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Reactivated and clinical *Toxoplasma gondii* infection in young lambs: Clinical, serological and pathological evidences

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ABSTRACT

In this study, fatal toxoplasmosis with severe neurological clinical signs was evaluated in 20 lambs of a sheep flock containing 90 Akkaraman sheep, 60 lambs and 3 rams. The clinical signs in infected lambs ($n = 20$) included incoordination, head shaking, tremors, shaking up, difficulty walking and subsequent death. No incidence of abortion observed in pregnant ewes. Two lambs with severe clinical signs were euthanized and necropsied following clinical and hematological examinations. Blood samples were also collected from the mothers of dead lambs and rams for Sabin-Feldman dye test to detect *Toxoplasma gondii*-specific antibodies. *T. gondii* infection was further confirmed by PCR analysis using *T. gondii* B1 gene specific primers. The histopathological findings included non-purulent myositis with *T. gondii*-like tissue cysts in the heart and skeletal musculature, severe necrotic vasculitis and multifocal necroses in the brain, liver and lungs. *T. gondii* immunoreactivity was present in the lungs, liver and spleen as well as tissue cyst-like structures. In differential diagnosis, *Neospora caninum* infection was excluded by immunohistochemical and PCR analyses. According to current literature, there has been no previous report on clinical toxoplasmosis in newborn lambs or goat kids, and the number of reports on clinical toxoplasmosis in small ruminants is limited to two adult goats with fatality following systemic toxoplasmosis. Our study indicated that toxoplasmosis occurs in lambs with severe neurological signs and subsequent death.

1. Introduction

Toxoplasma gondii is a pathogenic coccidian protozoon of *Apicomplexa* phylum that causes systemic and fatal infections in mammalian species (Radostits et al., 2000; Jubb et al., 2007; Dubey, 2009, 2010). *T. gondii* seropositivity rates in sheep flocks are very high, especially in European countries including Spain, Italy, France and England (Dubey, 2009). In sheep toxoplasmosis, the only clinical finding is abortion (Dubey, 2009, 2010). According to current literature, there has been no previous report on clinical toxoplasmosis in newborn lambs or goat kids, and the number of reports on clinical toxoplasmosis in small ruminants is limited to two adult goats with fatality following systemic toxoplasmosis (Mehdi et al., 1983; Radostits et al., 2000). Newborn lambs delivered by an experimentally infected ewes exhibited rare clinical signs and died within a month of their lives (Buxton et al., 1982). In contrast, some researchers indicated that toxoplasmosis could be a

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serious problem in newborn lambs as the fatality rate can incline to higher numbers especially in individuals with suppressed immune system (Buxton et al., 2007; Dubey, 2009, 2010). Stress factors including over-crowding, cold weather, immunosuppressive antibiotics and diet containing mycotoxins can reactivate subclinical *T. gondii* infection in lambs (Dubey, 2009, 2010). Toxoid vaccines have commonly been used to protect newborn animals against toxo-infectious diseases such as enterotoxemia, botulism, blackleg, tetanus, etc., which contain protein and antigenic fractions of the toxins (Radostits et al., 2000). However, commercial toxoid vaccines may still contain potent toxin traces that can initiate cytokine cascades and proinflammatory stress in the newborn animals (Radostits et al., 2000; Chandran et al., 2010). The use of new generations of recombinant toxoid vaccines in veterinary clinical practice is still under investigation (Chandran et al., 2010).

Sheep are mainly infected by the intake of feed and water contaminated with *T. gondii* oocysts from the surrounding environment. Thus, horizontal transmission is the main route for sheep toxoplasmosis (Buxton et al., 2006, 2007; Dubey, 2010). It has also been evidenced that the transplacental passage rate of *T. gondii* is higher than previously thought. There have been numerous field observations in Charolais and Suffolk sheep flocks confirming that toxoplasma infected newborns increase the prevalence (Duncanson et al., 2001; Morley et al., 2005; Williams et al., 2005). A study by Duncanson et al. (2001) reported that the transplacental passage rate in toxoplasmosis was 61% based on the PCR analyses. Morley et al. (2005) also reported that *T. gondii* infection rate among clinically healthy newborn lambs was 46.4%.

In this report, we aimed to describe the fatal outcome of clinical reactivated *T. gondii* infection in young lambs by means of clinical, pathological and PCR analyses and to discuss possible epidemiological and pathogenetical mechanisms of systemic toxoplasmosis in lambs.

2. Materials and methods

2.1. Animals and history

This study was conducted in a sheep flock in Province of Kirikkale, Turkey. The flock contained 90 Akkaraman sheep, 60 lambs and 3 rams. According to the history given by the animal owner and attending veterinarian, 3 rams were allowed for natural mating with the breeder 90 sheep. At the end of the subsequent gestation period, the delivered newborn lambs ($n = 60$) were vaccinated at the age of 20 days with a commercial toxoid vaccine against *Clostridium perfringens* Type A, B, D, *Clostridium septicum*, *Clostridium novyi*, *Clostridium tetani* toxins and *Clostridium chauvoei* (Coglavax, Ceva Animal Health). During the study period and following 2 years no incidence of abortion was observed. According to the history, 18 vaccinated newborn lambs died within 2 months of age following severe neurological signs including difficulty standing up, incoordination, ataxia, head shaking and tremors. At that time, none of the dead lambs was forwarded to a laboratory for etiologic diagnosis. In a later visit, we observed that two vaccinated lambs, a 25-day-old (lamb 1) and a 40-day-old (lamb 2), were exhibiting neurological clinical signs (Video file). These lambs were euthanized by administration of intravenous thiopental sodium (Pentothal, Abbott) at a dose of 1000 mg and tissue samples were collected systematically and processed for further analyses.

