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Acute Fatal Toxoplasmosis in Three Eurasian Red Squirrels (*Sciurus vulgaris*) Caused by Genotype II of *Toxoplasma gondii*

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ABSTRACT: *Toxoplasma gondii* parasites belonging to endemic genotype II caused fatal infection in three (16%) of 19 Eurasian red squirrels (*Sciurus vulgaris*) sent for necropsy in Finland between May 2006 and April 2009. The liver, spleen, and lungs were the organs most affected in all three cases, and high numbers of *T. gondii* parasites were visualized immunohistochemically in all the tissue samples available from them. The genotyping of the parasite strains was based on the results of analysis of length polymorphism at six microsatellite markers (B18, TUB2, TgM-A, W35, B17, and M33). The length of the PCR product at the additional seventh marker (M48) was 233 base pairs from the first two cases that were found dead together, suggesting a common infection source, and 215 base pairs from the third. Eurasian red squirrels may be exceptionally susceptible to *T. gondii* infection.

Key words: Genotyping, immunohistochemistry, proportional mortality rate, squirrel, toxoplasmosis, zoonosis.

Toxoplasma gondii is a zoonotic protozoan parasite that can infect Eurasian red squirrels (*Sciurus vulgaris*) and even kill them (Coles, 1914; Rodhain, 1950; Valtonen and Andersson, 1968; Duff et al., 2001). Eurasian red squirrels seem susceptible to the infection, even with parasites that show low virulence in other host species. The parasites isolated from three squirrel cases occurring in a zoo setting caused only chronic infections when bioassayed in mice, but proved fatal to a Eurasian red squirrel within 8 days of experimental inoculation (Rodhain, 1950). Low virulence in mice is typical of *T. gondii* belonging to genotype II that are endemic in Europe, including Finland (Maubon et al., 2008; Jokelainen et al., 2011). To identify the *T. gondii* genotypes that cause fatal infections in Eurasian red squirrels, we reinvestigated available archival cases diagnosed at Evira, the only wildlife pathology laboratory in Finland.

Of 19 Eurasian red squirrels examined postmortem between May 2006 and April 2009, three had died from generalized toxoplasmosis. In this material originating from the mainland of Finland, the proportional mortality rate was thus 16% (95% CI 4.2–37). Although the sample size is limited and this measure of disease incidence is subject to variation in the animals sent for examination, this result provides an impression of the relative importance of toxoplasmosis as a cause of death. Notably, the proportional mortality rate from toxoplasmosis appears higher in Eurasian red squirrels than in European brown hares (*Lepus europaeus*; 8.1%; difference not statistically significant) and mountain hares (*Lepus timidus*; 2.7%, $P<0.05$ Mid-P exact, $P=0.07$ Fisher exact) in Finland (Jokelainen et al., 2011).

The first two cases were found dead in December 2006 in a closet of a lakeside cottage located inland of southwestern Finland (61°29′N, 23°36′E). The weights of the squirrels were 327 and 238 g; their sexes were not recorded. The observations noted during routine necropsy of these squirrels were scarce. Multifocal reddish discoloration was detected in the lungs of both. The liver and the spleen of the larger squirrel had multifocal pale round spots 1 mm in diameter. Both squirrels had moderate flea infestations (*Monopsyllus sciurorum*). Bacteriologic cultures were negative for specific aerobic and microaerophilic bacteria, and fluorescent antibody tests were negative for *Francisella tularensis*. Tissue samples were fixed in 10% neutral buffered formalin, processed to paraffin-embedded tissue blocks,

sectioned, and stained with hematoxylin and eosin. Examination of histologic samples revealed multifocal necrotic inflammatory lesions with *T. gondii*–like protozoa in the liver and the spleen.

The third case, a juvenile female Eurasian red squirrel, was found dead in November 2007 near a garden feeder that it had been seen visiting regularly. The feeder was in the backyard of a home in a sparsely populated area inland of southwestern Finland (61°57′N, 23°30′E). Necropsy findings included a normal nutritional state and a weight of 305 g; marked pulmonary congestion; an excess of clear, blood-tinged serous fluid in the thoracic cavity; an uneven, yellowish color of the liver; and an uneven color of the spleen. The samples for bacteriologic testing were negative for specific aerobic bacterial infections. The main histopathologic findings were multifocal necrosis in the liver and the spleen, lymphoplasmacytic cellular infiltrations in the liver and lungs, and numerous *T. gondii* organisms in the liver, spleen, and lungs.

The diagnoses were retrospectively confirmed and the distribution of the parasites in tissues was evaluated by immunohistochemical staining (IHC) of sections of the formalin-fixed, paraffin-embedded tissue samples from the three squirrels. *Toxoplasma gondii* epitope-specific rabbit antibody (Thermo Fisher Scientific, Runcorn, UK) was the primary antibody for the automated IHC staining protocol (Jokelainen et al., 2011). As controls, we used sections without the primary antibody, known *T. gondii*–negative tissues from a Eurasian red squirrel that had died from head trauma, and positive tissues from a cat (*Felis catus*) that had died from generalized toxoplasmosis.

