Infection, Genetics and Evolution 92 (2021) 104895

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Cryptosporidium chipmunk genotype I – An emerging cause of human cryptosporidiosis in Sweden

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ARTICLE INFO

Keywords:
Cryptosporidiosis
Zoonotic transmission
Molecular characterisation
Epidemiology
Outbreak investigation

ABSTRACT

Most cases of cryptosporidiosis in humans are caused by *Cryptosporidium parvum* or *Cryptosporidium hominis*. However, more uncommon species are increasingly being recognised to cause infection in humans. Here we report that *Cryptosporidium* chipmunk genotype I, which has various rodents as its natural host, is the third most common source of human cryptosporidiosis in Sweden. We also describe the first small outbreak of cryptosporidiosis caused by *Cryptosporidium* chipmunk genotype I and report the first case of zoonotic transmission of *Cryptosporidium* chipmunk genotype I from a red squirrel to a human. *Cryptosporidium* chipmunk genotype I was identified in 20 human cases, including 16 sporadic cases, three outbreak-related cases, and one zoonotic case, as well as in two squirrel samples. *Gp60* subtyping which was successful for 19 human cases and two squirrel samples showed that all samples harboured the same subtype, XIVaA20G2T1. The work presented here suggests that red squirrel is a natural host of *Cryptosporidium* chipmunk genotype I and that infection with *Cryptosporidium* chipmunk genotype I is an emerging cause of domestic cryptosporidiosis in Sweden and a potential source of outbreaks.

1. Introduction

Cryptosporidium is a protozoan parasite that can cause disease in both humans and animals. The majority of human cryptosporidiosis cases are caused by either of two species, namely *Cryptosporidium hominis* and *Cryptosporidium parvum*. The latter has the broadest host range and infects several vertebrates, while *C. hominis* is mainly restricted to humans. *C. hominis* is the dominant species in developing countries, while both *C. parvum* and *C. hominis* are common in industrialised countries (Feng et al., 2018). These two species are responsible for the majority of all investigated outbreaks of cryptosporidiosis (Zahedi and Ryan, 2020). The distribution of less common *Cryptosporidium* species in humans also differs between industrialised and developing countries. *Cryptosporidium* chipmunk genotype I, *Cryptosporidium ubiquitum,* and *Cryptosporidium cuniculus* are more frequent in industrialised countries, while *Cryptosporidium meleagridis*, *Cryptosporidium felis*, *Cryptosporidium canis*, *Cryptosporidium viatorum,* and *Cryptosporidium muris* are more common in developing countries (Feng et al., 2018). In Sweden, *Cryptosporidium* infection has been a notifiable disease in humans since 2004, and in 2018 the Public Health Agency of Sweden initiated a microbiological surveillance programme using molecular typing in order to better understand the national epidemiology of domestic cases of cryptosporidiosis. Molecular typing has also been conducted within different studies and occasionally during outbreaks and source tracking. Most cases of *Cryptosporidium* acquired in Sweden are caused by *C. parvum* and to a lesser extent *C. hominis*, but less common species such as *Cryptosporidium* chipmunk genotype I also play a role in transmission. Between 2006 and 2017, eight sporadic cases of human infection with *Cryptosporidium* chipmunk genotype I were identified. The natural hosts of *Cryptosporidium* chipmunk genotype I are mainly squirrels, chipmunks, and deer mice (Guo et al., 2015).

The objective of the present study was to investigate the occurrence

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https://doi.org/10.1016/j.meegid.2021.104895
Received 10 February 2021; Received in revised form 3 May 2021; Accepted 4 May 2021
Available online 8 May 2021

and molecular epidemiology of *Cryptosporidium* chipmunk genotype I, the third most common source of human cryptosporidiosis in Sweden. We included a total of 20 *Cryptosporidium* chipmunk genotype I cases identified in 2018 and 2019. We investigated a small outbreak of *Cryptosporidium* chipmunk genotype I at a pre-school in Stockholm County as well as cases of suspected zoonotic *Cryptosporidium* transmission between animal caretakers and red squirrels at an animal rehabilitation centre.

2. Material and methods

2.1. Human samples

A total of 283 samples in 2018 and 599 samples in 2019 were sent to the Public Health Agency of Sweden as part of the microbiological surveillance programme or during outbreak investigations assessing human domestic *Cryptosporidium* cases. In total 21 human cases were included in the study; 16 sporadic cases of *Cryptosporidium* chipmunk genotype I detected during the microbiological surveillance programme, three *Cryptosporidium* chipmunk genotype I cases from a small outbreak investigation at a pre-school, and two cases (one *Cryptosporidium* chipmunk genotype I and one *C. parvum*) that were part of an investigation of zoonotic transmission at a small-animal rehabilitation centre (Table 1). The primary diagnosis had been done by light microscopy using modified Ziehl-Neelsen staining or multiplex real time PCR at local clinical microbiology laboratories. Each submitted *Cryptosporidium*-positive sample included information about the age, sex, and geographical location of the patient. Information concerning infections acquired abroad, routes of transmission, and when available, information about symptoms was retrieved from the national mandatory notifications system (SmiNet). Combined results of sample information, primary diagnosis, geographical location, and genetic characterisation are shown in Table 1.

Table 1
Patient information, primary diagnosis data, geographical location, and genetic characterisation of *Cryptosporidium* chipmunk genotype I and *C. parvum* samples.

