Acute toxoplasmosis in three wild arctic foxes (*Alopex lagopus*) from Svalbard; one with co-infections of *Salmonella* Enteritidis PT1 and *Yersinia pseudotuberculosis* serotype 2b

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Abstract

Acute disseminated toxoplasmosis was diagnosed in three wild arctic foxes (*Alopex lagopus*) that were found dead in the same locality on Svalbard (Norway). The animals included one adult female and two 4-months-old pups. The adult fox was severely jaundiced. Necropsy revealed multifocal, acute, necrotizing hepatitis, acute interstitial pneumonia, and scattered foci of brain gliosis, often associated with *Toxoplasma* tachyzoites. One pup also had *Toxoplasma*-associated meningitis. In addition, the latter animal was infected with *Yersinia pseudotuberculosis* serotype 2b and *Salmonella* Enteritidis phage type 1 (PT1), which may have contributed to the severity of the *Toxoplasma* infection in this animal. The diagnosis of toxoplasmosis was confirmed by positive immunohistochemistry and detection of anti-*Toxoplasma gondii* antibodies in serum of all foxes. The animals were negative for *Neospora caninum*, canine distemper virus, canine adenovirus, and rabies virus on immunolabelling of tissue sections and smears.

Keywords: *Alopex lagopus*; *Toxoplasma gondii*; *Salmonella* Enteritidis PT1; *Yersinia pseudotuberculosis* serotype 2b; Hepatitis; Pneumonia; Meningitis; Encephalitis

1. Introduction

Apart from rabies (Prestrud et al., 1992), little is known about mortality from infectious disease in wild arctic foxes (*Alopex lagopus*). The arctic fox has a circumpolar distribution, and inhabits the tundra and most Arctic islands, including the high Arctic archipelago of Svalbard, Norway, where it is the only terrestrial carnivore. In most of its range, the population shows large fluctuations caused by, and synchronised with, the 3–5 year cyclical fluctuations in microtine rodent numbers (Macpherson, 1969). In areas where small rodents are absent, such as most areas of Svalbard, the fox population appears to be more stable (Prestrud, 1992), although local fluctuations may occur (Fuglei et al., 2003).

Annual mortality in the arctic foxes is high, especially in young animals, and Prestrud (1992) estimated that...
only 26% survive their first year of life on Svalbard. Rabies is enzootic in arctic foxes on Svalbard, but contributes little to their mortality (Prestrud et al., 1992). Svalbard foxes have few predators, and starvation during the harsh Polar winter may be the main cause of death (Frafjord, 1992), but the factors regulating arctic fox numbers are not well understood.

In this report we describe three arctic foxes found dead on Svalbard, suffering from acute disseminated toxoplasmosis. In addition, one of the animals was infected with *Salmonella Enteritidis* PT1 and *Yersinia pseudotuberculosis* serotype 2b. To our knowledge, these infections have not been previously reported in wild arctic foxes.

2. Materials and methods

2.1. Animals and geographic area

In August and September 2000 three arctic foxes were found dead in Bjørndalen (78°10’N, 15°20’E), Svalbard, Norway, 6–7 km west of the main Norwegian settlement, Longyearbyen.

They were a 1–2 year old female (Case 1) and two pups, a male (Case 2) and a female (Case 3), both about 4 months old. The pup carcasses were found about 200 m below an arctic fox den. The age of the adult fox was estimated from its teeth. It had full adult dentition with little wear, and the uterus showed no sign of previous pregnancy. The pups still had a partly juvenile tooth set with both deciduous and permanent canines in the maxilla. The carcasses were kept frozen until necropsies were performed in 2002.

2.2. Histology

Samples of heart (Cases 1 and 3 only), brain, lung, liver, spleen and kidney tissue were fixed in neutral buffered 10% formalin, and embedded in paraffin wax, processed routinely, sectioned and stained with haematoxylin and eosin (H&E). Selected sections of liver, lung, heart and kidney were stained with van Gieson, Prussian blue, periodic acid–Schiff (PAS) and modified Gram’s staining technique.

2.3. Immunohistology

2.3.1. Rabies virus detection

Smears of brain tissue from the medulla oblongata, the hippocampus and the cerebral hemispheres of both sides were air dried, fixed in acetone and subjected to a direct immunofluorescence method (Meslin et al., 1996). FITC-labelled rabies nucleocapside-specific antibody was from Sanofi Diagnostics Pasteur (Marne-La-Coquette, France).

