DETECTION AND MOLECULAR CHARACTERISATION OF *CRYPTOSPORIDIUM PARVUM* IN BRITISH EUROPEAN HEDGEHOGS (*ERINACEUS EUROPAEUS*)

Lucy Sangster[1], Damer Blake[2]; Guy Robinson[3]; Timothy C. Hopkins[3]; Andrew A. Cunningham[1]; Rachel M Chalmers[3]; Becki Lawson[1]

[1] *Institute of Zoology, Zoological Society of London, Regents Park, London, NW1 4RY, United Kingdom*

[2] *The Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Hertfordshire, AL9 7TA, United Kingdom*

[3] *Cryptosporidium Reference Unit, Public Health Wales Microbiology ABM, Singleton Hospital, Sgeti, Swansea, SA2 8QA*

Corresponding author: Becki Lawson. Postal address: Institute of Zoology, Zoological Society of London, Regents Park, London, NW1 4RY, United Kingdom. Email: becki.lawson@ioz.ac.uk; Tel. +44 (0) 20 7449 6677; Fax +44 (0) 20 7483 2237.

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Abstract

Surveillance was conducted for the occurrence of protozoan parasites of the genus Cryptosporidium in European hedgehogs (Erinaceus europaeus) in Great Britain. In total, 108 voided faecal samples were collected from hedgehogs newly admitted to eight wildlife casualty treatment and rehabilitation centres. Terminal large intestinal (LI) contents from three hedgehog carcasses were also analysed. Information on host and location variables, including faecal appearance, body weight, and apparent health status, was compiled. Polymerase Chain Reaction (PCR) targeting the 18S ribosomal RNA gene, confirmed by sequencing, revealed an 8% (9/111) occurrence of Cryptosporidium parvum in faeces or LI contents, with no significant association between the host or location variables and infection. Archived small intestinal (SI) tissue from a hedgehog with histological evidence of cryptosporidiosis was also positive for C. parvum by PCR and sequence analysis of the 18S rRNA gene. No other Cryptosporidium species were detected. PCR and sequencing of the glycoprotein 60 gene identified three known zoonotic C. parvum subtypes not previously found in hedgehogs: IIdA17G1 (n=4), IIdA19G1 (n=1) and IIdA24G1 (n=1). These subtypes are also known to infectlivestock. Another faecal sample contained C. parvum IIcA5G3j which has been found previously in hedgehogs, and for which there is one published report in a human, but is not known to affectlivestock. The presence of zoonotic subtypes of C. parvum in British hedgehogs highlights a potential public health concern. Further research is needed to better understand the epidemiology and potential impacts of Cryptosporidium infection in hedgehogs.

Keywords: *Cryptosporidium parvum*, *Erinaceus europaeus*, European hedgehog, 18S rRNA, *gp60*.

Highlights

- *Cryptosporidium parvum* infection is present in wild hedgehogs in Great Britain.

- Glycoprotein 60 (*gp60*) subtypes IIdA17G1, IIdA19G1, IIdA24G1 and IIcA5G3j were detected.
- These *gp60* subtypes are known human pathogens and have not previously been reported in hedgehogs.
- Some of these glycoprotein 60 subtypes are known pathogens of livestock.
- Histological evidence of cryptosporidiosis associated with IIdA17G1 infection in one hedgehog.

1. Introduction

The European hedgehog (Erinaceus europaeus) is a small, nocturnal mammal that inhabits a variety of urban and rural habitats, often resulting in frequent direct and indirect contact with humans, domestic animals, and wildlife (Amori et al. 2008). There are a small number of reports of Cryptosporidium infection in this species in continental Europe and Great Britain (Sturdee et al. 1999; Enemark et al. 2002; Meredith & Milne 2009; Dyachenko et al. 2010; Barlow, *pers. comm.* 2), and in captive African hedgehogs (Ateletrix albiventris) in the U.S.A. (Graczyk et al., 1998) and Japan (Abe and Matsubara, 2015).

Infections with *Cryptosporidium* spp. protozoan parasites are ubiquitous in wildlife, domestic animals and humans (Gosling 2005; OIE 2008), but infection does not always cause disease (Xiao et al. 2004). A lack of morphological differences between different Cryptosporidium species and genotypes has often resulted in wildlife being incorrectly incriminated as a reservoir for human infection (Applebee et al., 2005), including Cryptosporidium parvum (OIE 2008; Xiao, 2010).