Patient	Time of diagnosis	Material	Primary diagnosis	Geographical location (county)	ssu rRNA	*gp60*
Swec946/Swec947[a] 41-year-old man, animal caretaker	2018 Jul/Aug	Faeces	Microscopy and Real time PCR	Stockholm	*C. parvum*[b]	IIdA16G1b[d]
Swec950 44-year-old woman, partner of animal caretaker	2018 Aug	Faeces	Microscopy	Stockholm	*Cryptosporidium* chipmunk genotype I[c]	Negative
Swec1037 70-year-old man	2018 Sep	Faeces	Real time PCR	Uppsala	*Cryptosporidium* chipmunk genotype I[c]	XIVaA20G2T1[e]
Swec1047 3-year-old boy	2018 Sep	Faeces	Real time PCR	Uppsala	*Cryptosporidium* chipmunk genotype I[c]	XIVaA20G2T1[e]
Swec1076 2-year-old boy	2018 Oct	Faeces	No information	Södermanland	*Cryptosporidium* chipmunk genotype I[c]	XIVaA20G2T1[e]
Swec1078 67-year-old woman	2018 Oct	Faeces	No information	Skåne	*Cryptosporidium* chipmunk genotype I[c]	XIVaA20G2T1[e]
Swec1088 11-year-old boy	2018 Oct	Faeces	Microscopy and real time PCR	Kronoberg	*Cryptosporidium* chipmunk genotype I[c]	XIVaA20G2T1[e]
Swec1100 9-year-old boy	2018 Nov	DNA	Real time PCR	Dalarna	*Cryptosporidium* chipmunk genotype I[c]	XIVaA20G2T1[e]
Swec1109 47-year-old man	2018 Dec	DNA	Real time PCR	Stockholm	*Cryptosporidium* chipmunk genotype I[c]	XIVaA20G2T1[e]
Swec1345 15-year-old boy	2019 Sep	Faeces	Real time PCR	Uppsala	*Cryptosporidium* chipmunk genotype I[c]	XIVaA20G2T1[e]
Swec1347 48-year-old man	2019 Sep	Faeces	Real time PCR	Kronoberg	*Cryptosporidium* chipmunk genotype I[c]	XIVaA20G2T1[e]
Swec1348 15-year-old boy	2019 Sep	Faeces	Real time PCR	Kronoberg	*Cryptosporidium* chipmunk genotype I[c]	XIVaA20G2T1[e]
Swec1349 53-year-old woman	2019 Sep	Faeces	Real time PCR	Uppsala	*Cryptosporidium* chipmunk genotype I[c]	XIVaA20G2T1[e]
Swec1394 17-year-old woman	2019 Oct	Faeces	Real time PCR	Uppsala	*Cryptosporidium* chipmunk genotype I[c]	XIVaA20G2T1[e]
Swec1401 41-year-old man	2019 Oct	DNA	No information	Stockholm	*Cryptosporidium* chipmunk genotype I[c]	XIVaA20G2T1[e]
Swec1402 43-year-old woman	2019 Sep	DNA	Real time PCR	Stockholm	*Cryptosporidium* chipmunk genotype I[c]	XIVaA20G2T1[e]
Swec1403 33-year-old man. Pre-school outbreak	2019 Oct	DNA	Real time PCR	Stockholm	*Cryptosporidium* chipmunk genotype I[c]	XIVaA20G2T1[e]
Swec1412 12-year-old girl	2019 Sep	Faeces	Real time PCR	Kronoberg	*Cryptosporidium* chipmunk genotype I[c]	XIVaA20G2T1[e]
Swec1413 39-year-old woman. Pre-school outbreak	2019 Oct	DNA	Real time PCR	Stockholm	*Cryptosporidium* chipmunk genotype I[c]	XIVaA20G2T1[e]
Swec1414 5-year-old girl. Pre-school outbreak	2019 Oct	DNA	Real time PCR	Stockholm	*Cryptosporidium* chipmunk genotype I[c]	XIVaA20G2T1[e]
Swec1642 8-year-old girl	2019 Oct	DNA	No information	Stockholm	*Cryptosporidium* chipmunk genotype I[c]	XIVaA20G2T1[e]

[a] Two samples from the animal caretaker with collection date July and August 2018.
[b] 100% identical to GenBank accession number KU892559.
[c] 100% identical to GenBank accession number JX978272
[d] 100% identical to GenBank accession number JX183808
[e] 100% identical to GenBank accession number KU852739.

2.2. Molecular investigation of human samples

DNA from faecal samples was extracted using the magLEAD 12gC instrument supplied with magDEA DX MV reagents (Precision System Science Co Ltd., Chiba, Japan). All extractions were performed according to the manufacturer's instructions. Prior to extraction, oocyst disruption was accomplished by bead beating using a Bullet Blender (Techtum, Sweden). Identification of *Cryptosporidium* species was done by amplification of the small subunit rRNA (ssu rRNA) gene by PCR followed by bi-directional sequencing of the PCR amplicons (Xiao et al., 2001; Xiao et al., 1999). Subtyping of *Cryptosporidium* chipmunk genotype I isolates was done by amplification of the 60 kDa glycoprotein (*gp60*) gene followed by bi-directional sequencing of the PCR products using the primers published in Guo et al. (Guo et al., 2015) as well as a recently designed forward primer CrChigp60Gt1Fw3: 5′-GGA AAA ATG AGA TTA ACG CTT ATC-3′. All isolates were also investigated by conventional *gp60* PCR using primers described by Alves et al. (Alves et al., 2003). Editing and analysis of sequences was done using the BioEdit sequence alignment editor (version 7.0.9.0) and CLC Main Workbench (Qiagen, Aarhus, Denmark, version 8). The obtained sequences were compared to isolates in the GenBank database using the Basic Local Alignment Search Tool (BLAST; NCBI www.blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Pre-school outbreak investigation

A smaller outbreak of cryptosporidiosis in the end of September 2019 was brought to the attention of the regional department of communicable disease control and prevention after reports of gastrointestinal symptoms in many of the children attending the pre-school, and an outbreak investigation was initiated. Three positive *Cryptosporidium* samples were analysed, including a 5-year-old girl (Swec1414) attending the pre-school, a 33-year-old man (Swec1403), and a 39-year-old woman (Swec1413) (Table 1). Possible sources were investigated through surveys and interviews with staff and parents at the pre-school.

2.4. Investigation of zoonotic transmission

At the end of July 2018, an animal caretaker at a small-animal rehabilitation centre housing squirrels, hedgehogs, and birds sought medical care and was later hospitalised. During interviews the patient, a 41-year-old man (Swec946/Swec947), also reported that he had been ill at the beginning of July coinciding with the occurrence of several sick squirrels at the rehabilitation centre. He also reported that about the same time his partner, a 44-year-old woman (Swec950), showed symptoms such as stomach pain, nausea, and later diarrhoea. Three human samples were analysed as part of the investigation, two from the animal caretaker and one from his partner (Fig. 1). In addition to this, two samples (Swesqu1 and Swesqu2) from the animal rehabilitation centre were sent to the National Veterinary Institute for analysis (Fig. 1). One sample (Swesqu1) was a deceased squirrel whereas the other was a faecal sample (Swesqu2) from an additional squirrel kept at the rehabilitation centre. The autopsy report showed that the squirrel was emaciated with a dilated intestine and signs of diarrhoea. The cause of the emaciation was suggested to be ongoing intestinal inflammation, and the animal eventually succumbed due to starvation in combination with fluid imbalances and bacterial overgrowth of *Esherichia coli* in the intestine. Both samples were analysed by microscopy using FITC-labelled monoclonal anti-*Cryptosporidium* antibodies (Waterborne Inc., New Orleans, LA, USA) according to the manufacturer's instructions. DNA was extracted using the DNeasy PowerLyzer PowerSoil Kit according to the manufacturer's instructions. (Qiagen, Hilden, Germany). Oocyst disruption was accomplished using an MP Fast Prep 24 Bead Beater. Identification of *Cryptosporidium* species was done by amplification of the ssu rRNA gene by PCR (Xiao et al., 2001; Xiao et al., 1999), and further subtyping was done by amplification of the *gp60* gene with the primers published in Alves et al. (Alves et al., 2003) for *Cryptosporidium* ferret genotype and the primers published in Guo et al. (Guo