2.3.2. Detection of canine distemper virus, canine adenovirus, *Neospora caninum* and *Toxoplasma gondii*

Formalin-fixed paraffin-embedded tissues were sectioned at 4 μm and mounted on poly-lysine coated microscope slides. Wax was removed from the sections with xylene and they were rehydrated through a graded ethanol series. To demask the antigen and inhibit non-specific binding, the sections for canine distemper virus (CDV) and *Toxoplasma gondii* labelling were placed in 0.1 M citrate buffer, pH 6.0, and boiled in a microwave oven for 2 × 5 min; then cooled to room temperature and incubated for 20 min with 30% skimmed milk powder, 3% bovine serum albumin (BSA), 0.1% sodium azide, 0.3% Tween 20 (Sigma Chemical Co, St. Louis, MO, USA) and 500 IE/ml heparin (Novo Industry, Copenhagen, Denmark) in distilled water. Sections for canine adenovirus labelling were treated with 1 mg/ml trypsin solution for 90 min at 37 °C and blocked with 5% BSA in 0.05 M Tris-buffered saline (TBS), pH 7.6, for 20 min. Sections for *Neospora caninum* labelling were incubated with 0.05% phenylhydrazine (Sigma, P6766) in phosphate buffered saline (PBS) for 40 min to inhibit endogenous peroxidase activity and blocked with 5% BSA in TBS for 20 min. After antigen retrieval and blocking procedures the sections were incubated at room temperature with the following antibodies (diluted in 2.5% BSA/TBS): (1) mouse anti-CDV (Biodesign International, Kennebunk, ME, USA), diluted 1:100, 30 min incubation, (2) mouse anti-canine adenovirus (HCC) (Biodesign), diluted 1:500, overnight incubation, (3) rabbit anti-*N. caninum* (Veterinary Medical Research and Development Inc., Pullman, WA, USA), diluted 1:4000, overnight incubation, and (4) rabbit anti-*T. gondii* (DAKO A/S, Glostrup, Denmark), diluted 1:800, 30 min incubation.

Biotinylated species-specific antibodies (all from DAKO) and streptavidin conjugated with either alkaline phosphatase (Amersham International, Little Chalfont Buckinghamshire, England; sections labelled for CDV, canine adenovirus, and *T. gondii*) or peroxidase (Boehringer Mannheim, Germany; sections labelled for *N. caninum*) were used to reveal positive immunolabelling. Alkaline phosphatase activity was detected using Fast red (Sigma), whereas peroxidase activity was detected using 3-amino-9-ethyl-carbazole (Sigma). Sections were counterstained with haematoxylin, and mounted in an aqueous mounting medium. Micrographs were taken in a Zeiss Axioskop photomicroscope equipped with a Nikon Coolpix 4500 digital camera.

Immunohistochemical examination was performed on the following organs; CDV, Case 1: brain, liver, spleen; Case 2: brain, lung, liver, spleen; Case 3: brain, lung, liver; *Canine adenovirus*; Cases 1 and 2: liver, spleen, and Case 3: liver, lung; *N. caninum*; Case 1: brain, liver, Case 2: brain, lung, liver, spleen, and Case 3: brain, lung, liver; *T. gondii*, Case 1: brain, lung,
liver, kidney, and Cases 2 and 3: brain, lung, liver, spleen.

Fixed, paraffin embedded tissue from a hare (Lepus europaes) with acute toxoplasmosis, and three dogs infected with canine adenovirus, CDV, or N. caninum, respectively, served as positive controls. Sections from the same tissue blocks stained with irrelevant mouse monoclonal IgG or nonimmune rabbit serum served as negative controls.

2.4. Serology

Haemolysed blood from the right ventricle was centrifuged (1000g, 15 min) and the serum stored at −20 °C until testing. The samples were assayed for IgG antibodies to T. gondii by a commercial direct agglutination test (DAT; Toxo-Screen, BioMérieux, Lyon, France) as recommended by the manufacturer. The sera were tested at dilutions 1:40 and 1:4000; then retested in serial dilutions in 3-fold steps beginning at 1:60. Titer 1:40 for T. gondii antibodies were considered positive (Jakubek et al., 2001).

The serum samples were also assayed for anti-Encephalitozoon cuniculi antibodies by a carbon immunoassay (CIA; Medicago, Uppsala, Sweden), as described (Åkerstedt, 2002). The CIA occasionally induces spontaneous agglutination of sera at lower dilutions, giving false positive reactions. From our experience, dilution 1:25 of hemolysed sera may cause spontaneous agglutination at a rather high rate, and the dilution 1:50 was therefore used.