Sequence analysis of the small sub-unit ribosomal RNA (18S rRNA) gene (Enemark et al. 2002), the 60 kDa glycoprotein (*gp60*), *actin* and 70 kDa heat shock protein (*hsp70*) gene fragments has been used to detect and characterise *Cryptosporidium* infection in European hedgehogs in Germany (Dyachenko et al. 2010) and Denmark (Enemark et al. 2002). Dyachenko and colleagues (2010) identified a genotype from captive *E. erinacei* which they considered to be hedgehog-specific, as *Cryptosporidium* sp*. gp60* subtype VIIa. This was later re-named *gp60* subtype XIII, and subsequently classified as the new species, *Cryptosporidium erinacei* by Kváč et al. (2014b), who also found it to infect *A. albiventris* through experimental exposure. *Cryptosporidium erinacei* infection has subsequently been identified in clinically healthy horses in Algeria (Laatamna et al. 2013) and in humans in the Czech Republic (Kvác et al. 2014a). These data suggest that it has a broader host range than initially suspected.

To investigate the occurrence of Cryptosporidium spp. in the free-living British hedgehog population, a survey was undertaken through testing voided faeces from hedgehogs recently admitted to wildlife centres and terminal large (LI) intestinal contents from hedgehogs found dead or euthanased. Isolates were characterised and associations assessed between Cryptosporidium infection and host and location variables.

2. Materials and methods

2.1. Collection of samples

From April-June 2014, a single voided faecal sample was collected from each of 108 individually housed hedgehogs newly admitted (most within 48 h of admission) to eight geographically dispersed wildlife casualty treatment and rehabilitation centres in Great Britain (Fig. 1). Maternally-dependent juveniles were excluded from the study. A standardised submission form provided basic information about the individual, including body weight, sex, approximate location found and reason for casualty presentation. Faecal consistency, colour and the presence of blood were recorded. Terminal LI contents were collected from three carcasses of freeliving hedgehogs during post mortem examination as part of the Garden Wildlife Health project (GWH, 2014).

Samples (minimum 1 g) were stored for up to one week at 4 ◦C, after which they were stored in 2.5% (w/v) potassium dichromate at 4 ◦C until processing. Additionally, a sample of small intestine (SI) was examined from a juvenile female hedgehog found in Worcestershire, England, in October 2012 and admitted to wildlife centre G (Fig. 1). During a period of circa three months in care, the hedgehog received antimicrobial and anthelminthic treatment and fluid therapy. It gained body weight from its admission at 173 g to a maximum recorded value of 411 g whilst in captivity. It had variable appetite throughout its time in care, but deteriorated to become inappetent and latterly anorexic, when it developed abnormal green faeces sometimes described as of 'runny' consistency. The hedgehog progressed to a recumbent state and was euthanased and submitted for post-mortem examination in February 2013. Histopathological examination showed evidence of intestinal cryptosporidiosis. An archived intestinal tract tissue sample (stored at −20 ◦C) was examined to characterise the Cryptosporidium species involved.

2.2. Faecal processing and DNA purification

To recover and enrich oocysts, faecal samples and terminal LI contents were centrifuged at 200 × g for five minutes, the potassium dichromate decanted and one gram of faeces subjected to saturated salt flotation (Kuczynska and Shelton, 1999). Four surface aliquots per sample were pooled, washed in phosphate buffered saline (PBS) by centrifugation at 4000 × g for one

minute, the sediment re-suspended in 200 l of PBS, boiled for five minutes and centrifuged at 10,000 × g for one minute (process adapted from Abe et al., 2002). DNA was extracted from the resulting supernatant using a Qiagen DNeasy Blood and Tissue Kit (Qiagen©, Germany) following the manufacturer's protocol. Samples were stored at −20 ◦C prior to ethanol precipitation (0.1 volumes sodium acetate, 3 M pH 5.2; 2.5 volumes ice cold 100% ethanol and 1 l glycogen as a carrier; centrifugation at 10,000 × g for 15 min; washed in one volume 70% ethanol and re-suspended in 20 l molecular grade water).

