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Succinctus

Zoonotic transmission of *Cryptosporidium meleagridis* on an organic Swedish farm ☆

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ABSTRACT

We believe that we present the first evidence of zoonotic transmission of the bird parasite, *Cryptosporidium meleagridis*. Despite being the third most common cause of human cryptosporidiosis, an identified zoonotic source has not been reported to date. We found *Cryptosporidium* oocysts in pigs, sheep/goats, hens and broiler chickens on a farm with suspected zoonotic transmission. By DNA analysis we identified *C. meleagridis* in samples from one human, three chickens and one hen. Sequencing of the ssrRNA and 70 kDa Heat Shock Protein (HSP) genes showed identical *C. meleagridis* sequences in the human and chicken samples, which is evidence of zoonotic transmission. The HSP70 sequence was unique.

Cryptosporidium meleagridis was first described in turkeys (*Meleagris gallopavo*) in 1955 (Slavin, 1955). Since then other birds such as poultry, finches and pet birds have been identified as natural hosts (Ryan and Xiao, 2008). Although considered an avian *Cryptosporidium* spp. it seems to have adapted to a wide host range. Experimental infection has been successful in mice, rats, rabbits, calves, gnotobiotic pigs and voluntary humans (Akiyoshi et al., 2003; Darabus and Olariu, 2003; Chappell et al., 2011). To date it is the only *Cryptosporidium* spp. that is known to infect different vertebrate classes. It is the third most common species in human cryptosporidiosis and the prevalence varies from 0.9% to 20.6% in different studies, with lower prevalence in industrialized countries than in developing countries (Gatei et al., 2002; Cama et al., 2003, 2008; Leoni et al., 2006; Iqbal et al., 2011; Insulander et al., 2012). Natural infection has been reported in both immunocompromised and immunocompetent humans (Cama et al., 2003, 2008; Insulander et al., 2012). In some studies of HIV-infected patients, *C. meleagridis* prevalence is similar to that of *Cryptosporidium parvum* (Gatei et al., 2002; Cama et al., 2007). Clinical *C. meleagridis* infection in humans is similar to *Cryptosporidium hominis* or *C. parvum* infection and symptoms include acute onset of watery diarrhoea, abdominal pain, nausea, vomiting and fever (Chappell et al., 2011; Insulander et al., 2012). Human cryptosporidiosis is notifiable in Sweden but species determination is not routinely included in diagnosis. When looking in more detail at human cryptosporidiosis cases in Stockholm County during 2006–2008, *C. meleagridis* constituted 5.7% (11 of 194) of all *Cryptosporidium* cases with successful speciation (Insulander et al., 2012). All *C. meleagridis* infections were acquired during travel to Asia or Africa (Insulander et al., 2012). In the current paper we not only describe the first known indigenous human *C. meleagridis* infection in Sweden, but to our knowledge also the first case of human *C. meleagridis* infection where a zoonotic source has been identified.

Three farm employees became ill with diarrhoea within a few days of each other in June 2011. On 28 June one of them, a 20-year old male, sought medical help due to abdominal pain and non-bloody diarrhoea that had lasted for 14 days. During the first few days of illness, the patient suffered from fever (~38 °C), but nausea or vomiting had not been present. The other two persons had suffered from acute, self-limiting diarrhoea. None of them had travelled abroad during the 3 weeks prior to the outbreak. The patient stated that the farm had cattle, pigs, horses, sheep, goats and poultry (laying hens and broiler chickens). It was reported that the eggs were washed in the kitchen where the personnel prepared their lunch. The human stool sample was analyzed for *Campylobacter*, *Salmonella*, *Shigella* and *Yersinia*, which are included in routine diagnostic tests. The sample was also screened for protozoan cysts and oocysts, as well as worm eggs, by light microscopy of wet smears after formol-ethyl acetate concentration. The presence of *Cryptosporidium* oocysts was confirmed by modified Ziehl–Neelsen staining. No virus analysis was done. The patient was diagnosed with *Cryptosporidium* and *Campylobacter* infections. Both infections are notifiable in Sweden and the case was thus

☆ Nucleotide sequence data reported in this paper are available in GenBank under accession numbers JX024759-JX024762.