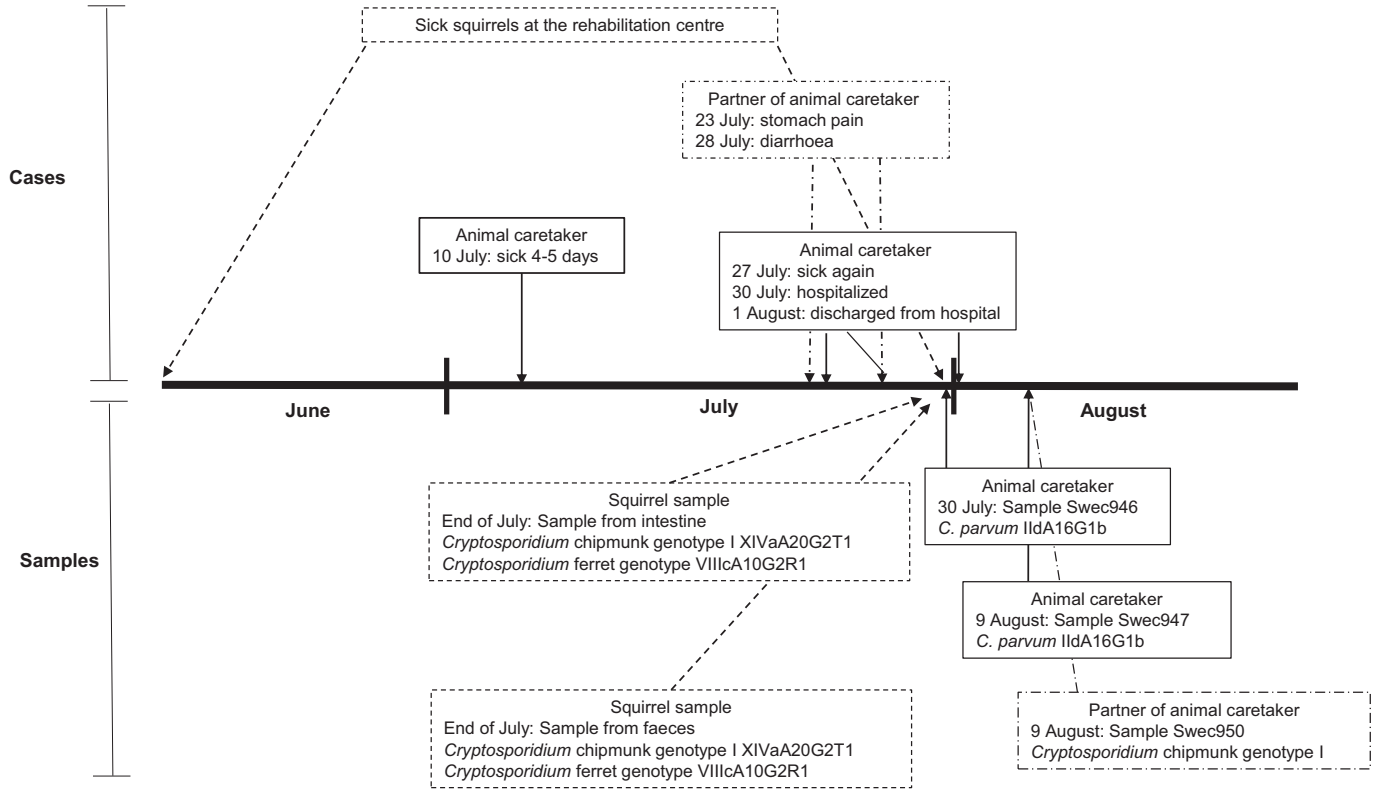

Fig. 1. Timeline for the investigation of zoonotic transmission.

et al., 2015) for *Cryptosporidium* chipmunk genotype I. The PCR fragments were sequenced with both Sanger and Illumina sequencing. Sanger sequencing was performed using both inner primers from the ssu rRNA PCR and *gp60* PCR for *Cryptosporidium* ferret genotype. For *Cryptosporidium* chipmunk genotype I, *gp60* sequencing was performed using the inner reverse primer as described by Guo et al. (Guo et al., 2015) for the reverse sequence and the new primer CrChigp60Gt1Fw3 for the forward sequence.

Further characterisation of the squirrel samples to exclude the presence of *C. parvum* was performed using Illumina deep sequencing. Amplicons generated in the ssu rRNA PCR were used as input for Nextera XT library preparation (Illumina) and sequenced on an Illumina MiSeq instrument as pair-end 2 × 250 bp generating a minimum of 100 k read pairs per sample. Data was trimmed using Trimmomatic 0.32 to remove leading and trailing bases with Q < 10 implementing a sliding 4 bp window from the 3′ end requiring a minimum of Q15, and finally requiring a trimmed minimum length of 50 bp. Because the reverse read quality was poor, only forward reads were used to count the occurrence of the unique sequence tags – CTA TAT ATT TTA GTA (representing the *C. parvum* sequence variant), CTA AAT TTT TGT TTG GTA (representing the *Cryptosporidium* ferret genotype sequence variant), and CTA TAT TTT TTA GTA (representing the *Cryptosporidium* chipmunk genotype I sequence variant) among the reads generated from each sample. Any counts corresponding to <1% of the total number of counts matching any of the three patterns were disregarded in order to avoid false positives from errors in de-multiplexing.

2.5. Phylogenetic tree analysis

Two phylogenetic trees were constructed. In the first tree the analysis was performed on the ssu rRNA gene sequences generated in the present study as well as sequences from known *Cryptosporidium* species/genotypes. In the second tree *Cryptosporidium* chipmunk genotype I *gp60* gene sequences from this study as well as published sequences from known *Cryptosporidium* chipmunk genotype I *gp60* subtypes were included. Phylogenetic trees were generated using the neighbor-joining method based on Kimura's 2-parameter model. To estimate robustness, bootstrap proportions were computed after 1000 replications. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

2.6. Accession numbers

Representative nucleotide sequences have been deposited in GenBank under accession numbers MW177561, MW177562, MW179498, MW179499, and MW179500.

3. Results

3.1. Human samples

None of the 21 patients had travelled outside Sweden within 14 days prior to infection. The majority of the cases were from the greater Stockholm area including Uppsala (11 samples, 55%), while the rest originated from various parts of Sweden (Fig. 2). Most cases appeared from August through December. There was no gender bias among the cases, and the ages ranged from 5 to 73 years with a mean of 31 years (median 33 years). Patient information, primary diagnosis, geographical location, and genetic characterisation are shown in Table 1.