Additionally, DNA was extracted from four 1 cm2 sections of archived SI tract collected from the hedgehog with histological evidence of cryptosporidiosis using a Qiagen DNeasy Blood and Tissue Kit (Qiagen©, Germany) following the manufacturer's instructions for tissue samples.

2.3 Polymerase chain reaction Cryptosporidium detection and genotyping

Purified DNA was tested for Cryptosporidium spp. using conventional PCR targeting a ~300 bp region of the Cryptosporidium 18S gene as described elsewhere (Morgan et al., 1997). C. parvum 18S rDNA derived from an infection in a domestic dog and cloned into pGEM-T easy (Promega, Southampton, UK) was used as a positive control and molecular grade water as a negative control. PCR ampli- fication was performed in a volume of 25 l. PCR amplicons were resolved by electrophoretic separation through 2% (w/v) agarose gel (UltrapureTM agarose powder in 0.5x Tris-Borate-EDTA buffer) stained with 0.01% (v/v) SafeView nucleic acid stain (NBS Biologicals, U.K.) and visualised under ultraviolet light using an U:Genius Image Capture gel documentation system (Syngene, U.K). Samples positive by 18S PCR were subtyped using a nested PCR targeting the Cryptosporidium gp60 gene (Alves et al., 2003; Dyachenko et al., 2010). PCR products of the anticipated size were purified using a Qiagen MinElute Purification Kit (18S) or QIAquick Gel Extraction Kit (gp60) as recommended by the manufacturer (Qiagen©, Germany). Purified PCR products were sequenced in each direction by GATC Biotech (Cologne, Germany) using the same primers as used for the original PCR. Sequences were analysed in CLC Main Workbench Version 5.7.1 using BLASTn against the National Centre for Biotechnology Information (NCBI) non-redundant nucleotide collection. The gp60 genotypes were identified according to sequence and serine repeat characteristics (Sulaiman et al., 2005).

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186 *2.4. Phylogenetic analysis*

187 To assess the relationship between *gp60* subtypes identified from the hedgehogs in this study
188 and those published in GenBank (reference accession numbers as shown in Fig. 3), a series
189 of phylogenetic trees were constructed, incorporating the reference sequences used by Kváč
190 et al. (2014b). All sequences were aligned using ClustalW and trimmed in CLC Main
191 Workbench using default parameters. The assembled sequences were analysed using the
192 maximum likelihood (ML), neighbour joining (NJ) and maximum parsimony (MP) methods. ML
193 analysis used the Kimura 2 + Gamma distribution model in MEGA 5.10, identified using the
194 Bayesian Information Criterion with 1000 bootstrap replicates. NJ and MP analyses used the
195 Kimura 2 model in MEGA 5.10 with 1000 bootstrap replicates.

196

197 *2.5. Statistical analyses*

198 For analysis of infection status and epidemiological variables, individuals were classed as
199 'apparently healthy' or 'apparently unhealthy' based on their reason for admittance. Hedgehogs
200 whose reason for admittance was described as 'underweight' by centre staff or that were found
201 'out during the day' were considered as 'apparently unhealthy' for the analyses since these
202 observations are known to be common in hedgehogs with significant disease when submitted
203 as wildlife casualties (Robinson & Routh 1999). Hedgehogs that were victims of road traffic
204 accidents and other physical reasons for presentation (e.g. entanglement) were considered
205 'apparently healthy' casualties as they presented as a result of acute trauma prior to which they
206 probably were not diseased. Hedgehogs that had been predated were assessed on an
207 individual basis for classification as 'apparently healthy' or 'apparently unhealthy' using body
208 weight and any supporting information.

209

210 Faeces were classified as 'normal' or 'abnormal'. Only samples that were solid, brown and free
211 of blood were considered normal. Samples with any other combination of descriptors were
212 classed as abnormal.

213

214 Statistical analyses were performed using 'R' (version 3.1.0) and significance was assigned
215 when $P < 0.05$. Possible associations between the presence/absence of Cryptosporidium by
216 18S PCR and the host and location variables sex, centre from which the sample originated,

apparent health status and faecal appearance, were tested by Fisher's exact test and mean body weight by Welch's t-test. The distribution of the sampling centres was mapped (QGIS 2.4; www.qgis.org) to demonstrate the sample and Cryptosporidium spp. and genotype distribution.

