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Morphological and molecular characterisation of a mixed *Cryptosporidium muris/Cryptosporidium felis* infection in a cat.

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Short Communication

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Abstract

To date *Cryptosporidium muris* has been identified by microscopy and genotyping in cats in two studies. We report morphological and genetic evidence of a mixed *C. muris* and *C. felis* infection in a cat and provide the first histological, immunohistochemical, *in situ* hybridisation and genetic confirmation of a *C. muris* infection in the stomach of a cat. The cat suffered persistent diarrhoea after the initial consultation, which remained unresolved, despite several medical interventions. Further studies are required to determine the range, prevalence and clinical impact of *Cryptosporidium* species infecting cats.

Keywords: *Cryptosporidium muris*; *Cryptosporidium felis;* cat, mixed infection; morphology; genotyping.

1. Introduction

Cryptosporidium is a genus of protozoan parasites whose members can cause diarrhoea in many hosts including humans and domestic animals. Currently 23 species of *Cryptosporidium* are accepted as valid including *C. muris,* which infects rodents as its primary host and *C. felis* in cats (Xiao, 2010; Fayer et al., 2010).

Cryptosporidium spp. infection is relatively common in cats and epidemiological surveys conducted worldwide have reported that the prevalence in cats ranges from 0 to 29% (Lucio-Forster et al., 2010). This apparent variation in the rate of infection might be due, in part, to the method of detection (e.g. concentration of oocysts and direct light microscopy versus microscopy of stained smears or PCR), as well as the population being sampled (animal age differences, owned animals, stray populations, shelter animals) and symptomatic versus asymptomatic animals (Lucio-Forster et al., 2010).

Genetic characterisation of oocysts recovered from faecal samples of cats have identified *C. felis* (Ballweber et al., 2009; Palmer et al., 2008; Huber et al., 2007; Thomaz et al., 2007; Fayer et al., 2006; Santin et al., 2006; Morgan et al., 1998; Sargent et al., 1998; Gasser et al., 2001; Ryan et al., 2003; Hajdusek et al., 2004) and *C. muris* in two studies (Santin et al., 2006; Pavlasek and Ryan, 2007). The identification of *C. muris* in cats in the latter two studies was based on genotyping of oocysts recovered from faeces. No histological studies were conducted and therefore it was not possible to determine if the cats were actually infected with *C. muris* or were merely acting as mechanical

vectors. In the present study, we report on genetic, morphological and histological characterisation a mixed *C. muris/C. felis* infection in a cat.

2. Materials, Methods and Results

In 2008, a 2 year old male neutered domestic long haired cat presented for investigation of chronic diarrhoea. The clinical signs were characteristic of small bowel diarrhoea with an increased frequency of defecation. Appetite was normal and weight loss and vomiting were not features of his initial clinical presentation. Physical examination at the time of initial presentation was unremarkable. Screening haematology, biochemistry and urinalysis identified a mild increase in creatine kinase activity (413 U/L; reference range 50 – 100 U/L). Fasting feline trypsin-like immunoreactivity was normal (30 ug/L; control reference 12 – 82 ug/L). The cat tested negative for feline leukaemia virus and feline immunodeficiency virus (Simplify, AGEN Biomedical; Brisbane, Australia). Initial symptomatic therapy consisted of cobalamin (Vitamin B12, Troy, Australia) at 200 mg/kg by subcutaneous injection weekly for 6 treatments and dietary modification to increase the content of soluble fibre, however there was little response to these interventions. Further symptomatic therapy was trialled, including metronidazole (Flagyl, Sanofi Aventis, Spain) at 9.4 mg/kg every 12 hours for 10 days and fenbendazole (Panacur 100, Virbac Animal Health, Australia) at 50 mg/kg once daily per os for 5 days.

The cat re-presented 13 months later with continuing diarrhoea and he had also begun to vomit most days. An abdominal ultrasound was performed and identified mild

mesenteric lymphadenomegaly, mildly irregular splenomegaly and normal gastrointestinal wall thickness and layering. Fine needle aspirate cytology of the mesenteric lymph nodes and spleen identified mild reactivity in both locations. Gastroduodenoscopy showed that there were areas of marked gastric mucosal oedema, however the duodenal mucosa was unremarkable. Mucosal pinch biopsies were collected from the stomach and duodenum. The cat was prescribed empirical amoxycillin-clavulanate (Clavulox; Pfizer, Australia) at 13.9 mg/kg every 12h per os and a novel protein diet trial whilst results were pending.

