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EXAMINATION OF RED FOXES (*VULPES VULPES*) FROM BELGIUM FOR ANTIBODY TO *NEOSPORA CANINUM* AND *TOXOPLASMA GONDII*

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Neospora caninum and *Toxoplasma gondii* are closely related protozoan parasites (Ellis and others 1994, Holmdahl and others 1994). The latter, which was first described in 1908, has a two stage asexual lifecycle with tachyzoites multiplying during an acute infection and bradyzoites in tissue cysts in the persistent form of the infection. Its full lifecycle, however, involving the production of oocysts by Felidae was not established until 1970 (Dubey and Beattie 1988). *N caninum*, on the other hand, was first described in 1984 when it was shown to be the cause of death in a group of puppies (Bjerkas and others 1984) and was named in 1988 (Dubey and others 1988). To date it is known to exist as bradyzoites in tissue cysts, and as tachyzoites ; a sexual lifecycle involving the production of oocysts has not been demonstrated, although it has been predicted that there is likely to be a definitive carnivore host with a role similar to the cat in toxoplasmosis (Dubey and Lindsay 1993). To study the distribution of *N caninum* in the environment sera from red foxes (*Vulpes vulpes*) caught in Belgium, which were sampled as part of the rabies control programme, were examined for evidence of antibodies to *N caninum* and *T gondii*.

Sera from 123 foxes were collected over a 12-month period , from February 1995 to January 1996, with 111 samples from adults, where age was estimated at over seven months, (52 females, 55 males and four where the sex was not recorded) and 12 from juveniles with an estimated age of less than seven months (sex not recorded). While 118 of the foxes were from rural areas (farmland, woods and open spaces) in the province of Luxembourg, five of the juveniles were from a suburban area near Brussels. IgG antibody to *N caninum* was tested with an immunofluorescent antibody test (IFAT), validated by monitoring the serological response in a group of experimentally infected sheep (Buxton and others 1997) before modifying it for use with fox sera. Selected fox sera were also examined for evidence of antibody to *N caninum* with a commercially available IFAT (YMRD ; Pullman).

Tachyzoites of the NCI isolate of *N caninum* were grown in Vero cells in Iscove's modified Dulbecco's medium (IMDM) containing 2 per cent horse serum, free from antibodies to *N caninum*, 100 units of penicillin per ml and 100 µg streptomycin per ml. After seven days infected cells were detached, rinsed three times in phosphate buffered saline (PBS) and resuspended in PBS containing formalin (0·2 per cent v/v) at a concentration of 1 x 10^7 tachyzoites per ml. After 24 hours at 4°C, aliquots were stored at -20°C until required, when they were thawed and 5 µI of suspension was placed in each well of a Multitest 15-well slide (ICN Biomedicals ; Ohio) and allowed to dry. The slides were then immersed in methanol for 10

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minutes and rinsed twice in PBS before test sera were applied in two-fold dilutions, from 1/64, to individual wells. After incubation at room temperature for 30 minutes, the slides were thoroughly rinsed with PBS before rabbitanti-canine whole IgG serum labelled with fluorescein isothiocyanate (FITC) (Sigma) was applied. Slides were viewed under an Olympus BX50 fluorescence microscope, fitted with a U-MNB filter cube, with a x40 objective. Detection of IgG antibody to *T gondii* was undertaken in the same way and as reported by Buxton and Finlayson (1986). Briefly, tachyzoites of the RH strain of *T gondii* were grown in the peritoneum of mice, harvested 72 hours after injection, rinsed three times in PBS and resuspended at a concentration of $1x10^7$/ml in PBS containing 0·2 per cent v/v formalin. After 24 hours at 4°C, aliquots were stored at -20°C until required, when they were thawed and the test carried out as above. Antibody titres to *N caninum* and *T gondii* are shown in Table I. Twenty-one samples (17 per cent) had antibody titres to N can inum greater than 1/64, with two at 1/1024 and one each at 1/2048, 1/4096 and 1/8192. All 12 juvenile foxes had titres of 1/64 or less. One hundred and twenty-one (98-4 per cent) of the serum samples had antibody titres to Toxoplasma of 1/128 or more. The largest number of foxes (31,7 per cent) had a titre of 1/1024 (Table 1). All 21 sera with antibody titres to *Neospora* of more than 1/128 also had Toxoplasma antibody titres of more than 1/128 with the titre to the latter parasite being the greater in 18, the same in one and less in the remaining two samples. *Neospora* antibody titres in selected fox sera examined with the commercially available IFAT gave largely similar results. Sera with titres of 1/64 or less by the IFAT described in this paper were uniformly negative by the commercial IFAT (defined as a titre of less than 1/50), while four sera - with titres of 1/256, 1/512, 1/1024 and 1/8192 - gave titres which were either equal to or one titre lower with the commercial!FAT. The canine negative and positive control sera supplied with the commercial test gave negative and positive results respectivel y with both *Neospora* IFATs.