3. Results

3.1. *Post-mortem examination*

Post-mortem examinations were performed on three hedgehogs from which LI contents were collected for testing; two had been euthanased with traumatic injuries apparently caused by predation while the third had a disseminated bacterial infection which was considered to be the cause of death. No other significant abnormalities were detected. The juvenile female hedgehog that was euthanased in a wildlife centre was in good body condition with ample fat deposits. Macroscopic examination revealed subcutaneous oedema and visceral congestion. Light microscopic examination of a saline-mount direct preparation of SI contents was negative for metazoan parasites. Microbiological examination of the liver and SI contents yielded no significant isolates. Histological examination revealed non-suppurative meningo-encephalitis and localised jejunal cryptosporidiosis. Numerous round bodies (2–3.5 m diameter and morphology characteristic of Cryptosporidium sp. parasites) were adherent to the intestinal epithelium and free within the lumen. These were associated with blunting and shortening of the villi in which there was interstitial oedema and a plasmalymphocytic inflammatory cell infiltrate (Fig. 2). Sparse parasitic infection was observed in a second SI section, whilst no evidence of infection was observed in three other sections of different regions of the small intestine.

3.2. *Detection of Cryptosporidium in hedgehogs by PCR*

Based on 18S PCR amplicon sequencing, nine of the 111 (8%; 95% CI: 3–13%) faecal and LI contents samples were positive for C. parvum; all were from hedgehogs <48 h in captivity. The SI tissue sample from the hedgehog examined post mortem which had been in captivity for circa three months was also positive.

No significant association was found between being positive for Cryptosporidium and any of the host and location variables examined (see Supplementary Table 1).

Of the 10 samples found to be positive using 18S PCR, seven (six faecal, one SI tissue) were also positive using gp60 PCR. Six gp60 PCR products were assigned to subtype family IId by BLASTn. Three different IId subtypes were identified (IIdA17G1, IIdA19G1, IIdA24G1). The seventhgp60 PCRproduct,froma faecal sample, was assigned to subtype family IIc, identified as subtype IIcA5G3j. The locations at which these different subtypes occurred are shown in Fig. 1. The sequences generated in this study have been submitted to GenBank with the accession numbers LN714778-87 (18S) and LN714788-94 (gp60).

3.3. *Phylogenetic analysis*

TheML tree for the gp60 gene (see Supplementary Fig. 1) showed that six of the seven sequences in this study form a clade with the annotated C. parvum IId GenBank sequence to which they are most similar on BLASTn analysis (JF727809), as well as AY738194, identified from human hosts in Kuwait (Sulaiman et al., 2005). This clade is separate to those sequences published for C. *erinacei* from the Czech Republic (KF612329) (Kvác˘ et al., 2014b) and Germany (e.g. GQ214081 and GQ259140) (Dyachenko et al., 2010). The remaining gp60 sequence annotated as IIc was identical to sequence GQ259136, isolated previously from a German hedgehog (Dyachenko et al., 2010). The NJ and MP methods produced phylogenies with comparable topologies (data not shown).

3.5. *Spatial distribution*

The number of samples positive per sampling centre and the geographical distribution of the different *gp60* subtypes identified is shown in Fig. 1, indicating a wide distribution of *Cryptosporidium* infection in hedgehogs. Subtype IIdA17G1 was found at multiple locations (centres B, C, F, G) and multiple subtypes (IIdA17G1, IIdA19G1 and IIdA24G1) were identified from centre B. Subtype IIcA5G3j was identified from centre G.

4. Discussion

We found *C. parvum* infection in ten European hedgehogs, of which six samples were identified as *gp60* subtype family IId. To our knowledge, this is the first report of this potentially zoonotic *C. parvum* subtype in hedgehogs. Our findings indicate that subtype IIdA17G1 appears widespread in British hedgehogs, but the sample size is too small to make conclusions on the distribution of the other subtypes found.

A seventh sample was confirmed as gp60 subtype IIcA5G3j, previously identified in the United Kingdom from humans (Chalmers et al., 2011a) and European hedgehogs in Germany (Dyachenko et al., 2010). Subtype IIcA5G3 was identified in a study of hedgehogs in the Netherlands (Krawczyk et al., 2015), but without the suffix or sequences deposited on GenBank it is not possible to compare this with that found in our study.