Faecal samples were collected and examined using malachite green staining as previously described (Eliott et al., 1999). Parasites were examined with the aid of an ocular micrometer in a Zeiss Axioskop microscope at 1000 × magnification and this revealed the presence of two different sized *Cryptosporidium* sp. oocysts; large oocysts which resembled *C. muris* in size and shape (8.0 × 5.8 µm, mean width/length ratio 1.4, n=30) and smaller oocysts which resembled *C. felis* in size and shape (4.6 × 4.0 µm width/length ratio 1.15, n=20) (Fig. 3).

Endoscopic biopsy specimens from the stomach and duodenum were fixed in 10% neutral buffered formalin for 24 hours, then processed routinely and embedded in paraffin. Histological sections were cut at 5 µm and stained with hematoxylin and eosin. Microscopic examination of the stomach biopsies revealed the presence of abundant *Cryptosporidium* sp. organisms within the gastric pits and within the lumina of fundic glands. The affected glands were frequently dilated and filled with numerous *Cryptosporidium* spp. organisms (Figure 1, A-B). There was a mild increase in fibrous tissue within some areas of the lamina propria, leading to mild separation of glands

accompanied by a mild, multifocal lymphoplasmacytic and neutrophilic inflammatory cell infiltrate. In the duodenum, the *Cryptosporidium* sp. stages were closely associated with the apical surface of enterocytes (Figure 1, C). There was a mild, patchy increase in lymphocytes and plasma cells in the lamina propria along with low numbers of scattered neutrophils and a mild, multifocal increase in intraepithelial lymphocytes.

Approximately 1 µg of purified PCR product DNA (~500 bp) from the *C. muris* 18S rRNA gene, from a rodent-derived *C. muris* isolate, was labelled with digoxigenin to produce DNA probes for *in situ* hybridisation using the DIG-Nick Translation Mix, according to the manufacturer's instructions (Roche Diagnostics). The digoxigenin-labelled DNA was added to a probe cocktail mixture consisting of 50% formamide, 10% dextran sulfate and 2× SSC buffer. Sections were deparaffinised, rehydrated, probed, washed, developed, counter-stained and mounted as previously described (Bennett et al., 2008). An irrelevant DNA probe for bandicoot papillomatosis carcinomatosis virus type 1 was used as a negative control.

For immunohistochemistry, histological sections were dewaxed in xylene and rehydrated through graded ethanols to water. Endogenous peroxidase activity was then blocked using 3% hydrogen peroxide. The primary antibody, mouse anti-*Cryptosporidium* (Serotec MCA-2571), was diluted 1:200 with antibody diluent (DakoCytomation) and applied to tissue sections for 30 minutes. Following thorough rinsing with phosphate buffered saline (PBS), primary antibody binding was detected using a horseradish peroxidase-labeled streptavidin biotin system (LSAB – Dako) according to the manufacturer's instructions. Slides were rinsed in tap water and the slide

was counterstained lightly with Harris' hematoxylin. Omission of the primary antibody was used as a negative control.

In situ hybridisation and immunohistochemical experiments confirmed that the organisms deep within the lumina of scattered gastric glands (Fig. 2a – 2c) were indeed members of the genus *Cryptosporidium*. Immunohistochemistry also confirmed the presence of *Cryptosporidium* organisms in the duodenal biopsies, whilst *in situ* hybridisation was unsuccessful at this anatomical site.

DNA was extracted from intestine and stomach paraffin embedded biopsies using a Qiagen DNeasy tissue kit (Qiagen, Germany). DNA was eluted in 50 µL of AE buffer to concentrate the DNA. DNA was amplified at the 18S and actin loci using a nested PCR as previously described (Ryan et al., 2003; Ng et al., 2006). The amplified DNA fragments from the secondary PCR product were separated by gel electrophoresis and purified using the freeze-squeeze method (Ng et al., 2006). Purified PCR products were sequenced using an ABI Prism™ Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions with the exception that the annealing temperature was raised to 58ºC for the 18S and 55°C for the actin sequencing reaction. Nucleotide sequences were analyzed using Chromas lite version 2.0 (http://www.technelysium.com.au) and aligned with reference genotypes from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) using ClustalW (http://www.clustalw.genome.jp). Partial sequence analysis of a ~580 and ~818 base pair section of the 18S rRNA and actin gene loci, respectively, identified the *Cryptosporidium* species in the intestine as *C. felis* and the species in the stomach as *C. muris* (100% identities).

