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Diversity of *Cryptosporidium* in common voles and description of *Cryptosporidium alticolis* sp. n. and *Cryptosporidium microti* sp. n. (Apicomplexa: Cryptosporidiidae)

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Abstract

Fecal samples from wild-caught common voles ($n = 328$) from 16 locations in the Czech Republic were screened for *Cryptosporidium* by microscopy and PCR/sequencing at loci coding small-subunit rRNA, *Cryptosporidium* oocyst wall protein, actin and 70 kDa heat shock protein. *Cryptosporidium* infections were detected in 74 voles (22.6%). Rates of infection did not differ between males and females nor between juveniles and adults. Phylogenetic analysis revealed the presence of eight *Cryptosporidium* species/genotypes including two new species, *C. alticolis* and *C. microti*. These species from wild-caught common voles were able to infect common and meadow voles under experimental conditions, with a prepatent period of 3–5 days post-infection (DPI), but they were not infectious for various other rodents or chickens. Meadow voles lost infection earlier than common voles (11–14 vs 13–16 DPI) and had significantly lower infection intensity. *Cryptosporidium alticolis* infects the anterior small intestine and has larger oocysts ($5.4 \times 4.9 \mu\text{m}$), whereas *C. microti* infects the large intestine and has smaller oocysts ($4.3 \times 4.1 \mu\text{m}$). None of the rodents developed clinical signs of infection. Genetic and biological data support the establishment of *C. alticolis* and *C. microti* as separate species of the genus *Cryptosporidium*.

Keywords

Experimental infection; molecular analyses; oocyst size; phylogeny; Rodentia; voles

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Conflicts of interest. None.

Introduction

Cryptosporidium is an apicomplexan protist parasite that primarily infects the gastrointestinal epithelium of a broad range of vertebrate species including humans (Lv *et al.*, 2009). Infections can be asymptomatic or can result in diarrhoea ranging from mild to severe. Disease severity depends mainly on the age and immune status of the host (Checkley *et al.*, 2015; Baneth *et al.*, 2016). Field studies have shown that genus *Cryptosporidium* is genetically diverse, with much of that diversity found in wildlife. Rodents are ubiquitous mammals comprising about 40% of mammalian diversity and occupying a wide range of habitats. Studies to date have shown that rodent species are predominantly parasitized with host-specific *Cryptosporidium* species and genotypes (Feng *et al.*, 2007; Foo *et al.*, 2007; Ziegler *et al.*, 2007a; Kvá *et al.*, 2008, 2013; Feng, 2010; Ng-Hublin *et al.*, 2013; Stenger *et al.*, 2015a, 2015b, 2018), although zoonotic species such as *C. parvum* and *C. ubiquitum* (Hajdušek *et al.*, 2004; Rašková *et al.*, 2013; Li *et al.*, 2014; Perec-Matysiak *et al.*, 2015) and livestock-specific species such as *C. scrofarum*, *C. andersoni* and *C. baileyi* (Ziegler *et al.*, 2007a; Lv *et al.*, 2009; Ng-Hublin *et al.*, 2013; Danišová *et al.*, 2017) have been reported. Despite a large number of studies, the diversity and biology of *Cryptosporidium* in several rodent hosts, including voles, have not been thoroughly characterized (Kvá *et al.*, 2014; Stenger *et al.*, 2018).

Early studies, relying on oocyst morphology to distinguish species, reported *C. parvum*, *C. muris* and *Cryptosporidium* sp. in voles (Chalmers *et al.*, 1997; Torres *et al.*, 2000; Sinski *et al.*, 1993, 1998; Bull *et al.*, 1998; Bajer *et al.*, 2002; Bednarska *et al.*, 2007). In more recent studies of voles, using more discriminatory genotyping tools to distinguish species, the prevalence of *C. parvum* was much lower than previously reported and *C. muris* was not detected. Additionally, common voles were not susceptible to *C. muris*, *C. proliferans* or *C. andersoni* under experimental conditions (Modrý *et al.*, 2012). In contrast, *Cryptosporidium* muskrat genotypes I and II and *Cryptosporidium* isolates closely related to muskrat genotypes I and II have been reported frequently (online Supplementary Table S1). In the most recent study, the largest to date, Stenger *et al.* (2018) reported greater diversity of *Cryptosporidium* spp. infecting North American and European voles than previously known. They identified at least 18 different *Cryptosporidium* spp. by sequencing of the partial sequence of the small ribosomal subunit rRNA and actin genes in European and North American voles, and most of these were identified for the first time. Phylogenetic analyses indicated the *Cryptosporidium* spp. infecting voles from the different continents remained closely related (Stenger *et al.*, 2018). Collectively, data from studies on voles show that they are host to at least 20 *Cryptosporidium* species and genotypes (see online Supplementary Table S1). Most of the genotypes lack biological data such as course of infection and host range.