With the aid of IHC, *T. gondii* parasites were identified in all the tissues available from the squirrels: liver, lung, cardiac muscle, cerebrum, and cerebellum from the first two cases, as well as spleen from one of them; and liver (Fig. 1), spleen, lymph node, pancreas, intra-abdominal fat tissue, cardiac muscle, duodenum, kidney, and haired skin from the third. The liver tissue was rich in parasites in all three cases, which is consistent with earlier descriptions of toxoplasmosis in Eurasian red squirrels that emphasize the liver as the organ with the greatest number of parasites (Coles, 1914; Rodhain, 1950).

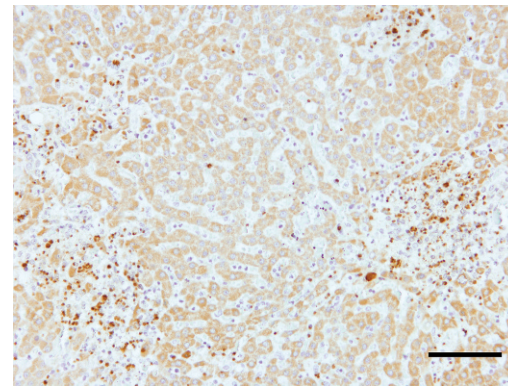

FIGURE 1. Numerous *T. gondii* organisms and multifocal necrotic lesions in the liver tissue of a Eurasian red squirrel (*Sciurus vulgaris*) in Finland. Immunohistochemical staining. Bar = 100 μm.

Since the parasites were abundant in the tissues of the three squirrels, we extracted DNA from their formalin-fixed, paraffin-embedded tissue samples and performed a direct genetic multilocus characterization of *T. gondii* (Jokelainen et al., 2011). In addition to six microsatellite markers (B18, TUB2, TgM-A, W35, B17, and M33) for genotyping, we used a seventh marker (M48) for further characterization (Blackston et al., 2001; Ajzenberg et al., 2005). DNA extracted from laboratory water, *Neospora caninum* strain NC1 (provided by Witold Stefański Institute of Parasitology, Warsaw, Poland), and the formalin-fixed paraffin-embedded tissues of the squirrel that had died from head trauma were included as negative controls. DNA from six *T. gondii* parasite strains served as positive controls and references: RH (genotype I; provided by the National Veterinary Institute, Uppsala, Sweden), and GIL (I), PSP-2007-TON (II), PRU (II), TOU-2004-FEU (III), and

NED (III; provided by Toxoplasma Biological Resource Centre, Limoges, France).

The analysis of length polymorphism was successful from DNA extracted from all three squirrels: from the liver tissue, a mixture of available tissues, or both. The results at the six genotyping markers were fully consistent with *T. gondii* genotype II; identical with the results obtained from the two genotype II reference strains. The length of the PCR product at the seventh marker (M48) was 233 base pairs from the first two cases, and 215 base pairs from the third. This result suggests that the first two cases, found dead together in the same place, may have had a common infection source.

The *T. gondii* parasites that caused the deaths of these three Eurasian red squirrels belonged to endemic genotype II. This genotype is typically nonvirulent in mice (Maubon et al., 2008). Our findings thus support the hypothesis that Eurasian red squirrels are exceptionally susceptible to *T. gondii* infections, even when caused by parasites of low virulence in some other host species. Our sample size is limited and previous reports on toxoplasmosis in Eurasian red squirrels are scarce; more studies are needed to understand this host–parasite interaction, especially with a focus on not only acute, fatal infections, but also chronic, latent infections.

In Finland, *T. gondii* belonging to genotype II has been identified as the cause of fatal infections in European brown hares and mountain hares (Jokelainen et al., 2011). Most hare cases, like these three squirrels, originated from southwestern Finland. Interestingly, 79% of the brown hare cases were diagnosed during the coldest winter months (November–February), and these three squirrels died in November and December. In this material, three of the five (60%) Eurasian red squirrels examined during the coldest winter months (November–February), but none of 14 (0.0%) necropsied in other months, had died from toxoplasmosis ($P<0.05$). Both of these host species thus often encounter the parasite and die from the infection during the harsh winters; the northern European winter conditions do not appear to hamper the lifecycle of *T. gondii*.

Omnivorous squirrels encounter *T. gondii* by ingesting food or water contaminated with oocysts or by eating the tissues of infected animals. The species-typical behavior of hiding food and drinking from several water sources, as well as living in the urban environment close to domestic cats, may increase the risk of infection from oocysts excreted into the environment by the definitive hosts, Felids. These three squirrels died from naturally acquired, acute, generalized toxoplasmosis and can be considered victims in the epidemiologic picture of *T. gondii*. However, as prey for other hosts, squirrels might also serve as an infection source.

Toxoplasma gondii is an important parasite of domestic animals, wildlife, and humans. Carcasses of wild animals harboring *T. gondii* may constitute a risk for humans skinning them (McDonald et al., 1990); as many as 10,200 Eurasian red squirrels are hunted for their fur in Finland annually (Finnish Game and Fisheries Research Institute, 2010). Humans could reduce the risk of squirrels encountering this parasite; for example, garden feeders for squirrels could be built in a way that minimizes the risk of contamination with cat feces.

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