Our results are consistent with previous published (Meredith & Milne 2009) and unpublished (Barlow, *pers. comm.* 2014) reports of *Cryptosporidium* infection in hedgehogs in Great Britain. *Cryptosporidium* infection has also been identified in European hedgehogs admitted to wildlife centres in Germany (Dyachenko et al. 2010) and Denmark (Enemark et al. 2002). The 8% occurrence in this study is similar to published values from the Netherlands (9%; 8/90) (Krawczyk et al., 2015), but considerably lower than in Germany (Dyachenko et al., 2010), where prevalence was estimated at up to 39.4% (45/114) in animals commencing treatment and rehabilitation (based on an immunoassay for coproantigen detection and microscopy of faeces). However, the study by Dyachenko et al. (2010) used a non-randomised population as individuals with diarrhoea were preferentially selected and the sample included juveniles. In other species, such as cattle, cryptosporidiosis is usually more common in juveniles than in adults (Constable, 2010). In the current study, sample collection was from maternally-independent hedgehogs newly admitted to the participating wildlife centres without further selection criteria.

Examination of a larger number of samples is required to robustly document the prevalence of Cryptosporidium infection in British hedgehogs and to explore whether seasonal or spatial variation occurs. Future application of a nested PCR approach targeting the multi-copy 18S rRNA gene would be expected to improve sensitivity of detection (Jiang et al., 2005).

The specific gp60 IId subtypes found in the current study have been previously identified in a range of species, including humans (see Supplementary Table 2). Subtype IIdA17G1 has been identi- fied in Spanish lambs and goat kids (Quílez et al., 2008), cattle in Portugal (Alves et al., 2006) and Sweden (Silverlås et al., 2013), and in immunocompetent humans in England andWales (Chalmers et al., 2011b). Subtype IIdA24G1 was identified in lambs in Spain (Quílez

et al., 2008) and has also been found in humans in Australia (Waldron et al., 2009) and Sweden (Gherasim et al., 2012). Subtype IIdA19G1 has previously been found in calves in China (Wang et al., 2011) and in Spanish lambs and goat kids (Quílez et al., 2008), as well as cattle in Hungary and Sweden (Plutzer and Karanis, 2007; Silverlås et al., 2013), and humans in Sweden (Insulander et al., 2013). Subtype IIdA19G1 has also been identified in HIV-positive humans in China (Wang et al., 2013) and Portugal(Alves et al., 2006) and in urban wastewater in China (Li et al., 2012). Infection (and associated disease) in humans with gp60 family IId has been seen in several countries butless frequently than gp60 family IIa (Nichols et al., 2014). C. parvum IIc subtypes are considered another important causative agent of cryptosporidiosis in humans (Xiao, 2010) and, until recently, this subtype family was considered to be humanspecific. Dyachenko et al. (2010) identified IIc subtypes in E. europaeus in Germany, including the subtype IIcA5G3j (GenBank Accession GQ259136), also identified in the current study. In the United Kingdom, this subtype has previously only been reported from humans (Chalmers et al., 2011b). The current study found no evidence of C. erinacei in British hedgehogs. This could relate to the small sample size, and further examination may reveal the presence of this parasite in the future. Alternatively, this could be due to parasite evolutionary divergence between Great Britain and mainland Europe. We found no evidence of an association between Cryptosporidium infection and any of the host or location variables tested. Histological evidence of localised cryptosporidiosis in a hedgehog infected with C. parvum subtype IIdA17G1 indicates that this subtype, at least, can cause disease in some animals. Since this hedgehog had been kept in captivity for a period of circa 3 months and had concurrent disease (meningoencephalitis), it was considered likely to have been in an immunocompromised state. Previous reports of cryptosporidiosis, some fatal, have been described in European hedgehogs held in captivity long term (Meredith and Milne, 2009; Barlow pers. comm. 2014). The extent to which cryptosporidiosis occurs as a primary disease of free-living hedgehogs is unknown. Future studies could combine molecular subtyping with microscopic examination of faecal smears or quantitative PCR in order to quantify infection intensities and identify active intestinal infection as opposed to parasite oocyst transport alone that could have resulted from ingestion of contaminated foodstuffs. The occurrence of C. parvum infection in the European hedgehog in the absence of clinical signs is noteworthy. To safeguard people against occupational zoonotic infection and prevent the spread of this parasite to uninfected animals, staff in wildlife centres should understand that infected