3.2. Molecular characterisation of human samples

All samples were successfully amplified at the ssu rRNA loci. Sequences from 20 cases showed complete identity to each other and to *Cryptosporidium* chipmunk genotype I sample Swec176 with GenBank accession number JX978272. The two samples from the animal caretaker (Swec946/Swec947) at the small-animal rehabilitation centre

Fig. 2. Geographical dispersion of *Cryptosporidium* chipmunk genotype I human cases in various counties in Sweden, including eight cases in Stockholm, five cases in Uppsala, four cases in Kronoberg, and one case each in Skåne, Södermanland, and Dalarna.

showed complete identity to *C. parvum* sample Swec402 with GenBank accession number KU892559. All samples were negative using the *gp60* primers described by Alves et al. (Alves et al., 2003) with the exception of the two samples from the animal caretaker (Swec946/Swec947). All *Cryptosporidium* chipmunk genotype I samples except for the animal caretaker's partner (Swec950), in total 19 samples, were successfully amplified using the *gp60* primers of Guo et al. (Guo et al., 2015). The samples were *gp60* subtype XIVaA20G2T1, and the sequences showed complete identity to sample Swec641 with GenBank accession number KU852739. The samples Swec946 and Swec947 from the animal caretaker were *gp60* subtype IIdA16G1b, and the sequences showed complete identity to the reference sequence with GenBank accession number JX183808. The combined results of the genetic characterisation are shown in Table 1.

3.3. Pre-school outbreak

The three samples (one child and two adults) investigated were all *Cryptosporidium* chipmunk genotype I, subtype XIVaA20G2T1 as described above. Both adults (Swec1403 and Swec1413) were parents of children attending the pre-school, but they were not part of the same family. Both parents reported that their respective child had shown similar symptoms prior to their own onset of symptoms, but neither of the two children were tested, hence the two adults were suspected to be secondary cases. After the first identified case of cryptosporidiosis connected to the pre-school, further testing was recommended for children, parents, and staff with ongoing gastrointestinal symptoms. Five additional cases were analysed, but none of them were positive for *Cryptosporidium*.

Several of the potential sources of the outbreak that were investigated were later excluded. Initially the tap water was a suspected source of infection. During routine water testing a couple of weeks prior to when the cases were diagnosed, green flakes were found in the drinking water which resulted in a temporary prohibition of water consumption for drinking purposes until the water samples had been analysed and showed no sign of contamination. Contaminated food was ruled out as a potential common source of infection for the confirmed cases because the child with the confirmed infection and the two symptomatic children of the confirmed adult cases had not shown symptoms during the same

weeks. This indicates the unlikeliness of a common single-point food contamination at the pre-school. Other possible sources were suspected such as swimming in lakes or small pools, animal contact, or farm visits, but no such events had taken place. It appeared that the children sometimes went to a grove nearby the pre-school where both squirrels and deer had been spotted. Squirrels were also frequently spotted in the yard of the pre-school. Interviews with employees at the pre-school regarding any new or different activities that had taken place before the time of the outbreak indicated that the employees and children had started cultivating beans and pumpkins in pallet collars with commercial soil in the yard of the pre-school. Some children had been seen tasting or eating the beans. Attempts were made to collect squirrel faecal samples on site, but none could be found.

3.4. Zoonotic transmission

As described in Section 3.2 the sample from the animal caretaker's partner (Swec950) showed that she was infected with *Cryptosporidium* chipmunk genotype I. She had also been in contact with sick squirrels at the rehabilitation centre. The *gp60* subtype could not be determined, and the PCR remained negative after several amplification attempts. The first sample from the animal caretaker (Swec946) showed that he was infected with *C. parvum* subtype IIdA16G1b (Table 1). Since it was suspected that he had been infected by sick squirrels, a new sample (Swec947) was taken for confirmation. This sample confirmed the previous result (Fig. 1).

Both squirrel samples were positive for *Cryptosporidium* sp., and microscopy revealed large numbers of oocysts. The samples were successfully amplified at the ssu rRNA and *gp60* loci. However, following sequence analysis of the ssu rRNA sequences, double peaks were detected indicating the presence of a concurrent infection with mixed genotypes. The presence of both *Cryptosporidium* chipmunk genotype I and *Cryptosporidium* ferret genotype was clearly observed, and the CryptoGenotyper tool in Galaxy (Afgan et al., 2016; Yanta et al., 2021) was used to extract the sequences of both species in the mixed infections. Both sequences were analysed using the 18S contig workflow, and subtyping revealed subtype XIVaA20G2T1 and VIIIcA10G2R1 (Fig. 1). To determine whether *C. parvum* was present in the samples, deep sequencing was performed. Only single reads showed the *C. parvum* unique sequence tag, leading to the conclusion that no *C. parvum* was present in the squirrel samples and that the single reads were instead due to sequencing errors (Table 2). Regarding the other two species, the percentage of reads corresponded well with observations from Sanger sequencing. See Table 2 for read counts of the ssu rRNA sequencing products.

3.5. Phylogenetic tree analysis

Two individual phylogenetic trees, ssu rRNA (Fig. 3) and *gp60* (Fig. 4), were generated. In the ssu rRNA tree all *Cryptosporidium* chipmunk genotype I sequences obtained in this study grouped with each other and with all publicly available *Cryptosporidium* chipmunk genotype I sequences from humans, various animals, and water. In the *gp60* tree two clusters were seen, and one contained all Swedish isolates and some of the human isolates from the US, while the other cluster contained isolates from various animals from Italy and the US, as well as human isolates from the US.

Table 2
Read counts of the ssu rRNA sequencing products from red squirrel (*Sciurus vulgaris)* samples.

Sample	*C. parvum*	*Cryptosporidium* ferret genotype	*Cryptosporidium* chipmunk genotype I
Faecal (Swesqu2)	1 (0%)	289 (30%)	682 (70%)
Intestinal (Swesqu1)	1 (0%)	480 (47%)	538 (53%)
Intestinal (Swesqu1)[a]	0	743 (47%)	826 (53%)

[a] Intestinal samples were run in duplicates.

4. Discussion

Most domestic human *Cryptosporidium* cases in Sweden are due to infection with *C. parvum* and to a lesser extent *C. hominis*. However, more uncommon species and genotypes also contribute to disease burden. In 2018 and 2019, *Cryptosporidium* chipmunk genotype I was shown, through the national microbiological surveillance programme, to be the third most common species accounting for around 3.4% of domestic cases. This is slightly higher than in two studies previously performed in Sweden. Those studies showed that from 2006 to 2008 1.8% of the patients infected in Sweden were infected with *Cryptosporidium* chipmunk genotype I (Insulander et al., 2013), and from 2013 to 2014 this number was 2.0% (manuscript submitted). Preliminary data from the national microbiological surveillance program of 2020 (August to November) supports an increasing trend with 23 cases identified.