2.2. Blood samples

Blood samples were collected from the mothers of dead lambs ($n = 13$, five ewes delivered twin lambs), 2 euthanatized lambs and 3 rams into silicon and EDTA containing vacuumed blood collection tubes. Anti-*T. gondii* antibodies were screened in serum samples using the Sabin-Feldman dye test (SFDT), in which live tachyzoites and methylene-blue staining were applied at the Laboratory of Parasitology, the Refik Saydam National Institute of Hygiene, Ankara. Using a light microscope, the SFDT data were recorded as positive if more than 50% of tachyzoites remained unstained. To eliminate the crossreactivity and false positive results, only 1/16 and higher titres were accepted as positive. Serum ALT, AST and ALP were analyzed spectrophotometrically (Shimadzu UV-1208).

2.3. Necropsy and histopathology

Tissue samples collected from the brain, lungs, liver, heart, skeletal muscles, mediastinal and mesenteric lymph nodes, kidneys, spleen and intestines were fixed in 10% buffered formalin for 48 h. Trimmed tissues were washed under running water for 12 h, dehydrated and embedded in paraffin wax. 5 μm thick sections were cut on a rotary microtome, stained with hematoxylin and eosin, and evaluated under an Olympus BX-51 light microscope. Digital photomicrographs were taken using an Olympus DP 25 digital camera.

2.4. Immunohistochemistry

Indirect streptavidin/biotin immunoperoxidase technique was used to demonstrate *T. gondii* antigens in tissue sections (Haziroglu et al., 2003). Endogenous peroxidase activity was quenched by incubating the slides with 3% hydrogen peroxide (H_2O_2) in absolute methanol for 15 min. The tissues were digested with 0.1% protease K for 10 min at 37 °C. Sections were rinsed in phosphate-buffered saline (pH 7.4) twice for 5 min. For protein blocking, sections were incubated in 2% normal goat serum for 5 min. Tissue sections were then incubated in a commercial polyclonal goat anti-*T. gondii* primary antibody (210-70-TOXO, VMRD Inc, Pulman, WA) at a dilution of 1:300 for 50 min in a humidity chamber. Sections were treated with biotin conjugated anti-goat IgG secondary antibody (Abcam, MA) for 15 min and then incubated in the streptavidin-peroxidase enzyme for 15 min at room temperature. Finally, aminoethyl carbasole (AEC) substrate chromogen solution (Zymed, USA) was applied for 5–10 min coloring reactions. Upon counterstaining with Mayer's hematoxylin for 1–2 min, sections then were dehydrated, and mounted with an aqueous mounting medium. For positive control, a brain tissue section of a chronically *T. gondii* infected mouse, which was containing *T. gondii* ME49 tissue cysts was used. For negative control, the primary antibody step was omitted and PBS was used instead. *Neospora caninum* immunoperoxidase test protocol was performed according to Kul et al. (2009). Briefly, a commercial *N. caninum* antibody (Pab-NC, VMRD Inc, Pulman, WA) was used at a dilution of 1:2000 and heart section of a calf with neosporosis was used as positive control tissue.

2.5. Nested-polymerase chain reaction

Tissue samples collected from the brain, skeletal muscles and heart were kept at −20 °C until tested. For genomic DNA isolation; 5 g of each frozen tissues were homogenized in a high speed tissue homogenizer (Omni-Th2). For elimination of cross contamination, disposable homogenizer blade and sterile 100 ml glass beaker were used for each sample. Following the kit protocol, genomic DNA was isolated using a semi-automated nucleic acid isolation system "Fujifilm QuickGene80™" and Fujifilm DNA Tissue Kit S. A nested-PCR protocol, previously described by Bowie et al. (1997) was applied to detect *T. gondii* B1 gene in tissue samples. In the first step; 194 bp part of B1 gene of *T. gondii* (Accession number, AF179871) was amplified using Toxo 1 for: 5′-GGAACTGCATCCGTTCATGAG-3′ and Toxo 2 rev: 5′-TCTTTAAAGCGTTCGTGGTC-3′. In the second step, a specific 97 bp part of the *T. gondii* B1 gene was targeted in previously amplified template DNA using Toxo 3 for: 5′-TGCATAGGTTGCCAGTCACTG-3′ and Toxo 4 rev: 5′-GGCGACCAATCTGCGAATACACC-3′ primers. At the first step of PCR, 25 μl PCR mixture containing 150 ng targeted DNA, 2 mM $MgCl_2$, 10× reaction solution (50 mM KCl, 10 mM Tris–HCl [pH 8.3], 0.1% Triton X-100), 10 pmol of each primer, 200 μM of each dNTP and 2 U of *Taq* DNA polymerase. The PCR conditions were as follows: following the first denaturation at 95 °C for 5 min, denaturation of 30 s at 95 °C for 35 cycles, annealing at 57 °C for 30 s, elongation at 72 °C for 60 s and the final elongation for 7 min at 72 °C. At the second step of the PCR, using 2 μl of the PCR product the PCR mixture was prepared at above ratios. The second stage PCR conditions were