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Occurrence of *Giardia* in Swedish Red Foxes (*Vulpes vulpes*)

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ABSTRACT: *Giardia duodenalis* is an intestinal protozoan capable of causing gastrointestinal disease in a range of vertebrate hosts. It is transmitted via the fecal-oral route. Understanding the epidemiology of *G. duodenalis* in animals is important, both for public health and for the health of the animals it infects. We investigated the occurrence of *G. duodenalis* in wild Swedish red foxes (*Vulpes vulpes*), with the aim of providing preliminary information on how this abundant predator might be involved in the transmission and epidemiology of *G. duodenalis*. Fecal samples (n=104) were analysed for *G. duodenalis* using a commercially available direct immunofluorescent antibody test. *Giardia duodenalis* cysts were found in 44% (46/104) of samples, with foxes excreting 100 to 140,500 cysts per gram of feces (mean, 4,930; median, 600). Molecular analysis, using PCR with sequencing of PCR amplicons, was performed on 14 samples, all containing over 2,000 cysts per gram feces. Amplification only occurred in four samples at the *tpi* gene, sequencing of which revealed assemblage B in all four samples. This study provides baseline information on the role of red foxes in the transmission dynamics of *G. duodenalis* in Sweden.

Key words: Anthropozoonotic, carnivore, disease, parasite, pathogen, protozoa, transmission, zoonotic.

Giardia duodenalis is an intestinal protozoan parasite capable of causing gastrointestinal disease in a range of vertebrate hosts. It is transmitted via the fecal-oral route. *Giardia duodenalis* annually causes clinical disease in over 200 million people in Asia, Africa, and Latin America, as well as being responsible for large-scale human waterborne outbreaks in developed countries, including in Scandinavia (Robertson et al. 2006; Feng and Xiao 2011). Of the eight different *G. duodenalis* assemblages (A–H), the two that are considered to have zoonotic potential (A and B) have been found to be infectious to a wide range of wildlife, including red foxes (*Vulpes vulpes*; Appelbee et al. 2005; Hamnes et al. 2007).

There is a lack of knowledge on the role of wildlife in transmission dynamics, and whether some wildlife species might be disease reservoirs for human infection with zoonotic *G. duodenalis* assemblages, or vice versa. In order to investigate the role of wildlife in the epidemiology of *G. duodenalis*, prevalence data in species living at the wildlife-human-domestic animal interface must be determined and isolates characterized at the molecular level.

The red fox has the widest geographical range of any member of the order Carnivora (Hoffmann and Sillero-Zubiri 2016). Being an opportunistic omnivore that is often found at high densities in urban areas, the red fox is exposed to a range of pathogens through its diet, and might act as a reservoir of pathogens between humans, domestic animals, and wildlife. Few studies have investigated the molecular epidemiology of *G. duodenalis* in European red fox populations (Hamnes et al. 2007; Beck et al. 2011; Onac et al. 2015), and in order to reduce the knowledge gap, we investigated the occurrence of *G. duodenalis* in wild Swedish red foxes.

Fecal samples (n=104), originating from 16 Swedish counties, were collected opportunistically by hunters between August and December 2014, as part of a national *Echinococcus multilocularis* survey. Samples were sent to the National Veterinary Institute in Sweden and frozen, unpreserved, at –80 C, then thawed and then refrozen at –20 C for 1 to 5 mo, prior to being transported to the Parasitology Department, Norwegian Univer-

sity of Life Sciences (NMBU) for analysis. At NMBU, samples were stored at 4 C.

Detection and quantification of *G. duodenalis* was by standard immunofluorescent antibody test with fluorescein isothiocyanate-labelled monoclonal antibody (Aqua-Glo, Waterborne Inc., New Orleans, Louisiana, USA) and 4′,6-diamidino-2-phenyl indole (DAPI) staining on 10 µL subsamples that had been prepared by resuspending 3 g of feces in water followed by sieving and centrifugation, before air-drying and methanol-fixing the subsamples to welled slides. The stained samples were examined by fluorescence microscopy (DM2700M, Leica Microsystems, Lysaker, Norway) at 200× and 400× magnification. Cysts were identified by standard fluorescence and morphological characteristics (US Environmental Protection Agency 2012). The total number of cysts on each slide were counted, and used to estimate the number of cysts per gram of feces. The theoretical limit of detection of this method is 100 cysts per gram (CPG) of feces.

Isolation of DNA was performed directly from the fecal pellet on 14 samples with over 2000 CPG using QIAmp DNA mini kit (Qiagen GmbH, Hilden, North Rhine-Westphalia, Germany). The protocol followed the manufacturer's instructions with slight modifications in the sample preparation: 0.1 g of fecal pellet was first mixed with 150 µL of tris-ethylene diaminetetraacetic acid buffer (100 mM tris and 100 mM ethylene diaminetetraacetic acid), incubated at 90 C for 1 h and an overnight proteinase K lysis step at 56 C before spin column purification. We eluted DNA in 30 µL of PCR-grade water, and stored it at 4 C.