149 The cat was subsequently treated with 5.3 mg/kg of azithromycin every 12h per os for
150 2 weeks. Tylosin, a commonly recommended treatment for cryptosporidiosis, was
151 temporarily unavailable at time of diagnosis. The vomiting resolved and the diarrhoea
152 improved but persisted. Rechallenge with the previous diet led to reoccurrence of severe
153 vomiting and the novel protein diet was recommenced. The cat continued to maintain
154 body weight. Follow up faecal analysis conducted 12 months after initial faecal analysis
155 and collection of endoscopic biopsies demonstrated oocysts resembling *C. muris* in
156 morphology, while *C. felis* oocysts were not identified at this time.
157

158 **4. Discussion**

159

160 In the present study, morphological and genetic characterisation has confirmed the
161 presence of a mixed *C. muris* infection in the stomach and a *C. felis* infection in the
162 intestine of a cat. This is the first histological, immunohistochemical, *in situ* hybridisation
163 and genetic confirmation of a natural *C. muris* infection in a cat and only the third report
164 of *C. muris* in cats. *Cryptosporidium muris* has been found in many rodents (mice, wood
165 mice, rats, bank voles, Syrian hamsters, desert hamsters, squirrels, and Siberian
166 chipmunks), a marsupial (bilbies), other mammals (Bactrian camels, mountain goats,
167 reticulated giraffe, ringed seals, cats, rock hyraxes, cynomolgus monkeys, dogs, and pigs)
168 (Warren et al., 2003; Santin et al., 2006; Pavlasek and Ryan, 2007; Lupo et al., 2008;
169 Kodadkova et al., 2009, Kvac et al., 2009; Feng, 2010), and birds (tawny frogmouth) (Ng
170 et al., 2006). It has also been identified in a few humans in developing countries (Palmer

et al., 2003; Gatei et al., 2006; Muthusamy et al., 2006). Experimental *C. muris* infections have been reported in dogs, rabbits, lambs and cats (Iseki et al., 1989).

Cryptosporidium felis has a much more restricted host range and has been confirmed using molecular techniques in cats, immunocompetent and immunocompromised humans and a cow (Bornay-Llinares et al., 1999; Lucio-Forster et al., 2010). In children in developing countries, *C. felis* is responsible for as much as 3.3% of overall cryptosporidiosis cases (Lucio-Forster et al., 2010). However, most human cases of cryptosporidiosis, worldwide, are associated with *C. hominis* and *C. parvum* (Xiao et al., 2010) and therefore *C. muris* and *C. felis* in cats are likely to be of low zoonotic risk to humans. It has also been suggested that some *C. felis* infections in humans were anthroponotically transmitted (Cama et al., 2006). In the present study, the source of infection in the cat is unknown as the cat was acquired as a stray and there were several other pets in the household. There was no clinical evidence of diarrhoea in any other household members.

As the cat was infected with both *C. muris* and *C. felis*, it is difficult to attribute the clinical presentations to either species. The presence of mild inflammation accompanied by mild fibrosis in the stomach and inflammation within the duodenum in association with the *Cryptosporidium* spp. is suggestive of an ongoing host response secondary to the presence of the organisms, however contribution from other factors (such as concurrent food hypersensitivity) cannot be ruled out especially given the partial response to a novel protein diet.

