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SEROLOGIC SURVEY FOR *TOXOPLASMA GONDII* INFECTION IN THE BROWN HARE (*LEPUS EUROPAEUS* P.) IN SWEDEN

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ABSTRACT: Serum samples from 176 brown hares (*Lepus europaeus* P.) shot in three areas of south and central Sweden during 1984 and 1985 were analyzed for antibodies to the protozoan parasite *Toxoplasma gondii* using several serological assays. No toxoplasma antibodies were detected in any of the sera, either by direct agglutination test or by ELISA, and selected sera were also negative in the IFAT and the Sabin Feldman dye test. Based on the high incidence of acute fatal toxoplasmosis in hares in Scandinavia, we propose that the brown hare is exceptionally susceptible to primary *T. gondii* infection.

Key words: *Toxoplasma gondii*, antibody, hare, ELISA, agglutination test, immunofluorescence test, dye test.

INTRODUCTION

The protozoan parasite *Toxoplasma gondii* is prevalent throughout the world. Felines are the only known definitive hosts, but most mammals and birds are potential intermediate hosts. *Toxoplasma gondii* infections usually are subclinical or give rise to only mild clinical signs in either the definitive or the intermediate host species. However, after a primary infection an animal will often harbor the parasite in a latent state in muscles and nervous tissues for long periods, sometimes for life. Such subclinically infected animals usually have circulating antibodies that can be demonstrated by different serological assays (Dubey and Beattie, 1988; Uggla and Buxton, 1990).

Among brown hares (*Lepus europaeus* P.), *T. gondii* infection often results in an acute and fatal disease (Hülphers et al., 1947; Gustafsson et al., 1988) quite unlike its effects in most other species. In Sweden the incidence of acute toxoplasmosis among free-living brown hares examined postmortem has been reported at 12% by Borg (1961) and 10% by Gustafsson et al. (1988). This high incidence raised the question of whether affected hares suffered from a primary infection with *T. gondii*, or from an activated subclinical infection. Our objective was to estimate prevalence of antibodies to *T. gondii* in brown hares in Sweden.

MATERIALS AND METHODS

Between August 1984 and November 1985, 176 apparently healthy brown hares were shot in three agricultural areas of south and central Sweden: Frötuna (59°54′N, 17°51′E), Sätuna (60°04′N, 17°35′E), and the island of Ven (55°54′N, 12°42′E). Domesticated as well as stray cats were prevalent on all sampling sites. During this period three to five hares were shot approximately once each month in each area. A blood sample was taken from the heart of each animal and the serum was stored at −20 C until analyzed. All hares were necropsied and tissues preserved in neutral buffered formalin for further possible histological studies.

An Ouchterlony immunodiffusion test and an adsorption test were performed to assess adequacy of using conjugated anti-rabbit immunoglobulin (Ig) preparations in indirect assays of enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescent antibody test (IFAT).

All samples were analyzed for *T. gondii* antibodies by a direct agglutination test (DAT) and by the ELISA. Forty sera also were tested with the IFAT and 23 with the Sabin and Feldman (1948) dye test (DT).

For the Ouchterlony technique, an agar gel consisting of 1% agarose (Difco Laboratories, Detroit, Michigan, USA) in 0.1 M tris hydroxymethyl aminomethane acetic acid buffer, pH 8.0 (Tris) (National Veterinary Institute, Uppsala, Sweden), with 2 mM ethylenediamine tetra-acetic acid (EDTA) was molded in a glass petri dish. After setting, seven 3 mm diameter

wells were punched in the gel as described by Hudson and Hay (1989). The central well was filled with 15 μl of sheep anti-rabbit IgG (Dakopatts, Copenhagen, Denmark), three of the surrounding wells were filled with 15 μl of undiluted laboratory rabbit sera and the other three with 15 μl of brown hare sera. The plates were incubated in a humid chamber at 20 C for 48 hr before being examined.

For the adsorption test, sheep anti-rabbit IgG preparation (Dakopatts) was mixed with undiluted hare serum, and with hare serum diluted 1:10 with phosphate buffered saline, pH 7.2 (PBS). The preparations were kept at 4 C overnight and tested in the ELISA the following day.

For the direct agglutination test, sera were screened at a dilution of 1:40 for antibodies against *T. gondii*, using U-shaped microtiter plates and commercial reagents from bioMérieux (Charbonnières-les-Bains, France). A 25 μl sample of serum diluted 1:20 in PBS was added to each well, followed by 25 μl of 0.2 M 2-mercaptoethanol diluted in PBS and 50 μl of formalin-treated *T. gondii* antigen diluted 1:5 in PBS. This kit included positive and negative control goat sera, and antigen was checked for spontaneous agglutination in wells in which PBS was substituted for test serum. Reagents were mixed and incubated at 20 C, and readings were taken after 5 and 18 hr.

A result was considered positive when *T. gondii* organisms were agglutinated in a mat covering at least half the well's base. Sera which gave borderline or positive reactions were further tested in serial three-fold dilutions of 1:60, 1:180, 1:540 and 1:1,620.

The ELISA was performed as described by Uggla et al. (1990), with the following modifications. Twenty of the sera were diluted 1:20 and all the sera were diluted 1:100. After the first incubation and repeated washings, 100 μl of a horseradish peroxidase (HRP) conjugated sheep anti-rabbit IgG preparation (Dakopatts), diluted 1:1,000, was added. The second incubation and further washings were followed by the introduction of 100 μl of the enzyme substrate 0.006% hydrogen peroxide and 0.10 mg/ml tetramethylbenzidine (Merck, Darmstadt, Germany) to each well and left to react for 1 hr at 37 C, after which the reaction was stopped by adding 50 μl of 2 M sulphuric acid. The optical densities (OD) of the samples were measured at 450 nm, using a titertec Multiscan spectrophotometer (Flow Laboratories, Irvine, Scotland). Samples with an OD value exceeding 0.1 were considered positive.