hedgehogs may appear clinically healthy with normalfaeces and should employ routine hygiene precautions when handling these animals or potentially-contaminated materials. The hedgehog also could be a reservoir or vector of C. parvum infection for livestock, the importance of which requires further investigation. Further work is needed to investigate the extent to which Cryptosporidium is a health threat to hedgehogs and what importance, if any, this parasite may have at a population-level.

5. Conclusion

We found infection with zoonotic subtypes of *C. parvum* to be widespread in the European hedgehog in Britain. These results should be taken into account by those handling this species, such as wildlife rehabilitators and appropriate hygiene measures should be taken. Similarly the possibility of hedgehogs acting as source of *C. parvum* infection of livestock should not be discounted. Surveillance of *Cryptosporidium* in free-ranging hedgehogs is warranted to further our knowledge of the epidemiology of this parasite in this species and our understanding of the individual and population impacts of infection on the hedgehog. The identification of novel subtypes in hedgehogs, and the difference between those found in the UK and mainland Europe, also warrant further investigation.

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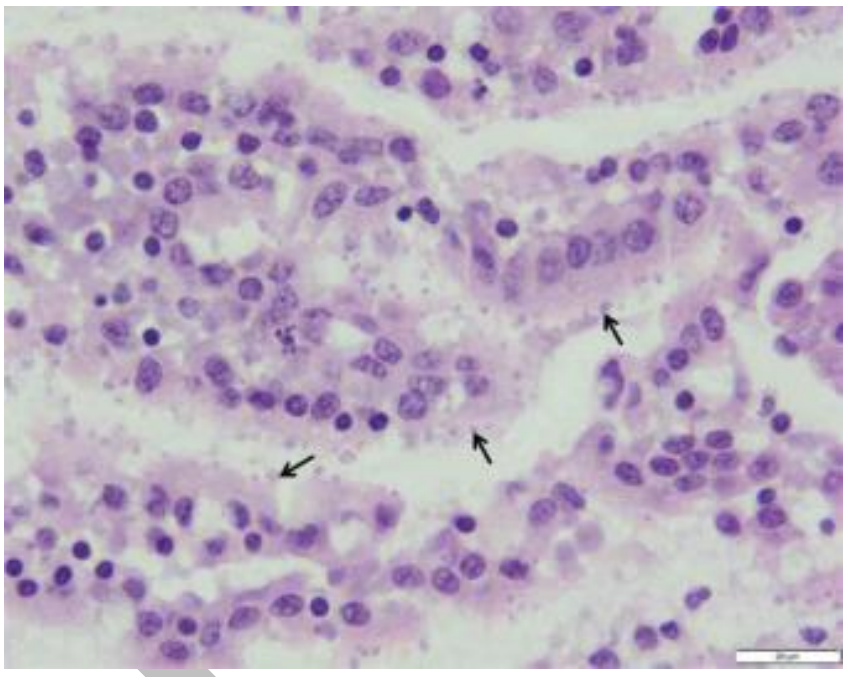
Figure legends

Figure 1. Map depicting Great Britain and the location of centres (A-I) where faecal samples were collected (black dots). Centres A-H are wildlife centres and centre I is the Institute of Zoology. The number of positive faecal samples over the total number of faecal samples screened from each centre is denoted in brackets. Pie charts indicate the percentage of faecal samples that were positive for *Cryptosporidium* based on a PCR targeting the 18S rRNA gene, confirmed by sequencing. The *gp60* subtypes found at each centre are also presented. The asterisk (*) indicates that this result is for the tissue sample taken from a hedgehog carcass at this centre.

561 **Figure 2**. European hedgehog (*Erinaceus europaeus)* cross-section of small intestine
562 (GI1XT0096-13) showing a large number of small round basophilic bodies (<2um diameter)
563 characteristic of *Cryptosporidium* oocysts lining the epithelial surfaces. Haemotoxylin and Eosin
564 stain. The black arrows indicate *Cryptosporidium* oocysts on the lining of the villi.



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