In the present study, azithromycin was unsuccessful in resolving the diarrhoea. Tylosin, which was temporarily unavailable at time of diagnosis, has been used

194 successfully in cats but requires a long course of treatment (Barr and Bowman, 2006).
195 Nitazoxanide has also been shown to reduce oocyst shedding in cats (Barr and Bowman,
196 2006). There was no overt evidence of immunosuppression in this cat as it was feline
197 leukemia virus and feline immunodeficiency virus negative, yet 9 months after the initial
198 faecal analysis, the cat was still shedding *C. muris* but apparently not *C. felis* indicating a
199 persistent infection, however the possibility of reinfection cannot be discounted.
200 The present study has confirmed that *C. muris* naturally infects the stomach of cats
201 and therefore cats are not merely acting as mechanical vectors. Further studies are
202 required to determine the range, prevalence and clinical impact of *Cryptosporidium*
203 species infecting cats, and the status of the host immune system in persistent or recurrent
204 *Cryptosporidium* spp. infections.
205

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209 professional services and to the owner of the cat for provision of samples.
210

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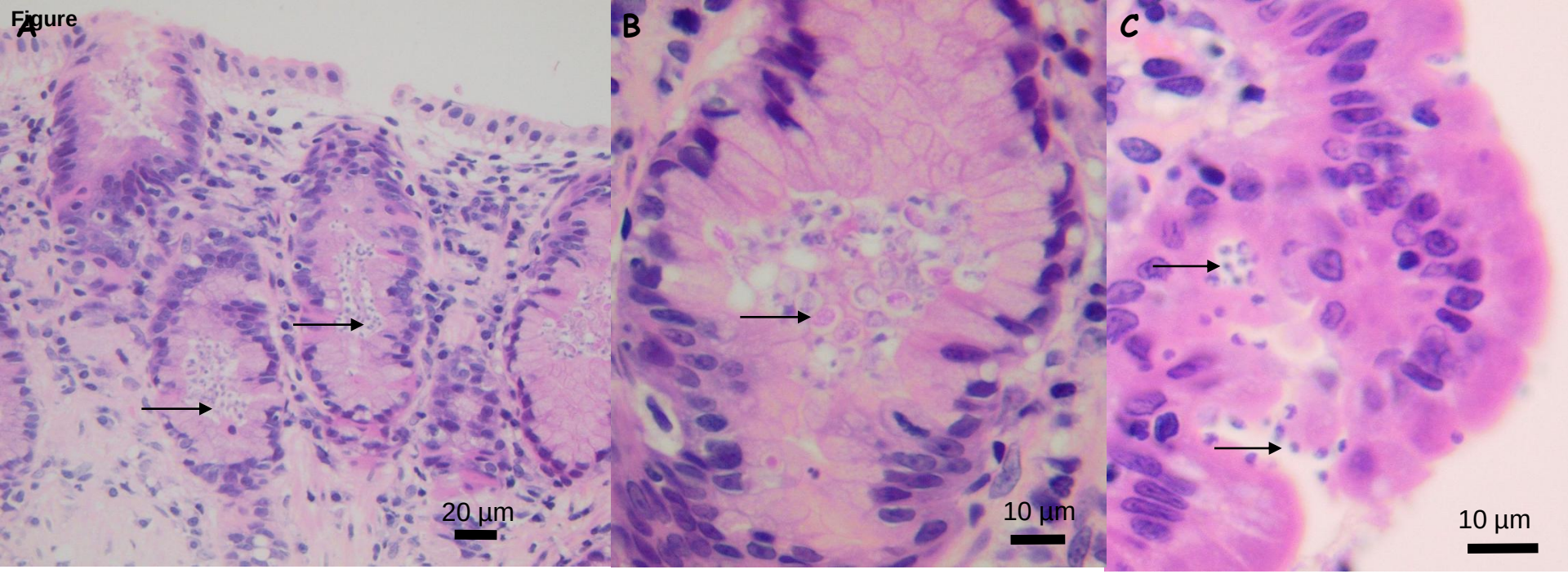
Figure 1. Hematoxylin & eosin stained sections of biopsies from the cat showing (A-B) clusters of *C. muris* stages located within the glands of the gastric mucosa; and (C) *C. felis* organisms along the enterocyte lining of the duodenum.

Figure 2. *In situ* hybridisation of digoxigenin-labelled *C. muris* 18S rRNA DNA probe on tissue sections from the cat stomach showing (A) parasitised and non-parasitised glands and (B) parasite stages deep within the glands. 2C. Immunohistochemistry on tissue sections from the cat stomach showing parasite stages deep within the glands.

Figure 3. Malachite green stained wet mount of cat faecal sample showing (A) *C. felis*-like oocysts and (B) *C. muris*-like oocysts.