Cryptosporidium chipmunk genotype I was initially identified in storm water in the state of New York as genotype W17 (Jiang et al., 2005). The same genotype was later identified in faecal samples in 2007 from rodents from the watershed of the New York City water supply and it was renamed to chipmunk genotype I (Feng et al., 2007). The first sporadic human cases were described by Feltus et al. in the state of Wisconsin (Feltus et al., 2006), and after that 19 additional cases were reported from the US (Guo et al., 2015). Except for the cases detected in Sweden, human infection with *Cryptosporidium* chipmunk genotype I has only been reported once in Europe, in France in 2008 (Network, 2010). In Europe, *Cryptosporidium* chipmunk genotype I has been found in red squirrels (*Sciurus vulgaris*) in Italy (Kvac et al., 2008). However, a later study also from Italy showed that red squirrels were exclusively infected with *Cryptosporidium* ferret genotype, while grey squirrels (*Sciurus carolinensis)* and Pallas's squirrels (*Callosciurus erythraeus*) were infected with *Cryptosporidium* chipmunk genotype I (Prediger et al., 2017). The latter species are not natural to Europe, and the authors suggested that *Cryptosporidium* chipmunk genotype I was introduced to Europe with grey squirrels from North America (Prediger et al., 2017). However, because Sweden only harbours red squirrels it is strongly suggested that these animals are natural hosts for *Cryptosporidium* chipmunk genotype I in Sweden.

Human infection with *Cryptosporidium* chipmunk genotype I has previously been shown to cause clinical disease in non-immunocompromised patients in Sweden (Lebbad et al., 2013). The aim of the present study was not to investigate clinical symptoms of *Cryptosporidium* chipmunk genotype I infections, and complete data regarding symptoms of all the patients was not collected. However, most likely all of the patients were sampled due to gastrointestinal symptoms or during investigations of outbreaks of gastrointestinal symptoms. Reported symptoms included stomach pain, diarrhoea, and other light gastrointestinal symptoms. One patient reported diarrhoea with an intensity of 5–7 times per day.

It is well established that the majority of *Cryptosporidium* outbreaks are associated with contaminated drinking water or recreational water, contact with farm animals, and consumption of contaminated food. Sweden has had two large outbreaks of *C. hominis* transmitted through the public water supply in 2010 and 2011 (Bjelkmar et al., 2017; Widerström et al., 2014) and several foodborne outbreaks associated with *C. parvum* infections (Gherasim et al., 2012; Insulander et al., 2013). Reported *Cryptosporidium* outbreaks associated with animals in Sweden include *C. parvum* outbreaks due to animal contact by veterinary students (Kinross et al., 2015) and cattle spring pasture events (Alsmark et al., 2018). Outbreaks of both *C. hominis* and *C. parvum* related to preschools or day care nurseries have also previously been seen in Sweden as well as in other countries (Chalmers et al., 2009; Insulander et al.,

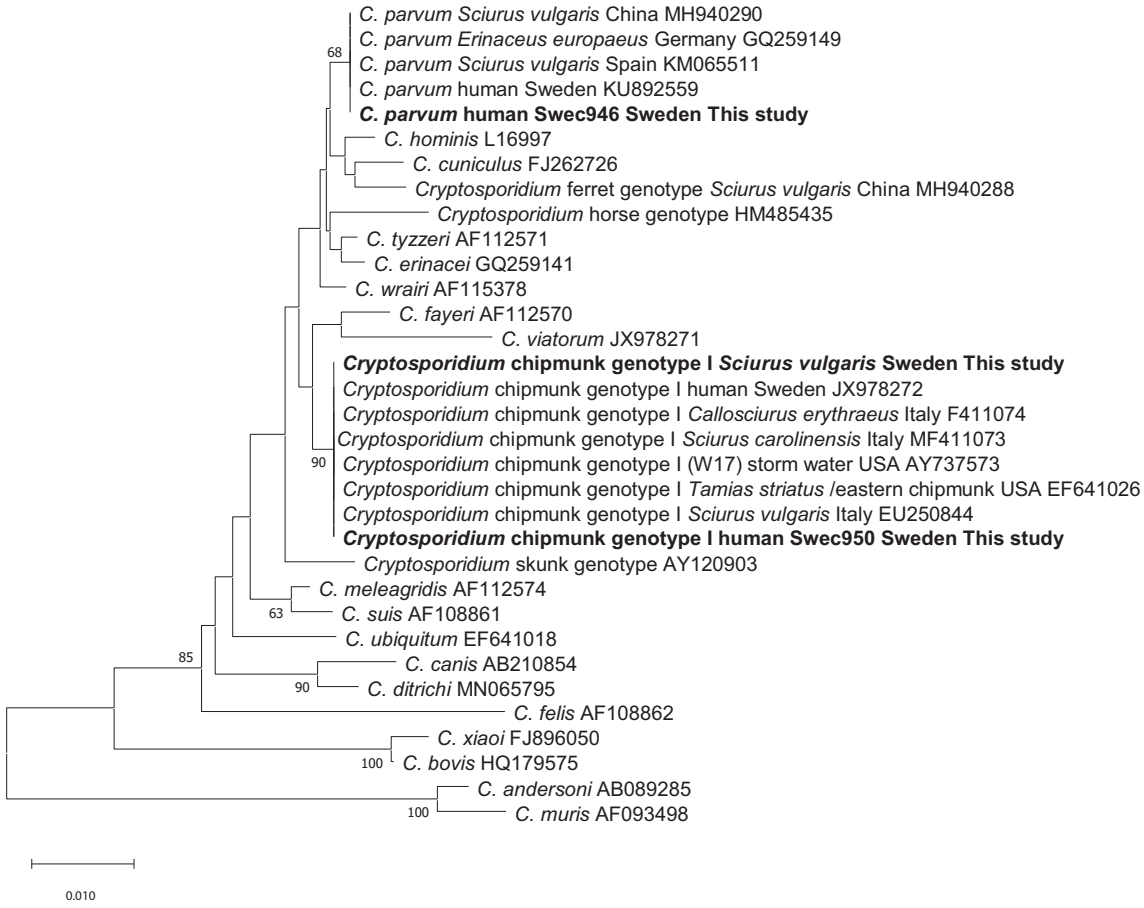

Fig. 3. Phylogenetic relationships between partial ssu rRNA *Cryptosporidium* sequences obtained in the present study and sequences retrieved from the GenBank database. The tree was constructed using the neighbor-joining method based on genetic distance calculated by Kimura's 2-parameter model as implemented in MEGA X version 10.1. There were a total of 544 positions in the final dataset. Bootstrap values ≥50% from 1000 replicates are indicated at each node. Samples from this study are in bold.