While this study shows that antibodies to *T gondii* detected with an IFAT are very common in foxes from the Belgian province of Luxembourg, it also shows that seroconversion to *N caninum* had occurred in a number of the animals studied. The proportion des- ignated as positive depends on the titre selected as indicating the presence of specific antibody. At the time of writing the authors were not aware of any similar surveys of red fox sera. With a Sabin-Feldman dye test, authors have selected titres of 1/16 or more and 1/20 in fox sera as indicating exposure to *T gondii* (Quinn and others 1976, Tizard and others 1976) and have shown that 53 per cent of 153 foxes and 84 per cent of 96 foxes in Ontario, Canada, were infected with *T gondii*. Mulley and others (1982) showed that eight red foxes from New South Wales were positive using a titre of 1/32 or more in a Toxoplasma haemagglutination test. In studies of canine sera Lindsay and others (1990) selected IFAT titres of 1/50 or more as indicating exposure to both parasites. For *N caninum*, however, Bjorkman and others (1994) used an IFAT titre of 1/80 or more and Trees and others (1993) a titre of 1/200 or more. More recently, Dubey and Lindsay (1996) affirmed that in their experience all parasitologically confirmed cases of canine neosporosis have had IFAT titres of 1/200 or more.

In view of the above variation in the titres selected and in an attempt to maximise sensitivity and minimise non-specificity it is considered that an IFAT titre of 1/128 or more is likely to indicate specific seroconversion to either parasite, although it must be accepted that a proportion of foxes with titres below this may also have been exposed to infection. On this basis, 98,4 per cent of the foxes in this study had been exposed to two negative animals being a cub and the other an

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adult female, and at least 17 per cent (21 animals) appear to have been infected with *N caninum*. The serological evidence also strongly suggests that there is no significant cross-reaction between the two tests, as a large number of foxes positive for Toxoplasma were negative for *Neospora* and two which were positive for *Neospora* (with titres of 1/8192 and 1/4096) had lower Toxoplasma titres (l/256 and 1/1024, respectively).

The high seroprevalence to Toxoplasma indicates the widespread occurrence of the parasite in the food supply of the foxes studied. While antibody to *Neospora* appears to be less common, a number of animals had encountered the parasite with titres of 1/1024 to 1/8196 suggesting that these animals may have experienced recent infection. While the majority of the foxes will have become infected with *T gondii* by ingesting small mammals and birds persistently infected with Toxoplasma tissue cysts, the source of *Neospora* can only be speculated upon until its full Iifecycle has been elucidated.

Neospora bradyzoites in tissue cysts have been shown to be resistant to HC1-pepsin solution, indicating that carnivorism may have a role to play in its lifecycle (Dubey and Lindsay 1996). While both parasites can exist as bradyzoites in tissue cysts and as tachyzoites, it is as yet unknown whether *Neospora* has a sexual Iifecycle in a definitive host range equivalent to members of the cat family in toxoplasmosis (Dubey and Lindsay 1993) or which species of intermediate host provides the source infection. If small vertebrates are involved, it is not certain whether the host is more restricted than for Toxoplasma, or whether infection occurs in a wide range of hosts but the incidence of infection is lower than for *T gondii*. Ingestion of bovine placenta infected with *N caninum* may also be a potential source of infection and, while in this tissue the parasite would mainly be present as tachyzoites, the experimental oral infection of cats and dogs with *Neospora* tachyzoites has induced seroconversion (Dubey and Lindsay 1996). Further epidemiological studies of fox and other wild animal sera will give an indication as to the likelihood of this and other sources of infection. At present it is not known if *N caninum* can persist in the environment for any length of time like *T gondii*, where contamination of animals' drinking water with oocysts can act as a source of infection. Nor is it known whether the vertical transmission of *N caninum* as described in dogs (Dubey and Lindsay 1993) can occur in foxes. The results of this study strongly suggest that *N caninum* does infect wild foxes in Belgium and that further research is required to define its extent and its significance.

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Table 1. *Reciprocal lgG IFAT titres to N caninum and T gondii in sera from 123 red foxes collected in Belgium of which IFAT titres of 1/128 or more were found in 98·4 per cent of foxes for T gondii and In 17 per cent for N caninum*

Reciprocal	*N caninum*	*T gondii*

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titre	n	percentage	n	percentage
<64	27	22·0	0	0·0
64	75	61·0	2	1·6
128	0	0	8	6·5
256	12	9·8	13	10·6
512	4	3.3	15	12·2
1024	2	1·6	39	31·7
2048	1	0·8	25	20·3
4096	1	0·8	17	13·8
8192	1	0-8	4	3.2

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