We undertook the present study to extend knowledge of the occurrence and diversity of *Cryptosporidium* spp. infecting the common vole (*Microtus arvalis*). We selected two isolates from wild-caught common voles and, in accordance with ICZN nomenclature rules and criteria established by the scientific community studying *Cryptosporidium* (Xiao *et al.*, 2004; Jirk *et al.*, 2008; Fayer, 2010), we describe the morphometry of oocysts, determine phylogenetic relatedness at multiple genetic loci and report on the infectivity for several

hosts (voles, laboratory and yellow-necked mice, laboratory rats and chickens) under natural and experimental conditions. Outcomes from the study support the conclusion that the *Cryptosporidium* isolates are genetically and biologically distinct from previously described species. We therefore propose them as new species named *Cryptosporidium alticolis* sp. n. and *Cryptosporidium microti* sp. n.

Material and methods

Area and specimens studied

From 2014 to 2017 (May to September each year), wild-caught common voles were trapped using snap traps baited with apple and peanut at 16 locations in the Czech Republic (Fig. 1). After trapping, we identified the species, measured body mass (± 1 g) and determined the sex of each individual. We estimated the age of each individual using body mass, such that an individual weighing <15 g was considered a juvenile and all other animals were considered adults. Following collection, we dissected each individual and collected a fecal sample from the colon. Fecal samples were stored at 4 °C without fixation. All fecal samples were screened for the presence of *Cryptosporidium* oocysts using the aniline–carbol–methyl violet (ACMV) staining (Miláček and Vítovec, 1985) followed by microscopic examination at 1000 \times magnification (light microscope Olympus BX51, Tokyo, Japan). During microscopic examination, we counted oocysts and we quantified the infection intensity as number of oocysts per gram of feces (OPG) according to Kváč et al. (2007).

Molecular characterization

DNA was extracted from 200 mg of feces by bead disruption for 60 s at 5.5 m s⁻¹ using 0.5 mm glass beads in a Fast Prep 24 Instrument (MP Biomedicals, Santa Ana, CA, USA) followed by isolation and purification using a commercially available kit in accordance with the manufacturer's instructions (PSP spin stool DNA Kit, Invitex, Stratec, Berlin, Germany). Purified DNA was stored at -20 °C prior to amplification by PCR.

A nested PCR approach was used to amplify a partial region of the small ribosomal subunit rRNA (SSU; ~830 bp; Xiao et al., 1999; Jiang et al., 2005), actin (~1066bp; Sulaiman et al., 2002), *Cryptosporidium* oocyst wall protein (COWP) (~550 bp; Spano et al., 1997) and 70 kilodalton heat shock protein genes (HSP70; ~ 1950 bp; Sulaiman et al., 2000).

The primary PCR mixtures contained 2 μ L of template DNA, 2.5 U of *Taq* DNA Polymerase (Dream Taq Green DNA Polymerase, Thermofisher Scientific, Waltham, MA, USA), 0.5 \times PCR buffer (SSU) or 1 \times PCR buffer (actin, COWP and HSP70; Thermofisher Scientific), 6 mM MgCl₂ (SSU) or 3 mM MgCl₂ (actin, COWP and HSP70), 200 μ M each deoxynucleoside triphosphate, 100 mM each primer and 2 μ L non-acetylated bovine serum albumin (BSA; 10 mg ml⁻¹; New England Biolabs, Beverly, MA, USA) in 50 μ L reaction volume. The secondary PCR mixtures were similar to those described above for the primary PCR, with the exception that 2 μ L of the primary PCR product was used as the template, the MgCl₂ concentration was 3 mM and no BSA was used. DNA of *C. parvum* and molecular grade water were used as positive and negative controls, respectively. Secondary PCR products were detected by 2% agarose gel electrophoresis, visualized by ethidium bromide