Figure



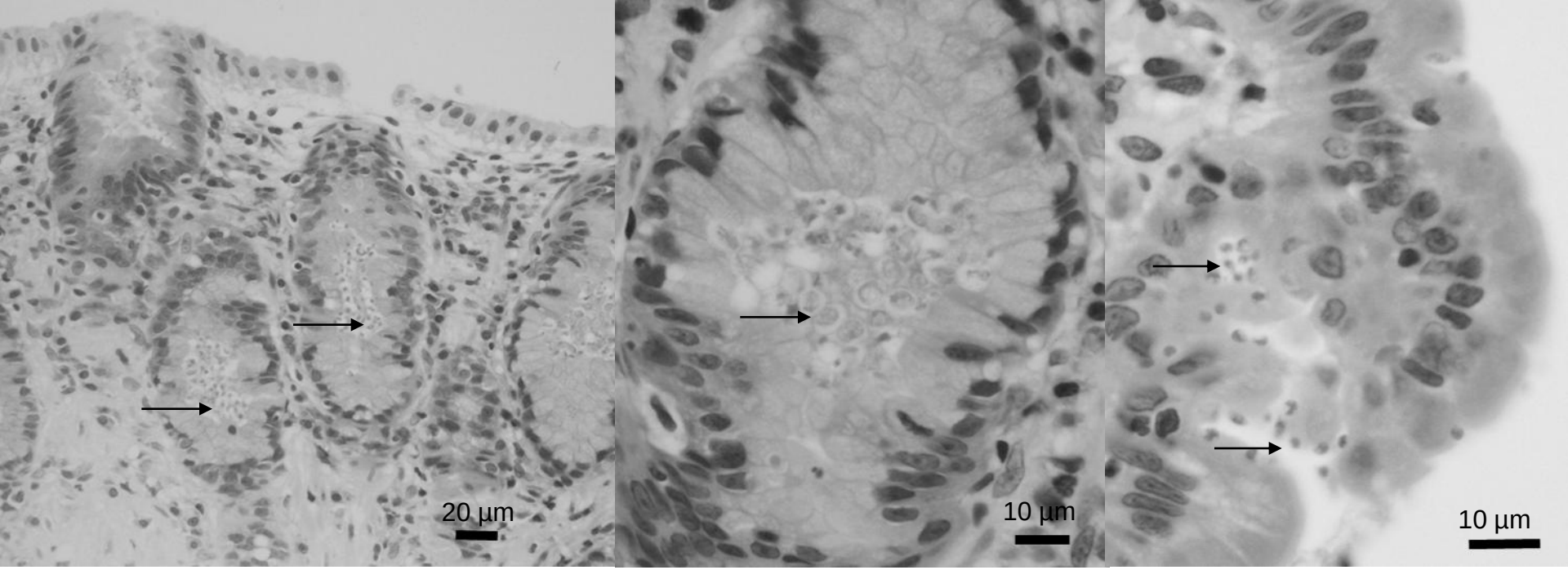

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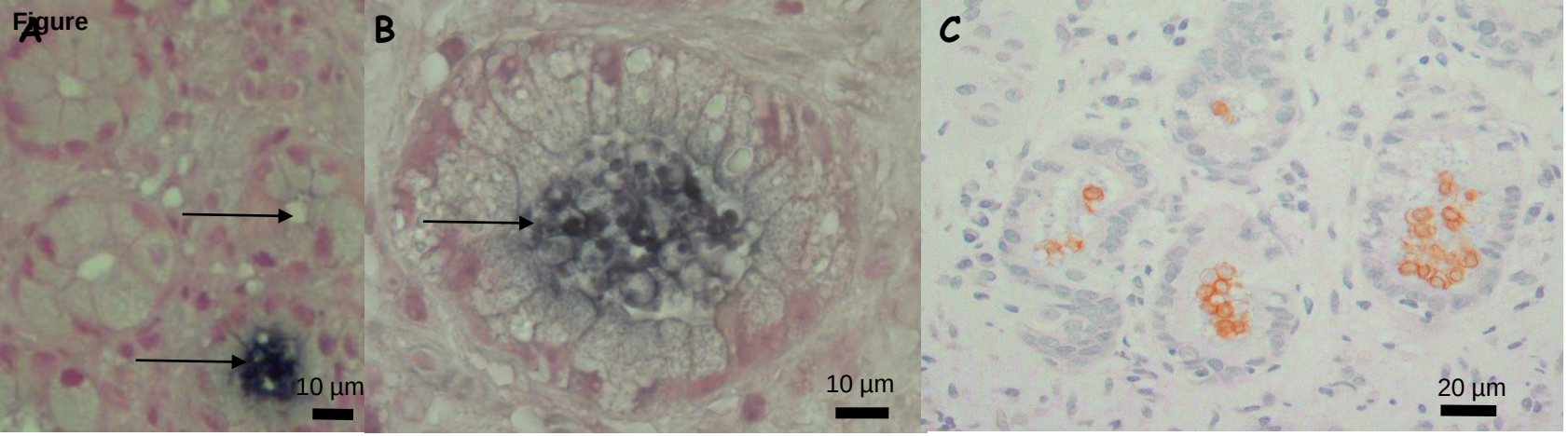
Figure