Conventional PCR was performed on *G. duodenalis* positive samples at the small subunit ribosomal RNA, glutamate dehydrogenase (*gdh*), triosephosphate isomerase (*tpi*), and β-giardin (*bg*) genes (Hopkins et al. 1997; Sulaiman et al. 2003; Read et al. 2004; Lalle et al. 2005). In all cases, PCR consisted of 8.3 µL PCR water, 1 µL forward and 1 µL reverse primer (at a final concentration of 0.4 mM), 0.2 µL bovine serum albumin (20 mg/L), 12.5 µL of 2× HotStartTaqMaster (Qiagen GmbH), and 2 µL of template DNA. PCR products were visualized by electrophoresis on 2% agarose gel with Sybr Safe stain (Life Technologies, Carlsbad, California, USA).

Positive samples were purified using a High Pure PCR Product Purification Kit (Roche, Oslo, Norway) and sequenced in both directions (GATC Biotech, Konstanz, Germany). Sequences were analysed using the program Geneious™ (Geneious 2016).

Examination of red fox fecal samples using immunofluorescent microscopy revealed the presence of *G. duodenalis* cysts in 44% (46/104) of samples. Foxes excreted 100 to 140,500 CPG (mean, 4,930; median, 600). In general, low numbers of cysts were shed, with only two samples containing over 10,000 CPG. All cysts examined directly by immunofluorescent antibody test were negative by DAPI for the presence of nuclei.

Of the 14 *G. duodenalis*-positive samples selected for molecular characterization, no amplification of DNA by PCR was seen at the *gdh*, small subunit ribosomal RNA, or *bg* genes. Four samples were positive at the *tpi* gene. Sequencing of these PCR products revealed assemblage B in all four samples (accession nos. KY304077–KY304080). All sequences were identical except for two ambiguous nucleotides in one isolate and a single nucleotide polymorphism in another isolate. All samples had identical protein translations. Nucleotide sequences were compared using BLAST in GenBank (National Center for Biotechnology Information 2016) that revealed 100% of the consensus region (498 base pairs) to be identical to *Giardia* isolates from a variety of sources (e.g., rhesus macaque in China, water from the US, and a human sample from Malaysia).

This study describes a high prevalence of low-intensity infections of *G. duodenalis* in wild red foxes in Sweden, with only assemblage B identified. *Giardia duodenalis* infection has previously been reported from a range of Swedish animals (Lebbad et al. 2010). However, this is the first report in Swedish red foxes and suggests that they might be important players in *G. duodenalis* epidemiology in this country. Infection prev-

alence was higher than reported prevalences in red foxes elsewhere in Europe: 4.5% (3/66) in Croatia, 4.6% (10/217) in Romanian, 4.8% (13/269) in Norway, 7.3% (9/123) in Bosnia and Herzegovina, and 19% (4/21) in Poland (Hamnes et al. 2007; Beck et al. 2011; Hodžić et al. 2014; Onac et al. 2015; Stojecki et al. 2015). Similar infection rates have been seen in other wild canids (Trout et al. 2006; Oates et al. 2012). The high prevalence found in the Swedish population might be due to innate differences in this population (e.g., associated with diet, proximity to farming or domestic animals, water sources, human contact, population densities). The low intensity of cyst shedding observed is important to consider when assessing zoonotic potential, because these animals would be less likely to lead to environmental contamination than animals with large excretion rates or higher fecal outputs.

In this study, PCR had limited success, similar to other studies trying to characterize *G. duodenalis* isolates from other canids (Sommer et al. 2015; Stojecki et al. 2015). The lack of DNA observed within the *G. duodenalis* cysts could indicate that the DNA was degraded or located free within the feces matrix. If this is the case, then the processing steps designed to remove fecal debris might have resulted in the loss of this DNA, thus causing false negatives. However, direct PCR on the feces, with and without DNA fishing, was attempted on some samples and did not provide any further results.

Red foxes have previously been reported to be infected with *G. duodenalis* assemblages A and B (Hamnes et al. 2007; McCarthy et al. 2008; Beck et al. 2011; Onac et al. 2015), whereas only one study has reported the canine-specific assemblage D in two samples (Ng et al. 2011), and no reports of assemblage C. In our study, Swedish red foxes were found to be infected with assemblage B. In contrast, the vast majority of *Giardia* isolates from dogs are assemblages C and D (Feng and Xiao 2011), even in environments where assemblage B is considered to predominate (Lebbad et al. 2008). It is therefore intriguing that assemblage B is apparently common in red foxes, but rarely establishes in dogs, suggesting a considerably different host-parasite relationship between these two canids. Finding assemblage B in Swedish red foxes might indicate that they act as a disease reservoir for zoonotic *G. duodenalis*. However, care must be taken when interpreting the zoonotic potential of these isolates based on a single gene locus, especially when taxonomic grouping can vary based on which genes are used for comparison (Lebbad et al. 2010).

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