

Seroprevalence of *Toxoplasma gondii* and *Neospora caninum* and Evidence of Microsporidia Infections in Rural Red Foxes

T.M. Murphy¹, J. Walochnik², A. Hassl², J. Moriarty¹, J. Mooney¹, D. Toolan³, C. Sanchez-Miguel⁴, A. O'Loughlin⁵ and A. McAuliffe¹

¹Central Veterinary Laboratory, Backweston Campus, Celbridge, Co Kildare

²Department of Medical Parasitology, Medical University of Vienna, Austria

³Regional Veterinary Laboratory, Kilkenny, Ireland

⁴Regional Veterinary Laboratory, Cork, Ireland

⁵Regional Veterinary Laboratory, Limerick, Ireland

Abstract

Thoracic fluid from 206 and 220 foxes were examined for antibodies to *Toxoplasma gondii* and *Neospora caninum* using indirect immunofluorescent antibody tests (IFAT). A total 115 (56%) and six (3%) foxes had antibodies to *T. gondii* and *N. caninum* respectively. The brains from 148 foxes were examined for histological lesions and pathological changes suggestive of parasitic encephalitis were observed in 33 (22.3%). Two thirds of these foxes had antibodies to *T. gondii* and one fox had antibodies to both *T. gondii* and *N. caninum*. PCR assays for *T. gondii* and *N. caninum*, on DNA extracted from the brains with histological lesions, were negative. However, microsporidian DNA was amplified from the brains of two of these foxes. Sequencing these amplicons revealed 100% homology with *Encephalitozoon (Septata) intestinalis* in one fox and *Encephalitozoon cuniculi* in the second fox. This is the first report of *Encephalitozoon* infections in wildlife in Ireland.

Introduction

Foxes are top of the wildlife food chain and are considered sentinel animals for whatever disease agent or pollutant is present in the environment. Their wide ranging diet and scavenging feeding habits results in them being reservoirs for a large number of helminth and protozoan parasites, many of which are pathogenic to man and animals.

The apicomplexan protozoa *Toxoplasma gondii* and *Neospora caninum* have an indirect life cycle and utilise a wide range of carnivorous and herbivorous intermediate hosts. They are very important economic parasites in livestock production as they cause foetal mortality and abortion in sheep and cattle respectively. They can also produce life-threatening disease in humans especially immunosuppressed individuals (Hill and Dubey, 2002; Lobato et al., 2006). The prevalence of these parasites amongst foxes varies depending on the country (Jakubek et al., 2001; Hamilton et al., 2005; Wanha et al., 2005).

Microsporidia are obligate parasites with a direct life cycle, which in recent years have emerged as important opportunistic pathogens. Several genera and species including *Encephalitozoon cuniculi* and *Encephalitozoon intestinalis* have been described as causing disease in man. They have been shown to be zoonoses and there is evidence that lagomorphs, foxes and rodents are reservoirs of *E. cuniculi* in the wild (Wilson, 1979; Hersteinsson et al., 1992).

In Ireland the most recent study on parasites of foxes was limited to foxes in the suburbs of Dublin the capital city (Wolfe et al., 2001). The present study was undertaken to determine the levels of protozoan infection amongst rural foxes.

Materials and methods

Thoracic fluid (consisting of clotted blood and pleural fluid) and brain tissue were collected from 220 and 148 foxes respectively. The foxes were part of a larger survey of Trichinosis in wildlife and had been shot during an annual vermin kill in January and February 2003 (Rafter et al., 2005). The samples of thoracic fluid were centrifuged at 1000g and the supernatant, which was assumed to contain serum proteins, was stored at -20°C until screened for evidence of infection with *N. caninum* and *T. gondii*. They were tested for the presence of antibodies to *N. caninum* using a commercial canine indirect fluorescence antibody test (IFAT, VRMD Inc, USA). Positive and negative canine sera were supplied with the kit and used as per the manufacturer's instructions. Reagents for the the *T. gondii* IFAT included rabbit anti canine IgG (FC+Fab) FITC (Autogen Bioclear Ltd., UK) used at 1:100 dilution, canine positive (Biobest, UK) and negative sera diluted 1:100 and *Toxoplasma* antigen substrate slides (VMRD Inc, USA).

Only brains from 148 foxes were suitable for examination. One half of the brain was fixed in buffered formalin and processed by standard histological procedures to H & E slides. The remainder of the brain was stored at -80°C until required for DNA extraction.

DNA for the *T. gondii* and *N. caninum* PCR was obtained from those brains with histological evidence of parasitic encephalitis using commercial mini-prep extraction kits (Sigma, UK; Quigen, UK). The *N. caninum* PCR was carried out using the primers and procedures described by Yamage et al. (1996). The *T. gondii* PCR was performed according to the methodology outlined by Burg et al. (1989). Amplification for both PCR reactions was performed in a PT-200 Programmable Thermal Controller (MU h, USA). The

amplicons were analysed on 2.0% agarose and visualised after staining with ethidium bromide.

