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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/km²). A surprisingly high prevalence of 40.78% (95% CI: 34.07%–6.05%) was observed in a previously published study using the same approaches where 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/km²). 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 (<0.1% of domestic cats may be shedding oocysts at 1·49% and 7·4% were found in *Mus domesticus*—108 per cat) that are environmentally persistent. 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, 2009a,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 (Hejlícek and Líterak, 1998). The assumption is that these natural populations of wild mammals are infected through interaction with cats.

Infected cats release large quantities of oocysts (10⁷–10⁸ 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 km² (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 km² (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
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 MgCl2, 500 mM KCl, 1% TritonX-100, 0.1% (w/v) stabilizer), 0.5 μl of dNTP mix (100 μM), 2.5 μl of (10 pM/μl) forward primer Fint (5′CAACTCTCACATTCCACCC3′) and reverse primer Rint (5′GCGCGTTGTTAGACCAAGACA3′) 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, 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 PCR was carried out using the same reaction and extension step of 10 min at 72 °C. Second-round amplification products (10 μl) were visualized by agarose gel electrophoresis on a 2% agarose gel. 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 MgCl2, 500 mM KCl, 1% TritonX-100, 0.1% (w/v) stabilizer), 0.5 μl of dNTP mix (100 μM), 2.5 μl of (10 pM/μl) forward primer Fint (5′CAACTCTCACATTCCACCC3′) and reverse primer Rint (5′GCGCGTTGTTAGACCAAGACA3′) 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, 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 PCR was carried out using the same reaction and extension step of 10 min at 72 °C. Second-round PCR was carried out using the same reaction and cycling conditions as the PCR was carried out using the same reaction and extension step of 10 min at 72 °C. Second-round PCR was carried out using the same reaction and cycling conditions as the PCR was carried out using the same reaction and extension step of 10 min at 72 °C.
found ($\chi^2 = 0.003$, d.f. = 1, $P = 0.957$). 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 ($\chi^2 = 0.153$, d.f. = 1, $P = 0.6953$) 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* ($P = 0.621$) or *M. domesticus* ($P = 0.622$).

**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 ($\chi^2 = 0.412$, d.f. = 1, $P = 0.52$). 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 ($\chi^2 = 0.046$, d.f. = 1, $P = 0.83$) 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

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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; Hejlick et al. 1997; Hejlick 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 (Hejlick 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 woodmouse in this study.

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**REFERENCES**

Toxoplasma infection in Apodemus sylvaticus