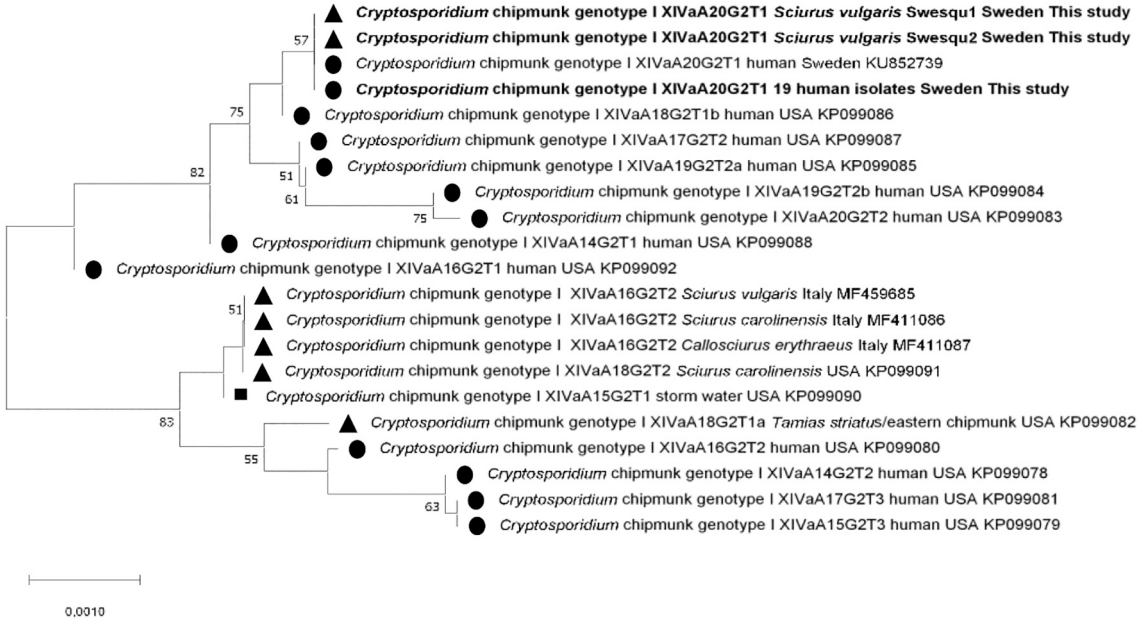

Fig. 4. Phylogenetic relationships between partial *gp60 Cryptosporidium* chipmunk genotype I sequences obtained in the present study and sequences retrieved from the GenBank database. The tree was constructed using the neighbor-joining method based on genetic distance calculated by Kimura's 2-parameter model as implemented in MEGA X version 10.1. There were a total of 885 positions in the final dataset. Bootstrap values ≥50% from 1000 replicates are indicated at each node. ▲ subtypes identified in animals ● subtypes identified in humans ■ subtype identified in waste water. Samples from this study are in bold.

2013).

Almost all reported zoonotic *Cryptosporidium*-outbreaks in humans are due to direct or indirect contact with *C. parvum*-infected cattle or sheep. For the first time we have shown that also squirrels harbouring *Cryptosporidium* chipmunk genotype I may be a potential reservoir for smaller human outbreaks. The cases in the small pre-school outbreak reported in this paper were only linked to each other through the pre-school. Two of the cases were suspected to be secondary cases because they were parents of children who had shown similar symptoms during the outbreak period and who attended the same pre-school as the confirmed child. This suggests a common source at or close to the pre-school. Squirrels had been spotted around the pre-school and its garden and were suspected to be the source of infection because no other common source was found. One can speculate that infected squirrels could have contaminated the beans and pumpkins that the children had been seen eating and touching, but also potentially that squirrels contaminated the sand or something else in the yard of the pre-school. Sampling of squirrel faeces was unsuccessful as previously mentioned, and we could not confirm this as the source.

We also present an investigation of an animal caretaker, his partner, and red squirrels at a small-animal rehabilitation centre. Red squirrels were early on suspected as the source of infection because the centre had several sick red squirrels at the time that both the animal caretaker and his partner showed symptoms of cryptosporidiosis. Both humans proved to be positive for *Cryptosporidium* sp., but molecular typing showed that the animal caretaker was infected with *C. parvum* subtype IIdA16G1b while his partner was infected with *Cryptosporidium* chipmunk genotype I, which was also detected in sick squirrels from the animal rehabilitation centre. This discrepancy led to resampling of the animal caretaker in order to exclude the possibility of sample mix-up, and the second sample confirmed infection with *C. parvum*. To determine whether the squirrels could still be the source of infection, squirrel samples were analysed using deep sequencing, but presence of *C. parvum* could not be detected. Only a few cases of *C. parvum* infections in red squirrels are known, namely in two pet red squirrels from China (Deng et al., 2020) and in a red squirrel from Spain (GenBank accession number KM065511) (unpublished). Unfortunately, there are no *gp60* sequences available from those samples. With regards to this it is unlikely that the squirrels were the source of the animal caretaker's infection. Subtype IIdA16G1b has previously been found in Sweden in calves with diarrhoea (Silverlås and Blanco-Penedo, 2013) and in five human samples (manuscript submitted). The animal caretaker had no history of close contact with cattle, but the rehabilitation centre also harboured hedgehogs, which could be a potential source because hedgehogs have previously been shown to be infected with *C. parvum* IId subtypes (Hofmannová et al., 2016; Sangster et al., 2016). Other animals present at the rehabilitation centre were also their pet animals like ducks, hens, cats, and dogs. The source of the animal caretaker's infection with *C. parvum* remains unknown. The animal caretaker had been sick two weeks prior to being sick again and hospitalised. One can speculate that he was initially infected by *Cryptosporidium* chipmunk genotype I when no sampling was done and later became infected with *C. parvum* from another source. Remaining gastrointestinal irritation from the first infection could be the reason for the more severe symptoms with the need for hospitalisation in the second round of infection. In the beginning of the investigation the partner was thought to be a secondary case to the animal caretaker; however, molecular typing could not confirm this because she was shown to be infected with *Cryptosporidium* chipmunk genotype I. She herself had taken care of sick squirrels at the rehabilitation centre, thus proposing that she was infected with *Cryptosporidium* chipmunk genotype I directly by sick squirrels. As previously mentioned, the *gp60* subtype could not be determined even after several amplification attempts. The sample was taken several weeks after she first showed symptoms, and it is likely that the oocyst count was low and thus probably the reason why subtyping was not successful.