2.5. Microbiology

In all cases, samples of lung, liver, spleen and kidney tissues were taken at necropsy, and plated on 5% bovine blood agar and bromthymole blue lactose sucrose agar (Stuve et al., 1992). Cultures were incubated at 37 °C under aerobic and anaerobic conditions, and examined daily for growth. Colonies were sub-cultured for purity, and identified to genus or species level. From Case 3, samples of caudal jejunum and mesenteric lymph node were analysed for Salmonella by plating on brilliant green agar after enrichment of the samples in tetraethionate broth (Difco, Becton–Dickinson and Co, MA, USA) at 42 °C.

3. Results

3.1. Macroscopic findings and histopathology

The carcasses were well preserved, with no sign of scavenging. Case 1 was in good condition for the season, with considerable subcutaneous and abdominal fat but had marked jaundice. Carcass weight without fur coat was 2.25 kg. Cases 2 and 3 were dehydrated and had no visible subcutaneous fat, but had small amounts of fat in the abdomen and epicardium; the carcass weights were 2.15 and 2.30 kg, respectively.

Grossly, the livers were yellow- (Case 1), to orange-brown (Cases 2 and 3), with multiple small pale spots seen throughout the parenchyma of Cases 2 and 3. In all animals, the stomach and intestine were almost empty. In Case 2, an intussusception was found in the caudal jejunum, with moderate congestion and oedema of the affected segment. The apposed serous membranes were only slightly adherent, suggesting that this was an agonal event. The colon contents of this animal were darkly bloodstained, with a loose and slimy consistency. The faecal consistency in Cases 1 and 3 was normal. In all animals, foam was present in the trachea and bronchi, and the lungs were glistening, congested, firm and non-collapsing. Case 1 had a dilated right ventricle and a persistent foramen ovale.

The liver sections in all cases showed multifocal to coalescing hepatic necrosis, without a clear zonal distribution (Fig. 1A). The necroses were acute, lytic and coagulative, with no fibrosis and few inflammatory cells. Toxoplasma tachyzoites were present both in the necrotic foci (Fig. 1B), and in adjacent hepatocytes and vessel walls. In Case 1, the hepatocytes in non-necrotic areas showed severe fatty degeneration, and there was stasis in the biliary canaliculi. In Case 2, hemosiderin-loaded Kupffer cells appeared in the periphery of the necrotic areas, as revealed by Prussian blue staining.

Focal necrosis was also seen in the spleen in Cases 2 and 3. In Case 1, however, autolysis was too pronounced in the spleen and lung for proper evaluation. Sections of lung tissue from Cases 2 and 3 showed increased numbers of leukocytes in the blood vessels and multiple foci of acute, interstitial inflammation (Fig. 1C) with fibrin exudation and foci of necrosis. Tachyzoites were seen in the necrotic foci and in alveolar walls and in macrophages throughout the lungs (Fig. 1D). Tachyzoites were also seen in heart tissue of Cases 1 and 3; heart tissue of Case 2 was not examined. Brain sections from all cases showed scattered foci of gliosis, some with co-localizing Toxoplasma tachyzoites (Fig. 1G), but a few groups of protozoa were not associated with inflammation. In addition to gliosis, Case 2 had focal areas of acute meningitis with numerous tachyzoites associated with the inflamed tissue (Fig. 1E and F). A few colonies of gram-negative short- to medium-sized rod-shaped bacteria were seen in lung, liver, and spleen sections of Case 2 only.

The protozoa seen in the present cases did not stain with either PAS or modified Gram’s stain and they were found either as single cells, or in small groups (Fig. 1B and D-G). Some larger groups or cyst-like structures with 30–50 parasites were seen, but few were larger than 20 μm in diameter. Single cells
Fig. 1. Histopathological lesions of liver, lung and brain. (A) Liver, Case 2, showing focal necroses (N). H&E stain. Scale bar: 100 μm. (B) Liver, Case 2, higher magnification showing *Toxoplasma* tachyzoites (arrows) in necrotic lesions. H&E stain. Scale bar 20 μm. (C) Lung, Case 2, illustrating focal interstitial pneumonia. H&E stain. Scale bar: 200 μm. (D) Lung, Case 2, higher magnification showing tachyzoites (arrows) in the wall and lumen of alveoli. H&E stain. Scale bar 20 μm. (E) Brain, Case 2, illustrating meningeal inflammation with large numbers of tachyzoites (small dark dots). H&E stain. Scale bar 100 μm. (F) Brain, Case 2, higher magnification showing numerous tachyzoites (arrows) in the meninges. H&E stain. Scale bar: 20 μm. (G) Brain, Case 3, showing positive immunolabelling for *Toxoplasma gondii* (red stained; arrows). Streptavidin–biotin complex method, haematoxylin counterstain. Scale bar: 10 μm.
predominated in the tissues of Cases 2 and 3, whereas small groups were more frequent in Case 1. In many of the protozoan groups or cyst-like structures, the nuclei seemed to be situated toward the centre of the cells. However, the exact intracellular location of the nuclei could be difficult to determine because of autolysis and freezing artefacts. Toxoplasma tissue cysts are PAS-positive and have bradyzoites with terminal nuclei, whereas tachyzoites have a more centrally located nucleus and few or no PAS-positive granules (Dubey et al., 1998). Because the protozoa in the groups or cyst-like structures were PAS-negative, and often had centrally located nuclei, we concluded that they were predominantly tachyzoites.

