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Cryptosporidium Infection in Domestic Geese (*Anser anser f. domestica*) Detected by In-situ Hybridization

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Summary

An in-situ hybridization (ISH) procedure was developed for the detection of *Cryptosporidium* sp. in paraffin wax-embedded tissues with a digoxigenin-labelled probe targeting the 18S rRNA. This technique was used in addition to traditional methods, such as haematoxylin and eosin staining, periodic acid-Schiff reaction, transmission electron microscopy and the polymerase chain reaction, to examine the bursa of Fabricius (BF), conjunctiva and other tissues from 20 domestic geese aged 16–36 days for the presence of cryptosporidia. Positive signals were found to a moderate or marked extent in both conjunctival samples (89%) and BF samples (88%) but not in other tissues. Sequencing of the PCR amplification product revealed identity with *Cryptosporidium baileyi*. The infected geese showed no clinical signs and only scanty histological lesions. These results confirm reports showing that young waterfowl are especially vulnerable to cryptosporidium infection and indicate that the BF and conjunctiva are the preferred sites for the presence of the protozoon. ISH proved a good method for detecting and identifying even small numbers of cryptosporidia in tissue sections.

Keywords: Cryptosporidium baileyi; goose; parasitic infection

Introduction

Cryptosporidia are protozoan parasites within the phylum Apicomplexa which develop in epithelial cells. They are pathogens of human beings (Ungar, 1990; Mead *et al.*, 1999; Joachim, 2004) and many other vertebrate species (Jervis *et al.*, 1966; Rehg *et al.*, 1979; Tzipori *et al.*, 1981; Snodgrass *et al.*, 1984; Bennet *et al.*, 1985; Quilez *et al.*, 1996; Fayer *et al.*, 1997), and are typically found in neonatal and young animals. Infection may be symptomless, but cryptosporidia have frequently been associated with clinical enteritis. First reports of cryptosporidia in avian species date back to 1929 (Tyzzer, 1929). Cryptosporidia have been reported in birds of different orders, such as Galliformes, Columbiformes, Passeriformes, Struthioniformes and Anseriformes, in which they may induce respiratory and intestinal diseases (Lindsay *et al.*, 1987; Lindsay and Blagburn, 1990; Goodwin and Davis, 1993; Sréter and Varga, 2000). Most studies on avian cryptosporidiosis have focused on chickens (Goodwin, 1988; Goodwin and Brown, 1988; Goodwin *et al.*, 1990; Rhee *et al.*, 1997). The first report of cryptosporidia in domestic geese described the parasites in the epithelial microvilli of the large intestine (Proctor and Kemp, 1974). Later, cryptosporidian oocysts were found in scrapings from the cloaca and bursa of Fabricius (BF) of a dead gosling (Palkovič and Pecka, 1989). Experimental inoculation of geese with oocysts of *Cryptosporidium baileyi* showed numerous endogenous stages in epithelial cells of the BF only (Current *et al.*, 1986). In addition to the BF, intestines and cloaca, cryptosporidia were found, albeit to a lesser extent, in tissue scrapings

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of the conjunctiva, sinus infraorbitalis, trachea and lung from naturally infected ducks and geese (Richter *et al.*, 1994).

Identification of cryptosporidia is usually based on the use of acid-fast stains (e.g. Kinyoun staining), transmission electron microscopy, indirect immunofluorescent antibody procedures, enzyme-linked immunosorbent assay (ELISA), and the polymerase chain reaction (PCR) (Current, 1990; Sréter and Varga, 2000).

This report describes the high prevalence of cryptosporidia in the epithelia of the conjunctiva and BF in young geese (*Anser anser f. domestica*), as shown by electron microscopy, the PCR (including subsequent sequencing of the amplification product) and in-situ hybridization (ISH), which was introduced as a reliable method for detecting various developmental stages of cryptosporidia in paraffin wax-embedded tissue samples.

Materials and Methods

Tissue Samples

At histological examination of paraffin wax-embedded tissues from an experimental study on the pathogenicity of Usutu virus (USUV) for geese, we frequently observed, as an incidental finding, cryptosporidia in the BF and conjunctiva of the experimental birds. It was therefore decided to evaluate this phenomenon in more detail. The tissue samples were from 20 domestic geese which were obtained from a local breeder at the age of 1 day and kept in isolation cabinets located in a biosafety level 2 facility thereafter. At 14 days of age 11 of the geese were infected with USUV and then observed daily for clinical signs of disease. Five uninoculated geese (in-contact controls) were placed in the same isolation cabinet. An additional four uninoculated geese were housed separately as a control group. The geese were humanely killed or died due to intercurrent bacterial infection at the age of 16, 17, 19, 24, 30 or 36 days. All geese were subjected to post-mortem examination and samples of brain, heart, liver, spleen, kidney, lung, intestine, eye, pancreas, BF and thymus were collected and fixed in 7% buffered formalin for histopathological examination. Usutu virus infection did not cause clinical disease in the experimental geese and viral antigen was not detectable immunohistochemically in any tissue. However, low-level viral replication was demonstrated by reverse transcriptase (RT)-PCR and there was evidence of transient viraemia and subsequent seroconversion in some of the experimental animals.

Histology and Periodic Acid-Schiff (PAS) Reaction

Tissue samples were embedded in paraffin wax, processed by routine methods to haematoxylin and eosin (HE)–stained tissue sections, and examined by light microscopy. The BF and eyes (with conjunctiva) were additionally stained with PAS.

ISH

This technique was used to identify *Cryptosporidium*-specific 18S ribosomal RNA (rRNA) sequences in paraffin wax-embedded tissues. After dewaxing and rehydration, the tissues were incubated in Tris-buffered saline with proteinase K (Roche, Basel, Switzerland) 2.5 µg/ml at 37 °C for 30 min. After rinsing, the slides were covered with hybridization mixture, 100 µl of which contained 50 µl formamide, 20 µl of 20×standard saline citrate buffer (SSC), 2 µl Denhardt's solution, 10 µl dextran sulphate (50% w/v), 5 µl boiled herring sperm DNA (50 mg/ml), 12 µl distilled water and 1 µl *Cryptosporidium*-specific digoxigenin labelled oligonucleotide probe (MWG Biotech, Ebersberg, Germany) with a final concentration of 25 ng/ml. The probe sequence was: 5′-GTGCTGAAGGAGTA-9#AGGAACAACCTCCAATCTCTAGTTGG-3′. This sequence is complementary to a segment of 18S rRNA of all *Cryptosporidium* species and exhibits sufficient nucleotide differences from other related protozoa of the phylum Apicomplexa. Thus this probe is highly specific for cryptosporidia and cross-hybridization to other protozoa is unlikely to occur. The slides were hybridized for 16 h in a humid chamber at 40 °C and then washed in 2× SSC, 1×SSC and 0.1×SSC (10 min each) at room temperature. For detection of digoxigenin-labelled hybrids, the slides were incubated for 60 min with anti-digoxigenin-AP Fab fragments (dilution 1 in 100) (Roche). The slides were rinsed twice and the signal was "visualized" with BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (4-nitro blue tetrazolium chloride) (Roche). After 30 min, the colour development was stopped with TE buffer (pH 8.0), the slides were counterstained with haemalum, dehydrated, and mounted under coverslips with Aquatex (VWR, Vienna, Austria).

Negative controls consisted of tissue sections containing protozoa of the genera *Eimeria* (in intestine and liver), *Sarcocystis, Toxoplasma, Histomonas, Trichomonas, Encephalitozoon, Entamoeba* and fungi of the genera *Candida* and *Aspergillus*.