DNA for the microsporidia PCR was extracted from the brains with histological lesions using QiAmp DNA mini kit (Qiagen GmbH, Germany), The PCR reaction using primers Mic3U and Mic421U was carried out according to Kock et al. (1997). The amplified products were visualised by silver staining after polyacrylamide electrophoresis (Amersham Pharmacia, Austria) and sequenced by direct sequencing (ABI Prism BigDye sequencing kit, Applied Biosystems, Germany) and an automatic sequencer (310ABI PRISM, PE, Applied Biosystems, Germany). Sequences were obtained from both strands.

Results

Thoracic fluid was available for testing from foxes in 16 counties. The number of foxes tested in each county varied from two to 18. A titre of 1:100 was taken to indicate seroconversion to either parasite. On this basis the overall prevalence of *N. caninum* and *T. gondii* was 2.7% [95% confidence interval (CI): 1.1-6.1%] and 55.8% [95% CI: 51.8-65.2%] respectively. Antibodies to *N. caninum* were found in six foxes, two each from counties Tipperary and Wexford and one fox in each of the counties Westmeath and Wicklow. Four of these foxes were also seropositive to *T. gondii*. The prevalence of *T. gondii* infection varied from county to county and ranged from 18.7 to 88.9%. The counties with the highest prevalence included Kildare (88.9%, 95% CI: 54.2-99.4), Limerick (82.4%, 95% CI: 57.3-95.3), Louth (78.6%, 95% CI: 50.4-94.3%) and Clare (75.0%, 95% CI: 26.8-98.6%).

Pathological changes suggestive of mild parasitic encephalitis caused by protozoa were observed in 33 brains (22.3%, 95% C.I.: 16.0-30.0). These lesions ranged from focal to multifocal unilateral perivascular cuffing to foci of mononuclear cellular infiltration around an area of neurophil necrosis. In a few of the brains there was a diffuse infiltration of mononuclear cells in the cerebrum. Nearly two thirds of these foxes had antibodies to *T. gondii* and one fox had antibodies to both *T. gondii* and *N. caninum*. Thoracic fluid was available from 80 animals with no brain pathology and of these 55 (68.8%, 95% C.I. 57.4-78.4) had antibodies to *T. gondii*. One fox (1.3%, 95% C.I.: 0.1-7.7) was seropositive to *T. gondii* and *N. caninum* and the remaining 24 (30.0% 95% C.I.: 20.5-41.4) were negative for both parasites.

The PCR assays for *N. caninum* and *T. gondii* were negative in all 33 foxes tested. However, fragments of microsporidian rRNA gene were amplified from the brains of two foxes giving a 421 bp amplicon in fox no.1231 and a 419 bp product in fox no. 1268. Sequencing these amplicons revealed a 100% homology with *E. intestinalis* in fox no. 1231 and with *E. cuniculi* in fox no. 1268. In fox no. 1231 a focal area of gliosis was observed in the cerebrum and it was seronegative for antibodies to *T. gondii* and *N. caninum*. There were no significant histological changes in the brain of fox no. 1268 although it was seropositive for *T. gondii*.

Discussion

The presence of specific antibodies in wild carnivores is a sign that *T. gondii* and *N. caninum* are present in the environment and also emphasises the danger of infection to farm animals and humans. The results suggest that there is a high level of *T. gondii* circulating in rural Ireland and contaminating the diet of foxes. The prevalence of 56% reported here was comparable to a prevalence of 47% reported previously for a smaller cohort of 51 foxes caught in and around metropolitan Dublin (Wolfe et al., 2001). These results are slightly higher than Austrian, British and Swedish studies in which 20%, 35% and 38% respectively of foxes had antibodies to *T. gondii* (Jakubek et al., 2001; Hamilton et al., 2005; Wanha et al., 2005).

Three percent of foxes in this study had antibodies to *N. caninum*. Previous serological surveys in Ireland and the U.K., two countries with similar rural environment and animal husbandry practices, have recorded a seroprevalence of 1.4% and 0.9% respectively (Wolfe et al., 2001; Hamilton et al., 2005). The infection rate amongst foxes in continental Europe varied between zero in Austria and Sweden to 17% in Belgium (Buxton et al., 1997; Jakubek et al., 2005, Wanha et al., 2005). This study also continued the trend that there is a lower prevalence of antibodies to *N. caninum* in wild canids than to *T. gondii* (Buxton et al., 1997; Wolfe et al., 2001; Jakubek et al., 2005, Wanha et al., 2005).

