Experimental Parasitology 120 (2008) 255–260

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Neospora caninum: Detection in wild rabbits and investigation of co-infection with *Toxoplasma gondii* by PCR analysis

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ARTICLE INFO

Article history:
Received 9 May 2008
Received in revised form 22 July 2008
Accepted 23 July 2008
Available online 30 July 2008

Keywords:
Apicomlexa
Neospora caninum
Toxoplasma gondii
PCR
Co-infection
Rabbit
Wildlife
DNA, deoxyribonucleic acid
ELISA, enzyme linked immunosorbent assay
IFAT, indirect florescent test
PCR, polymerase chain reaction
SAG, surface antigen gene
TE, Tris–EDTA buffer

ABSTRACT

Neospora caninum is an important pathogen of cattle causing significant economic loss. There is much current interest in wild animal reservoirs for this parasite. The role of the rabbit in this is currently unknown. DNA samples from the brains of wild rabbits (*Oryctolagus cuniculus*) collected from the Malham area of the Yorkshire dales were investigated by species-specific PCR for the presence of *N. caninum* and *Toxoplasma gondii*. We found prevalences of *N. caninum* of 10.5% (6/57) and *T. gondii* of 68.4% (39/57) with 8.8% (5/57) co-infected. Strain typing of *T. gondii* positive rabbits revealed strain types I–III were present in this population. Investigation of tissue distribution determined *N. caninum* DNA was most often detected in the brain and heart, less often in the tongue and not in the liver. To our knowledge this is the first report of *N. caninum* detection in naturally infected wild rabbits.

1. Introduction

Neospora caninum is an obligate intracellular protozoan that causes abortion and economic loss in the cattle industry. There are still many aspects of the lifecycle of *N. caninum* that remain unknown. At present only the dog and coyote are proven definitive hosts (McAllister et al., 1998; Gondim et al., 2004) but the recent finding of oocysts in the faeces of the red fox suggests this may also be a definitive host (Wapenaar et al., 2006). There is currently much speculation about the role wildlife may play as a reservoir of infection (e.g. Gondim, 2006). Recent findings indicate that small mammals such as mice (*Mus domesticus* and *Mus musculus*), rats (*Rattus norvegicus*) and wood mice (*Apodemus sylvaticus*) may be natural intermediate hosts (Huang et al., 2004; Hughes et al., 2006; Jenkins et al., 2007; Ferroglio et al., 2007; Barratt et al., 2008). Investigation of risk factors associated with bovine neosporosis in Normandy (France) found the presence of rabbits and/or ducks were a risk factor for seropositivity in dairy cattle (Ould-Amrouche et al., 1999). To our knowledge there has been no investigation of the prevalence of *N. caninum* in wild rabbits in the UK, and only one previous serological study in rabbits in Spain (Almería et al., 2007). In this latter study no evidence of exposure to the parasite was detected in rabbits. The closely related parasite *Toxoplasma gondii* has been more extensively studied and has been found to be a naturally occurring infection of wild rabbits in Europe (e.g. Hejlicek and Literak, 1994; Almería et al., 2004; Figueroa-Castillo et al., 2006). The objectives of this study were to investigate the prevalence and co-infection of *N. caninum* and *T. gondii*, using PCR techniques, in a naturally infected population of wild rabbits from Northern England; the tissue distribution of *N. caninum* in rabbits and the range of strain types of *T. gondii* present in this sample of rabbits.

2. Materials and methods

2.1. Collection of samples and DNA extraction

Rabbits (*Oryctolagus cuniculus*) $n = 57$ were collected from within 2 km of Malham Tarn in the Yorkshire Dales over a three

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doi:10.1016/j.exppara.2008.07.011

year period. All rabbits were necropsied at Malham Tarn Field Centre and tissue was taken from five areas of the brain, one each from the fore, mid and hind cerebrum, one from the cerebellum and one from the corpora quadrigemina. In some rabbits ($n = 42$) samples were also collected from the heart, liver and tongue. In all cases samples were collected using clean, sterile instruments with great care being taken to exclude contamination either between samples or from the environment as described previously (Williams et al., 2005). Approximately 1 g of tissue was placed in a sterile tube containing lysis buffer, and transferred to the laboratory for DNA extraction. DNA was extracted as described by Williams et al. (2005) using a proteinase K lysis followed by phenol chloroform extraction and ethanol precipitation method. DNA was resuspended in 100 μl of TE buffer, pH 8.0 and stored at 4 °C.

2.2. PCR detection of Neospora caninum

Detection of *Neospora caninum* was carried out by a nested PCR amplification of the Nc5 region as described in Hughes et al. (2006). The Nc5 region has been shown to be highly specific to *N. caninum* and is clearly distinguishable from *T. gondii* (Kaufmann et al., 1996; Yamage et al., 1996). This method has been shown to be extremely sensitive when used for detection of *N. caninum* DNA in the presence of host DNA in naturally occurring infections (Hughes et al., 2006). All PCRs were repeated a minimum of three times at three different dilutions to ensure a comprehensive sampling of the test DNA. PCR reactions were conducted carefully to minimise contamination as previously described (Hughes et al., 2006). The PCR product from one positive example of each individual was extracted from the gel using the GENECLEAN 2 kit (Q-BIOgene) cloned using the TOPO TA cloning® kit (Invitrogen), sequenced (Lark technologies Inc.) to confirm the specificity of the PCR and also, based on minor sequence differences, to confirm that amplified products are not derived from a contamination source.