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reported to the Department of Communicable Disease Control and Prevention in Stockholm and to the Swedish Institute for Communicable Disease Control (SMI). Because zoonotic transmission was indicated by both the pathogens and the case history, an investigation of the farm animals was initiated to trace the infection source.

The farm was sampled on 7 July, 2011. It was registered as a 4H Club farm but was run as a regular organic animal and crop farm. 4H is an international organization for children and youth. Activities include camping, fishing, playing games and education about animals and nature. There was a café and a farm shop. The following animal species and numbers were present at the time of inspection: five adult horses, 35 Highland cattle cows with 20 pre-weaned calves, six 8 month old Angus calves, five adult sheep and three lambs, nine adult goats and two kids, 10 4.5 month old pigs, approximately 150 laying hens, a few roosters and approximately 100 broiler chickens aged 5 weeks. According to the farm manager, there were no health issues with the animals. However, when directly questioned about diarrhoea in the animals, he confirmed that the broiler chickens had diarrhoea. All employed personnel took care of all animals. There were no routines present to prevent the spread of infection between different groups of animals. The horses were kept in a large grazing field and there were no fresh faecal pats within visible range when entering the field, so horses were excluded from sampling. The Highland cattle herd grazed in a large 2 ha field and none of the staff had been in physical contact with them during the previous 2 months, so they were not considered a possible infection source and were excluded from sampling. The Angus calves were kept on deep litter in an open barn. The goats had arrived 10 days before sampling and were thus not a possible infection source. However, they were kept with the sheep in a large semi-forested paddock and were included in the sampling group. The pigs were kept in a tree-free paddock with dirt ground, a mud puddle and some grass. The laying hens were kept in a grass paddock with two wagons containing nests for resting and laying eggs. The chicken flock was kept in a small house with a grass paddock outside.

Faecal samples, as fresh as possible, were collected from the environment. In total, 27 samples were collected as follows: Angus calves ($n = 4$ from six pats), lamb ($n = 1$, rectal), sheep/goat ($n = 3$), pig ($n = 3$), hens ($n = 13$, nine from the wagons, four from outdoors), chickens ($n = 3$ from five pats). Animal faecal samples were cleaned and concentrated by a saturated sodium chloride flotation method and examined for *Cryptosporidium* oocysts by epifluorescence microscopy after staining with an fluorescein isothiocyanate (FITC)-conjugated anti-*Cryptosporidium* monoclonal antibody as previously described (Silverlås et al., 2009).

Oocyst DNA was extracted as described by Lebbad et al. (2008). Eluted samples were stored at −20 °C until further use. All samples positive for *Cryptosporidium* by microscopy were analysed with a nested PCR-restriction fragment length polymorphism (PCR-RFLP) ssrRNA protocol (Xiao et al., 2001) and a nested PCR protocol to amplify the complete 70 kDa heat shock protein (HSP70) gene (~1,950 bp) (Sulaiman et al., 2000). Samples that failed to amplify with the latter protocol were reanalysed, targeting a smaller part (~400 bp) of the HSP70 gene (Morgan et al., 2001). The human and chicken samples were also analysed by a PCR-RFLP protocol targeting the *Cryptosporidium* oocyst wall protein (COWP) gene (Spano et al., 1997). *Cryptosporidium meleagridis* samples ($n = 11$) isolated from humans in a previous study (Insulander et al., 2012) were analysed at the HSP70 locus for comparison. The 60 kDa glycoprotein (GP60) gene was targeted using a nested PCR protocol to produce ~800 bp amplicons (Chalmers et al., 2005) with additional primers described by Glaberman et al. (2001). All ssrRNA, HSP70 and GP60 amplicons were sequenced by standard Sanger sequencing and the resulting contigs were compared with sequences published in GenBank using BLAST (Basic Local Alignment Search Tool, NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi)).