3.2. Immunohistology

Direct immunofluorescence for rabies virus antigen in brain tissue was negative.

Immunohistochemical labelling for T. gondii resulted in positive staining of the parasites (Fig. 1G) in sections of all the organs examined, except the kidney of case 1. Immunolabelling for CDV, N. caninum, and canine adenovirus was negative.

3.3. Serology

Anti-T. gondii antibodies were detected in all serum samples (Case 1, 1:14,580; Case 2, 1:60; Case 3, 1:180). Serological tests for antibodies to E. canisculi were negative (titres < 1:50).

3.4. Microbiology

Bacteria were cultured from the lung, liver, kidney and spleen of all cases. From Case 1, β-haemolytic and non-haemolytic Staphylococcus strains were isolated from all tissues, and identified as Staphylococcus aureus using STAPH-ZYM® (Rosco, Taastrup, Denmark).

From Case 2, Y. pseudotuberculosis was grown from all tissues. In addition, Salmonella Enteritidis was recovered from liver and spleen, and Escherichia coli was recovered from lung. E. coli and Y. pseudotuberculosis were identified using the API 20E® system (Bio-Merieux). Salmonella Enteritidis was identified using Salmonella polyvalent and monovalent sera (Statens Serum Institut, Copenhagen, Denmark). The Yersinia strain from the spleen and the Salmonella strain from the liver were sent to the National Institute of Public Health, Oslo, and identified as Y. pseudotuberculosis serotype 2b and Salmonella Enteritidis PT1, respectively.

In Case 3, bacteriological examination showed growth of Pseudomonas spp. from lung, liver and kidney, whereas spleen was negative. Pseudomonas spp. was identified using the API 20NE® system (Bio-Merieux). The caudal jejunum and mesenteric lymph node from Case 3 were analysed for Salmonella by the enrichment procedure, with negative results.

4. Discussion

The three arctic foxes had lesions compatible with acute disseminated toxoplasmosis (Barker and Van Dreumel, 1985). Histology revealed multifocal to coalescing necrotizing hepatitis, interstitial pneumonia with scattered necrosis, and multifocal meningitis and/or gliosis, with numerous tachyzoites associated with the lesions. The diagnosis was confirmed by immunohistochemical labelling using specific T. gondii antibody, and positive serology.

Experimental studies in farmed foxes (A. lagopus) suggest that arctic foxes are highly susceptible to Toxoplasma infections (Bjerkås, 1990; Novinskaya, 1977). Bjerkås (1990) reported high mortality in litters born by vixens that were inoculated 10–16 days before delivery with the highly virulent T. gondii RH-strain and in pups that were inoculated 15 days postnatally with the same strain. The postnatally infected pups all died within 8 days, whereas the infected vixens appeared healthy. The affected animals developed focal necrosis in many organs, including severe lesions in the central nervous system. Natural infections in farmed blue foxes (A. lagopus) have also been reported (Novinskaya, 1977; Kopczewski et al., 2001), but the present case study is to our knowledge the first report of fatal toxoplasmosis in wild arctic foxes.

It is uncertain whether the cases represent a recently acquired or a relapsing Toxoplasma infection. However, tachyzoites, seen as single cells or in small groups, were numerous in the tissue sections, but there were few aggregations (cyst-like structures) larger than 20 μm, supporting the idea that the cases are primary infections. Since cysts form during the early phases of primary infections, their presence does not exclude an acute infection (Van Loon, 1989; Dubey, 1998), but the size is dependent on cyst age and the type of host cell parasitized (Dubey et al., 1998). Case 1 had a very high serum titre for anti-T. gondii antibodies, which may support a primary infection, although a high titre in a single serum sample does not have definitive diagnostic value. The low positive titres in Cases 2 and 3 can be explained either by death early in primary infection, or by reactivation of a latent infection (Van Loon, 1989), as well as suboptimal serum quality. Reactivation of latent infection is common in congenital Toxoplasma infections, and in immunodeficiency diseases (Bhopale, 2003). Primary Toxoplasma infections are usually asymptomatic in immunocompetent individuals, but may be fatal in animals with underlying immunological defects (Davidson et al., 1993). The immunosuppressive effect of canine distemper is well documented in dogs (Dungworth,
suggesting that it may predispose to toxoplasmosis in foxes (Møller and Nielsen, 1964; Reed and Turek, 1985). In the present study, however, immunohistochemistry for CDV, canine adenovirus, rabies virus and N. caninum were negative, and no predisposing factors were found in Cases 1 and 3. Death from acute *Toxoplasma* infection without known concurrent infection has been reported in a wild red fox (*Vulpes vulpes*) from Pennsylvania, USA (Dubey et al., 1990) and a grey fox (*Urocyon cinereoargenteus*) from Mississippi, USA (Dubey and Lin, 1994).