In 2015, Guo et al. published a *gp60* subtyping method for *Cryptosporidium* chipmunk genotype I (Guo et al., 2015). Fifteen different subtypes have been identified so far, all belonging to the same subtype family – XIVa. The subtypes are differentiated mainly by the number of trinucleotide repeats (TCA, TCG, or TCT) in the serine repeat and in some cases by single nucleotide polymorphisms (SNPs) in the non-repeat region. In the study by Guo et al., 11 of these subtypes were found in 19 patients from four US states. The two subtypes that were found in wildlife samples did not appear in any human samples (Guo et al., 2015). *Gp60* subtyping has also been performed on *Cryptosporidium* chipmunk genotype I samples from three different squirrels species from Italy (Kvac et al., 2008; Prediger et al., 2017), where all three species had subtype XIVaA16G2T2, a subtype also reported from humans in Maine in the US (Guo et al., 2015). However, a closer analysis of those sequences revealed one SNP difference in the non-repeat region of the squirrel sample compared to the human sample, thus the subtypes are not identical. In Sweden 100% identical sequences, corresponding to subtype XIVaA20G2T1, have been detected in all human samples analysed so far as well as in two red squirrel samples (Fig. 4). The cause for the finding of only one subtype in human cases is not well understood. It might be due to a limited number of hosts for this *Cryptosporidium* species in Sweden where red squirrels are the most likely source of infection; the other described host animals; chipmunks, grey squirrels, and deer mice are not native to the country (Guo et al., 2015). Investigations of more samples from squirrels as well as from other Swedish animals that might serve as hosts for *Cryptosporidium* chipmunk genotype I have been initiated and will hopefully clarify this issue.

Another interesting aspect is why *Cryptosporidium* chipmunk genotype I – which seems to be an emerging cause of cryptosporidiosis in Sweden has not been reported in humans in other European countries, with the exception of one case from France (Network, 2010). Although red squirrels have declined significantly in some European countries following the introduction of grey squirrels, even the latter are a potential source of human *Cryptosporidium* chipmunk genotype I infection (Prediger et al., 2017). The sporadic cases in our study all appeared during late summer to autumn months, a period when Swedes traditionally spend a lot of time working in the garden and picking berries and mushrooms in the forest, locations they might share with squirrels. Increased awareness of domestic *Cryptosporidium* infections in Sweden after the large-scale outbreaks (Bjelkmar et al., 2017; Widerström et al., 2014) and the shift in diagnostic procedures from microscopy to molecular-based methods are factors that may influence the detection level in Sweden. Most cases in our study were detected by real time PCR (Table 1), and such molecular methods are generally considered more sensitive than microscopy for detection of *Cryptosporidium* spp. (McHardy et al., 2014). However, none of the mentioned factors can explain the growing number of reported *Cryptosporidium* chipmunk genotype I cases in Sweden and the almost total absence of reported cases from the rest of Europe.

Cryptosporidium diagnostics based on PCR are usually considered highly sensitive. However, this might not be the case for some diagnostic PCRs designed for human diagnostics. Several diagnostic multiplex real time PCR assays, including both in-house and commercially available assays, have been designed to detect infection with species within the *C. hominis/C. parvum* complex, which means that the primers may have less or no affinity for genetically different species such as *Cryptosporidium* chipmunk genotype I, and this will influence the sensitivity for these more distant species (Autier et al., 2018; Bruijnesteijn van Coppenraet et al., 2009). *Cryptosporidium* chipmunk genotype I was not evaluated specifically, but a study comparing three commercial multiplex real time PCR assays showed that only one of them was able to detect *C. canis* and *C. felis*, two other species that are genetically distinct from *C. hominis* and *C. parvum* (Autier et al., 2018). Maybe this inability of some PCR assays to detect certain species could be the reason for the uneven distribution of *Cryptosporidium* chipmunk genotype I cases in Sweden (Fig. 2). We also speculate that this might explain why some of the symptomatic cases from the pre-school outbreak investigation

showed negative results for *Cryptosporidium*. Those samples were analysed at another clinical laboratory in the region, which used a different PCR assay than the laboratory that detected the initial positive cases. Clinical microbiological laboratories performing primary diagnosis of *Cryptosporidium* should consider the choice of assay in order not to get false negative results for less common but still important human pathogenic species.

5. Conclusions

Infection with *Cryptosporidium* chipmunk genotype I, even though accounting for only a minority of cases, is an emerging cause of human cryptosporidiosis in Sweden. In the present study, sick squirrels were associated with human disease and thus represent a reservoir of human cryptosporidiosis. We also describe the first small outbreak of cryptosporidiosis caused by infection with *Cryptosporidium* chipmunk genotype I.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors are grateful to the animal caretaker and his partner for their participation in the investigation. The authors would also like to thank Harri Ahola for help with molecular work on the squirrel samples. The project was funded by the Public Health Agency of Sweden and the Swedish Civil Contingencies Agency.

References

Afgan, E., Baker, D., van den Beek, M., Blankenberg, D., Bouvier, D., Cech, M., Chilton, J., Clements, D., Coraor, N., Eberhard, C., Gruning, B., Guerler, A., Hillman-Jackson, J., Von Kuster, G., Rasche, E., Soranzo, N., Turaga, N., Taylor, J., Nekrutenko, A., Goecks, J., 2016. The galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. Nucleic Acids Res. 44, W3–W10.

Alsmark, C., Nolskog, P., Angervall, A.L., Toepfer, M., Winiecka-Krusnell, J., Bouwmeester, J., Bjelkmar, P., Troell, K., Lahti, E., Beser, J., 2018. Two outbreaks of cryptosporidiosis associated with cattle spring pasture events. Vet. Parasitol. Reg. Stud. Rep. 14, 71–74.

Alves, M., Xiao, L., Sulaiman, I., Lal, A.A., Matos, O., Antunes, F., 2003. Subgenotype analysis of Cryptosporidium isolates from humans, cattle, and zoo ruminants in Portugal. J. Clin. Microbiol. 41, 2744–2747.

Autier, B., Belaz, S., Razakandrainibe, R., Gangneux, J.P., Robert-Gangneux, F., 2018. Comparison of three commercial multiplex PCR assays for the diagnosis of intestinal protozoa. Parasite 25, 48.

Bjelkmar, P., Hansen, A., Schönning, C., Bergström, J., Löfdahl, M., Lebbad, M., Wallensten, A., Allestam, G., Stenmark, S., Lindh, J., 2017. Early outbreak detection by linking health advice line calls to water distribution areas retrospectively demonstrated in a large waterborne outbreak of cryptosporidiosis in Sweden. BMC Public Health 17, 328.

Bruijnesteijn van Coppenraet, L.E., Wallinga, J.A., Ruijs, G.J., Bruins, M.J., Verweij, J.J., 2009. Parasitological diagnosis combining an internally controlled real-time PCR assay for the detection of four protozoa in stool samples with a testing algorithm for microscopy. Clin. Microbiol. Infect. 15, 869–874.