It is often difficult to detect pathological changes induced by protozoa in larger animals because the number of parasitic cysts may be low and the size and weight of tissue that can be analysed by histology is small. Detection of circulating antibodies is more straightforward and appears to give a more accurate indication of infection and prevalence rates. In this study a limited number of brains was examined and over a third of the foxes had no significant histological changes in their brains but had antibodies to *T. gondii*. PCR analysis of the brains with lesions also failed to detect any *T. gondii* or *N. caninum* DNA.

This is the first account of *E. cuniculi* and *E. intestinalis* infection in wild mammals in Ireland. To our knowledge it is also the first identification of *E. intestinalis* in foxes in general. The importance of *Encephalitozoon* species in Veterinary Medicine appears to be increasing especially amongst animals reared in intensive production systems. At present the route and transmission of these parasites is uncertain and it is important that efforts continue to be made to identify domestic and wildlife reservoirs and sources of environmental contamination of these organisms.

References

- Burg, J.L., Grover, C.M., Pouletty, P., Boothroyd, J.C., 1989. Direct and sensitive detection of a pathogenic protozoan *Toxoplasma gondii*, by polymerase chain reaction. J. Clin. Microbiol. 27, 1787-1792.
- Buxton, D., Maley, S.W., Pastoret, P.P., Brochier, B., Innes, E.A., 1997. Examination of red foxes (*Vulpes vulpes*) from Belgium for antibody to *Neospora caninum* and *Toxoplasma gondii*. Vet. Rec. 141, 308-309.

- Hamilton, C.M., Gray, R., Wright, S.E., Gangaadharan, B., Laurenson, K., Innes, E.A., 2005. Prevalence of antibodies to *Toxoplasma gondii* and *Neospora caninum* in red foxes (*Vulpes vulpes*) from around the UK. *Vet. Parasitol.* 130, 169-173.
- Hersteinsson, P., Gunnarsson, E., Hjartardottir, S., Skirnisson, K., 1993. Prevalence of *Encephalitozoon cuniculi* antibodies in terrestrial mammals in Iceland. *J. Wildl. Dis.* 29, 341-344.
- Hill, D., Dubey, J.P., 2002. *Toxoplasma gondii*: transmission, diagnosis and prevention. *Clin. Microbiol. Infect.* 8, 634-640.
- Jabubek, E.B., Brojer, C., Regnersen, C., Uggla, A., Schares, G., Bjorkman, C., 2001. Seroprevalence of *Toxoplasma gondii* and *Neospora caninum* in Swedish red foxes (*Vulpes vulpes*). *Vet. Parasitol.* 102, 167-172.
- Kock, N.P., Petersen, H., Fenner, T., Sobottka, I., Schmetz, C., Deplazes, P., Pieniazek, N.J., Albrecht, H., Schottelius, J., 1997. Species-specific identification of Microsporidia in stool and intestinal biopsy specimens by polymerase chain reaction. *Eur. J. Clin. Microbiol. Infect. Dis.*, 16, 369-376.
- Lobate, Janaina, Silva, D.A.O., Mineo, T.W.P., Amaral, J.D.H.F., Segundo, G.R.S., Costa-Cruz, J.M., Ferreira, M.S., Borges, A.S., Mineo, J.R., 2006. Detection of immunoglobulin G antibodies to *Neospora caninum* in humans: high seropositivity rates in patients who are infected by human immunodeficiency virus or have neurological disorders. *Clin. Vaccine Immunol.* 13, 84-89.
- Rafter, P., Marucci, G., Brangan, P., Pozio, E., 2005. Rediscovery of *Trichinella spiralis* in red foxes (*Vulpes vulpes*) in Ireland after 30 years of oblivion. *J. Infect.* 50, 61-65.
- Wanha, K., Edelhofer, R., Gabler-Eduardo, C., Prosl, H., 2005. Prevalence of antibodies against *Neospora caninum* and *Toxoplasma gondii* in dogs and foxes in Austria. *Vet. Parasitol.* 128, 189-193.
- Wilson, J.M., 1979. *Encephalitozoon cuniculi* in wild European rabbits and a fox. *Res. Vet. Sci.*, 26, 114.
- Wolfe, A., Hogan, S., MacGuire, D., Fitzpatrick, C., Vaughan, L., Wall, D., Hayden, T.J., Mulcahy, G., 2001. Red foxes (*Vulpes vulpes*) in Ireland as hosts for parasites of potential zoonotic and veterinary significance. *Vet. Rec.*, 149, 759-763.
- Yamaga, M., Flechtner, O., Gottstein, B., 1996. *Neospora caninum*: specific oligonucleotide primers for the detection of brain "cyst" DNA of experimentally infected nude mice by the polymerase chain reaction (PCR), *J. Parasitol.*, 82, 272-279.