Case 2, however, had concurrent infections with *Y. pseudotuberculosis* serotype (O) 2b and *Salmonella Enteritidis* PT1, which may have contributed to the severity of the disease. It has been shown that virulent *Y. pseudotuberculosis* can cause immunosuppression in laboratory mice (Simonet and Berche, 1986; Sing et al., 2002). *Yersinia pseudotuberculosis* is widely distributed in cold regions, and is classified as serotypes O1 to O14, in which the serotypes O1 to O5 are almost all pathogenic to humans (Fukushima et al., 2001). The bacteria can infect many mammals and birds, with formation of characteristic caseonecrotic foci and pyogranulomatous lesions in viscera and lymphoid tissue (Valli, 1985). The most common mode of transmission is by food or water contaminated with faecal material, and by predation of carrier animals (Wanger, 1998).

Neither *Salmonella* spp. nor *Y. pseudotuberculosis* have hitherto been reported in the wild fauna or in domestic animals of Svalbard. However, *Salmonella Enteritidis* is one of the most frequently reported human *Salmonella* variants worldwide, and infection may be lethal (Rabsch et al., 2001).

Like *Y. pseudotuberculosis* and *Salmonella Enteritidis*, the source of *Toxoplasma* in the Svalbard fauna is unknown. *Toxoplasma* is a global pathogen that infects about one-third of the world’s human population (Grigg and Suzuki, 2003), and affects most species of warm-blooded animals, including birds (Dubey, 2002). The ingestion of food or water contaminated with oocysts from cat faeces or tissue cysts from undercooked meat are the major routes of postnatal transmission (Dubey, 1998). On Svalbard, cats occur in the Russian settlement of Barentsburg but the *Toxoplasma* status of these animals is unknown.

Sexual stages of *Toxoplasma* are only found in felid species, but *Toxoplasma* is also capable of indefinite asexual propagation in which predation or scavenging by intermediate hosts can effectively maintain the life cycle (Grigg and Suzuki, 2003). On Svalbard, arctic foxes are associated with all the main groups of birds and mammals, being a top predator and scavenger depending on both terrestrial and marine food webs (Prestrud, 1992; Frafjord, 1993, 2002). Important prey animals in summer are migratory seabirds, whereas reindeer carcasses and food remnants, such as seal carcasses, left by polar bears are important in winter. Cannibalism has also been observed (Prestrud, 1992; Frafjord, 1993, 2002). Svalbard has no native species of voles or lemmings, but there is a small population of introduced voles (*Microtus rossiaemeridionalis*), located in a restricted area of Grumant (78°10′N, 15°09′E), 7 km South-West of Bjørndalen, where the fox carcasses were recovered. The foxes were found in an area with bird cliffs housing at least 50,000 migrating seabirds during summer (SCRIB, 2003).

New infectious agents may also be introduced to Svalbard foxes via domestic dogs and humans, and from Northwest Russia and Greenland by the fox itself. Arctic foxes may travel more than 1000 km during seasonal migrations in search of food and also cross the pack ice (Pulliainen, 1965; Eberhardt and Hanson, 1978). As an example, one arctic fox, live trapped and ear tagged in Ny-Ålesund, Svalbard (78°55′N, 11°56′E), was shot on the island of Novaya Zemlya (about 75°N, 55°E; Henttonen et al., 2001), a direct line distance of about 1200 km.

In conclusion, acute disseminated *T. gondii* infection was identified in three wild arctic foxes found dead in the same geographic area of Svalbard. One of the animals was also infected with *Salmonella Enteritidis* PT1 and *Y. pseudotuberculosis* serotype 2b. None of these infections has been previously reported in the Svalbard fauna. The present information will be valuable for future studies on arctic foxes both on Svalbard, and throughout its circumpolar range.

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