Oocysts were identified in 16 of 27 animal samples and in all sampled animal groups except cattle (Table 1). The chicken samples contained high numbers of oocysts, whereas samples from pigs, laying hens and sheep/goats had moderate or few oocysts. By morphology, *C. meleagridis* was the species identified in chickens, and both *C. meleagridis* and *Cryptosporidium galli* were identified in hen samples. Only *C. parvum* -like oocysts were identified in pigs and sheep/goats (Table 1).

In the human case and the three chicken samples we could identify *C. meleagridis* with both the COWP-RFLP and ssrRNA-RFLP. In one of the hen samples *C. meleagridis* was also identified but only with the ssrRNA-RFLP. The poultry samples as well as the human case sample were identified as *C. meleagridis* ssrRNA genotype I (GenBank ID AF12574) (Table 1). The three pig samples were positive by ssrRNA PCR and identified as *Cryptosporidium* pig genotype II (GenBank ID DQ182600) (Table 1).

The human case and all three chicken samples were positive at the HSP70 locus using the full-length PCR protocol (Sulaiman et al., 2000). Only two out of the eight *Cryptosporidium*-positive hen samples were positive with the HSP70 protocol and then only with the protocol targeting a shorter fragment (Morgan et al., 2001). Nine *C. meleagridis* samples previously identified from humans (Insulander et al., 2012) were successfully amplified with the full-length HSP70 PCR and five were sequenced.

Thirteen *C. meleagridis* sequences for the full HSP70 gene, belonging to 10 genotypes, were present in GenBank. Most variation is found in the 3′ end of the gene, where there is a repetitive region of a 12 bp segment ($n = 7–10$) with single nucleotide polymorphisms (SNPs) at the third and sixth base of the segment (GG(T/C)GG(T/A)ATGCCA) (Fig. 1). Scattered SNPs can also be found closer to the 5′ end of the gene (Fig. 1). Genotype 6 was used as a consensus because this sequence has the largest number of repeats. The longest contigs produced were 1,923 bp, and chicken sequences were identical to the human case. Our isolate was not identical to any previously reported genotypes, and the human and chicken HSP70 sequences have been deposited in GenBank as genotype 11 (ID JX024759 and JX024760). There were nine repeats, and the sequence was most similar to genotype 5 (GenBank ID AF402283) with a non-synonymous SNP at bp 857 (from bp 1 in genotype 2 (Fig. 1)). This SNP causes a change from valine to isoleucine in the deduced amino acid (aa) sequence. The short HSP70 sequences in the two hens (308 bp) were identical to the corresponding part in human and chicken sequences. Of the five human *C. meleagridis* samples from Insulander et al. (2012) that were sequenced, three belonged to genotype 6 and two were novel genotypes. Isolate SMI030 (genotype 12, GenBank ID JX024761) was most similar to genotype 5 with an SNP at bp 1,690, and isolate SMI063 (genotype 13, GenBank ID JX024762) was most similar to genotype 3, with an SNP at bp 433 (Fig. 1). Both of these SNPs are silent mutations. Pig samples were analysed with the short fragment HSP70 protocol and a single sample was positive. The isolate (311 bp) was identical to *Cryptosporidium* pig isolate Eire V zz67 (GenBank ID DQ833282).

The GP60 protocol produced faint bands of approximately the correct size for the human and chicken samples but sequencing produced multiple peaks throughout the sequences and data could not be interpreted.

This is the first identified indigenous human *C. meleagridis* case in Sweden but for several reasons it is safe to assume that it might not be the first case to occur. Domestic *Cryptosporidium* cases are most likely under-diagnosed in Sweden because people who have acquired diarrhoea abroad are more prone to seek help compared with individuals infected at home. Also, parasitic investigations are more often requested in samples from travellers and less