2.3. PCR detection of Toxoplasma gondii

Detection of *Toxoplasma gondii* was carried out using nested PCR amplification of the Surface Antigen Gene 1 (SAG1) (Savva et al., 1990) as modified by Morley et al. (2005). All samples were tested a minimum of 3 times at three different dilutions to ensure a comprehensive sampling of the test DNA. PCR reactions were conducted carefully to minimise contamination as described previously (Williams et al., 2005; Morley et al., 2005). An example PCR product was sequenced to confirm the correct amplification of the SAG1 gene (GenBank Accession No. EU700308).

All PCR negative samples underwent a tubulin PCR (Terry et al., 2001), targeting host DNA, to confirm the sample was suitable for PCR.

Table 1
Frequency of infection and co-infection of *Neospora caninum* and *Toxoplasma gondii* in the brain tissue of naturally infected rabbits

	Toxoplasma positive (%)	*Toxoplasma* negative (%)	Total (%)
Neospora positive (%)	5 (8.8%)	1 (1.8%)	6 (10.6%)
Neospora negative (%)	34 (59.6%)	17 (29.8%)	51 (89.4%)
Total (%)	39 (68.4%)	18 (31.6%)	57 (100%)

2.4. Strain typing of Toxoplasma gondii

All samples shown to be *Toxoplasma gondii* positive by SAG1 PCR were retested using a SAG3 PCR to determine the strain type. This nested PCR method allows the differentiation of the amplification product into one of three strain types by restriction enzyme digestion of the PCR product. SAG 3 PCR was carried out as described by Grigg et al. (2001) with minor modifications as described below. Each 50 μl reaction contained 5 μl of 10× supertaq buffer (HT technologies), 0.5 μl dNTP mix (100 mM), 25 pM each primer and 2.5 units Biotaq polymerase (Bioline) In the 1st round primers primers *SAG3*, F_{ext}, CAACTCTCACCATTCCACCC, R_{ext}, and GCGCGTTGTTAGACAAGACA, and in the 2nd round primers F_{int}, TTGTCGGGTGTTCACTCA, R_{int}, CACAAGGAGACCGAGAAGGA were used. One microlitre of 1st round product was used as template for the second round. Cycling conditions were identical for both rounds of PCR and were as follows: an initial denaturing step of 94 °C for 5 min was followed by 35 cycles of 94 °C for 40 s, 60 °C for 40 s, 72 °C for 60 s and a final extension step of 10 min at 72 °C. Samples were amplified on a Robocycler 96 (Stratagene). Amplification products (10 μl) were electrophoresed on an ethidium bromide stained 2% agarose gel and visualised on an Alpha imager 1220. Positive PCR reactions were further analysed by restriction enzyme digestion with NciI and AlwNI as follows, 13 μl PCR product, 1.5 ul buffer 4 (NEB) and 0.5 μl enzyme (NEB) were incubated at 37 °C for 4 h to overnight. Products were electrophoresed on a 2.5% agarose, ethidium bromide stained, gel visualized on an Alpha imager 1220.

3. Results

The prevalence of *Neospora caninum* infection by PCR analysis can be seen in Table 1. *N. caninum* DNA amplified successfully from brain tissue from 6 of the 57 rabbits tested giving a prevalence of 10.5%. To ensure that *Neospora* negativity was not due to a failure of PCR amplification, all samples were tested using a mammalian tubulin PCR reaction to ensure that successful amplification could be achieved—in all cases successful amplification was achieved (data not shown). The results of 1 *Neospora* PCR amplification experiment is shown in Fig. 1. Sequence analysis of cloned PCR products confirmed the amplification of the Nc5 region in

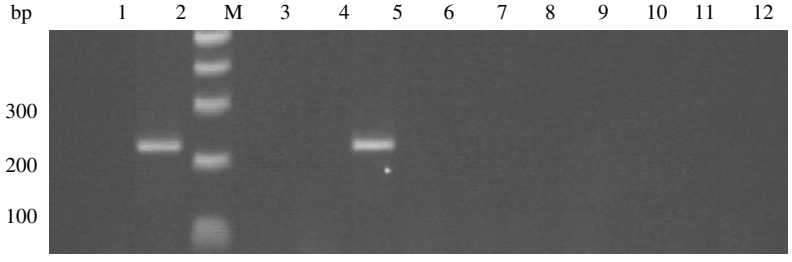

Fig. 1. Amplification of *N. caninum* DNA by nested PCR. (Lane 1) negative control, (lane 2) positive control, (lane M) 1 kbPlus marker (invitrogen) (lanes 3–12) 10 different rabbit brain samples. Note the positive PCR amplification in (lane 5).