences seen in our studies on UK species of *Apodemus* and *Mus*. Geographical variance, host species behaviour or methods of detection (e.g. serology *vs* PCR) could all be factors. There is a growing body of evidence that suggests that serological methods may not efficiently detect *T*. *gondii* infection in mice – especially if that infection is congenitally derived. This was first noticed by Beverley (1959), where serological detection was inefficient despite high mortality in congenitally infected mice. This has also been reported subsequently (Dubey *et al*. 1997; Owen and Trees, 1998; Araujo *et al*. 2010; Rejmanek *et al*. 2010).

The closely related parasite *N*. *caninum* is clearly capable of efficient vertical transmission and can be maintained in its hosts by endogenous transplacental transmission (Cole *et al*. 1995; Trees and Williams, 2005; Williams *et al*. 2009). Little is known about the mechanisms of endogenous transplacental transmission in *T*. *gondii*. One possibility is that the parasite is able to manipulate the host immune system during transplacental transmission such that it is either recognized as 'self' or not recognized at all. Further research in this area may be important if vertical transmission of *T*. *gondii* is relevant to natural populations of animals such as the woodmice in this study.

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