Chalmers, R.M., Elwin, K., Thomas, A.L., Guy, E.C., Mason, B., 2009. Long-term Cryptosporidium typing reveals the aetiology and species-specific epidemiology of human cryptosporidiosis in England and Wales, 2000 to 2003. Euro Surveill. 14.

Deng, L., Chai, Y., Luo, R., Yang, L., Yao, J., Zhong, Z., Wang, W., Xiang, L., Fu, H., Liu, H., Zhou, Z., Yue, C., Chen, W., Peng, G., 2020. Occurrence and genetic characteristics of Cryptosporidium spp. and Enterocytozoon bieneusi in pet red squirrels (Sciurus vulgaris) in China. Sci. Rep. 10, 1026.

Feltus, D.C., Giddings, C.W., Schneck, B.L., Monson, T., Warshauer, D., McEvoy, J.M., 2006. Evidence supporting zoonotic transmission of Cryptosporidium spp. in Wisconsin. J. Clin. Microbiol. 44, 4303–4308.

Feng, Y., Alderisio, K.A., Yang, W., Blancero, L.A., Kuhne, W.G., Nadareski, C.A., Reid, M., Xiao, L., 2007. Cryptosporidium genotypes in wildlife from a New York watershed. Appl. Environ. Microbiol. 73, 6475–6483.

Feng, Y., Ryan, U.M., Xiao, L., 2018. Genetic diversity and population structure of Cryptosporidium. Trends Parasitol. 34, 997–1011.

Gherasim, A., Lebbad, M., Insulander, M., Decraene, V., Kling, A., Hjertqvist, M., Wallensten, A., 2012. Two geographically separated food-borne outbreaks in Sweden linked by an unusual *Cryptosporidium parvum* subtype, October 2010. Euro Surveill. 17.

Guo, Y., Cebelinski, E., Matusevich, C., Alderisio, K.A., Lebbad, M., McEvoy, J., Roellig, D.M., Yang, C., Feng, Y., Xiao, L., 2015. Subtyping novel zoonotic pathogen Cryptosporidium chipmunk genotype I. J. Clin. Microbiol. 53, 1648–1654.

Hofmannová, L., Hauptman, K., Huclová, K., Květoňová, D., Sak, B., Kváč, M., 2016. Cryptosporidium erinacei and C. parvum in a group of overwintering hedgehogs. Eur. J. Protistol. 56, 15–20.

Insulander, M., Silverlås, C., Lebbad, M., Karlsson, L., Mattsson, J.G., Svenungsson, B., 2013. Molecular epidemiology and clinical manifestations of human cryptosporidiosis in Sweden. Epidemiol. Infect. 141, 1009–1020.

Jiang, J., Alderisio, K.A., Xiao, L., 2005. Distribution of cryptosporidium genotypes in storm event water samples from three watersheds in New York. Appl. Environ. Microbiol. 71, 4446–4454.

Kinross, P., Beser, J., Troell, K., Axén, C., Björkman, C., Lebbad, M., Winiecka-Krusnell, J., Lindh, J., Löfdahl, M., 2015. Cryptosporidium parvum infections in a cohort of veterinary students in Sweden. Epidemiol. Infect. 143, 2748–2756.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35, 1547–1549.

Kvac, M., Hofmannova, L., Bertolino, S., Wauters, L., Tosi, G., Modry, D., 2008. Natural infection with two genotypes of Cryptosporidium in red squirrels (Sciurus vulgaris) in Italy. Folia Parasitol. 55, 95–99.

Lebbad, M., Beser, J., Insulander, M., Karlsson, L., Mattsson, J.G., Svenungsson, B., Axen, C., 2013. Unusual cryptosporidiosis cases in Swedish patients: extended molecular characterization of Cryptosporidium viatorum and Cryptosporidium chipmunk genotype I. Parasitology 140, 1735–1740.

McHardy, I.H., Wu, M., Shimizu-Cohen, R., Couturier, M.R., Humphries, R.M., 2014. Detection of intestinal protozoa in the clinical laboratory. J. Clin. Microbiol. 52, 712–720.

Network, Anofel Cryptosporidium National, 2010. Laboratory-based surveillance for Cryptosporidium in France, 2006-2009. Euro Surveill. 15, 19642.

Prediger, J., Horcickova, M., Hofmannova, L., Sak, B., Ferrari, N., Mazzamuto, M.V., Romeo, C., Wauters, L.A., McEvoy, J., Kvac, M., 2017. Native and introduced squirrels in Italy host different Cryptosporidium spp. Eur. J. Protistol. 61, 64–75.

Sangster, L., Blake, D.P., Robinson, G., Hopkins, T.C., Sa, R.C., Cunningham, A.A., Chalmers, R.M., Lawson, B., 2016. Detection and molecular characterisation of Cryptosporidium parvum in British European hedgehogs (Erinaceus europaeus). Vet. Parasitol. 217, 39–44.

Silverlås, C., Blanco-Penedo, I., 2013. Cryptosporidium spp. in calves and cows from organic and conventional dairy herds. Epidemiol. Infect. 141, 529–539.

Widerström, M., Schönning, C., Lilja, M., Lebbad, M., Ljung, T., Allestam, G., Ferm, M., Björkholm, B., Hansen, A., Hiltula, J., Långmark, J., Löfdahl, M., Omberg, M., Reuterwall, C., Samuelsson, E., Widgren, K., Wallensten, A., Lindh, J., 2014. Large outbreak of Cryptosporidium hominis infection transmitted through the public water supply, Sweden. Emerg. Infect. Dis. 20, 581–589.

Xiao, L., Morgan, U.M., Limor, J., Escalante, A., Arrowood, M., Shulaw, W., Thompson, R.C., Fayer, R., Lal, A.A., 1999. Genetic diversity within Cryptosporidium parvum and related Cryptosporidium species. Appl. Environ. Microbiol. 65, 3386–3391.

Xiao, L., Bern, C., Limor, J., Sulaiman, I., Roberts, J., Checkley, W., Cabrera, L., Gilman, R.H., Lal, A.A., 2001. Identification of 5 types of Cryptosporidium parasites in children in Lima, Peru. J. Infect. Dis. 183, 492–497.

Yanta, C.A., Bessonov, K., Robinson, G., Troell, K., Guy, R.A., 2021. CryptoGenotyper: a new bioinformatics tool for rapid Cryptosporidium identification. Food Waterborne Parasitol. 23, e00115.

Zahedi, A., Ryan, U., 2020. Cryptosporidium - an update with an emphasis on foodborne and waterborne transmission. Res. Vet. Sci. 132, 500–512.