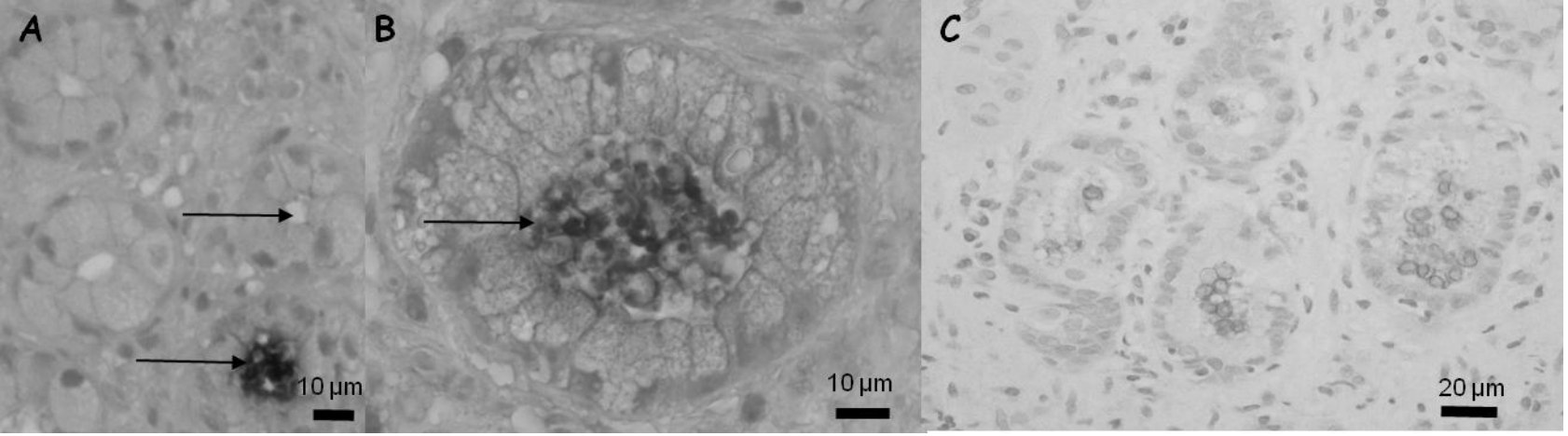

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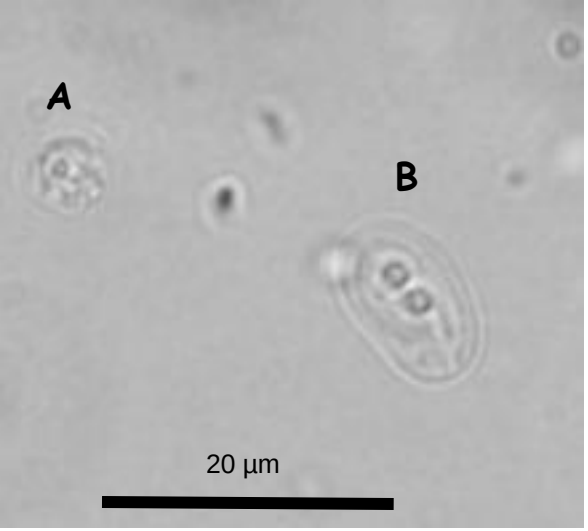
Figure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