Forty hare sera selected at random were diluted 1:20 in PBS and examined in a *T. gondii* IFAT as described by Uggla and Hjort (1984). The antigen consisted of whole *T. gondii* tachyzoites dried onto microscope slides (bioMérieux) and the conjugate was a fluorescein-labelled sheep anti-rabbit IgG preparation (Dakopatts) diluted 1:600 in PBS containing a 1:10,000 dilution of Evans blue (bioMérieux). Buffered glycerine (Hudson and Hay, 1989) was added as a mounting medium before the slides were read in a BHT Olympus fluorescence microscope (Olympus Corporation, Tokyo, Japan). An unbroken peripheral line of fluorescence around the majority of the organisms was taken to indicate a positive reaction.

Twenty-three brown hare sera selected at random were analyzed by the dye test according to Sabin and Feldman (1948).

For controls, tissue fluid preparations were obtained from two brown hares that died of acute toxoplasmosis confirmed immunohistochemically (Gustafsson et al., 1988). Approximately 0.5 cm^3 of intact lung tissue was held in 2 ml of PBS overnight at 4 C. After centrifugation, supernatants were stored at −20 C until they were used as positive controls in each batch of serological assays of ELISA and DAT. In addition, serum from one specific pathogen-free rabbit and serum from one rabbit experimentally infected with *T. gondii* were included as negative and positive controls, respectively, in each group of different tests employed.

RESULTS

The precipitation lines formed in the Ouchterlony test gels fused smoothly together, thus the IgG antigens from both rabbits and brown hares reacted identically with the sheep anti-rabbit IgG antibodies. After the sheep anti-rabbit IgG preparation had been mixed with undiluted hare serum or with hare serum diluted 1:10 in PBS the absorbance values in the ELISA were reduced by 23% and 40%, respectively, in comparison with unadsorbed sera.

In the ELISA, none of the brown hare sera diluted 1:100 showed absorbance values exceeding 0.1 (mean ± SD = 0.01 ± 0.01). In contrast, undiluted tissue fluid preparations from two hares that died of acute toxoplasmosis had absorbance values between 0.55 and 0.85. These controls also reacted positively in the DAT, both giving a titer of 1:1,620. None of the hare sera examined by the DAT, IFAT or Sabin Feldman dye test showed positive reactions.

DISCUSSION

Antibodies to *T. gondii* were not detected in any of the 176 brown hares shot in three agricultural areas of south and central Sweden. The results were consistent in all the tests employed despite that antigen preparations used in the four serological assays were different. Live *T. gondii* tachyzoites were used in the Sabin Feldman dye test, and intact formalin fixed tachyzoites in the direct agglutination test and the IFAT. These assays primarily detect antibodies directed against the parasite's cell surface. Freeze-thawed and sonicated tachyzoites used in the ELISA also react to cytoplasmic antigens, some of which may be less specific (Uggla and Buxton, 1990). However, all sera gave uniform results in the DAT, a method of high specificity, and in the ELISA, a method of high sensitivity. Antigenic similarity and cross-reactivity of rabbit and hare immunoglobulins, essential in the ELISA and IFAT used, were clearly demonstrated by Ouchterlony diffusion and adsorbtion tests. Furthermore, using the ELISA and DAT, we demonstrated significant levels of *T. gondii* antibodies in tissue fluid preparations from hares with acute toxoplasmosis. We conclude therefore that negative samples did not contain antibodies against *T. gondii*.

There is not always a correlation between *T. gondii* antibodies and the demonstrable parasites in a host, and different results have been reported in different host species (Work, 1967; Hagiwara, 1977). No report of this relationship has been found in brown hares. However, antibodies to *T. gondii* were demonstrated in tissue fluid preparations obtained from two hares with acute toxoplasmosis diagnosed immunohistochemically. Parasites also can be readily isolated by mouse inoculation (Rasín 1970a; Dubey and Beattie, 1988) from such cases (Gustafsson and Uggla, unpubl.).

Previous studies on antibodies to *T. gondii* in brown hares using different serological assays indicate a very low prevalence in Australia, Austria and the Netherlands (Polman, 1959; Munday, 1972; Werner et al., 1973; Kutzer et al., 1976; Cox, 1981). Poli et al. (1987) reported a prevalence of only 6.4% using an indirect hemagglutination test. Yet, high prevalences (23 to 45%) were found in Romania (Elias, 1966) and in Czechoslovakia (Havlik and Hübner, 1958; Rasín, 1970b; Catar, 1972). However, relevant controls were not always used in these studies, and in the Romanian survey species of rabbits (*Oryctolagus* sp.) and hares (*Lepus* sp.) were not distinguished.

In countries like Sweden and Denmark where acute fatal toxoplasmosis is common among brown hares, the disease is widespread (Christiansen and Siim, 1951; Gustafsson et al., 1988). Provided that the infection is dispersed evenly throughout the population and that it gives rise to a persistent antibody response, we estimate a prevalence of $<2\%$ within 95% confidence limits (Cannon and Roe, 1982).

From our results the prevalence of subclinical *T. gondii* infection among brown hares in Sweden was remarkably low, as found in other countries where relevant controls have been applied to the serological assays used. Based on the low prevalence of latent *T. gondii* infection, meat from clinically healthy hares may be unimportant as a potential source of human toxoplasmosis. In view of the high incidence of acute fatal toxoplasmosis observed in brown hares in Sweden (Gustafsson et al., 1988), we believe that brown hares may be exceptionally susceptible to primary *T. gondii* infection.

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