staining and extracted using GenElute™ Gel Extraction Kit (Sigma-Aldrich, St. Louis, MO, USA). Purified secondary products were sequenced in both directions with an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the secondary PCR primers and the BigDye1 Terminator v3.1 cycle sequencing kit (Applied Biosystems) in 10 μ L reactions.

Phylogenetic analysis

The nucleotide sequences of each gene obtained in this study were edited using the ChromasPro 2.4.1. (Technelysium, Pty, Ltd, South Brisbane, Australia) and aligned with each other and with reference sequences from GenBank using MAFFT version 7 online server using the Q-INS-I algorithm (<http://mafft.cbrc.jp/alignment/software/>). Alignment adjustments were made manually to remove artificial gaps using BioEdit 7.0.5.3 (Hall, 1999). Phylogenetic analyses were performed and the best DNA/protein phylogeny models were selected using the MEGA7 software (Guindon and Gascuel, 2003; Tamura *et al.*, 2013) and Geneious v7.1.7 (<http://www.geneious.com>). Phylogenetic trees were inferred by maximum likelihood (ML) method, with the substitution model that best fits the alignment selected using the Bayesian information criterion. ML analysis of SSU, actin, COWP and HSP70 alignments was done in the MEGA7 software and concatenated SSU–actin–COWP alignment was done in RAxML v7.2.8 implemented in Geneious. The General Time Reversible model was selected for SSU, actin, HSP70 and concatenated SSU–actin–COWP alignment and the Tamura 3-parameter model was used of COWP alignment. All models were used under an assumption that rate variation among sites was γ distributed with invariant sites.

Bootstrap support for branching was based on 1000 replications. Phylograms were edited for style using CorelDrawX7. Sequences have been deposited in GenBank under the accession numbers (Acc. nos.) MH145308–MH145335.

Origin of specimens for transmission studies

Isolates of *C. alticolis* sp. n. and *C. microti* sp. n. were obtained from wild-caught common voles trapped at Da ice and Radimovice, respectively, in the Czech Republic. Oocysts from each species were used to infect a 6-month-old common vole (vole 0). Oocysts from vole 0 were purified using caesium chloride gradient centrifugation (Arrowood and Donaldson, 1996) and used for analysis of oocyst morphometry and to infect other animals (see below).

Transmission studies

We experimentally determined the infectivity and pathogenicity of *C. alticolis* sp. n. and *C. microti* sp. n. for 6-month-old common voles, meadow voles (*Microtus pennsylvanicus*) and yellow-necked mice (*Apodemus flavicollis*); 2-month-old SCID (severe combined immunodeficiency), BALB/c and C57BL/6J mice (*Mus musculus*) and brown rats (*Rattus norvegicus*); and 3-day-old chickens (*Gallus gallus* f. *domestica*). Common voles and yellow-necked mice used for infectivity studies were obtained from captive colonies maintained at the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, eské Bud jovice, Czech Republic. Laboratory (i.e. house mouse) mice and rats were purchased from Charles River Laboratories, Sulzfeld, Germany. Chickens

originated from International Testing of Poultry, Ústřední, Tábor, Czech Republic. Meadow voles were obtained from a captive colony maintained at Smith College, Northampton, Massachusetts, USA and used in transmission studies at North Dakota State University, USA. All other experiments were performed at the Biology Centre of the Academy of Sciences of the Czech Republic. In determining infectivity and pathogenicity, we used five individuals from each species/group. A week prior to inoculation, fecal samples from all individuals were screened daily for the presence of *Cryptosporidium* oocysts and specific DNA of *Cryptosporidium* spp. using parasitological and molecular tools (SSU) as described above. Individuals were housed separately in plastic cages with sterilized bedding and supplied with a sterilized diet and water *ad libitum*. Each animal was inoculated orally by gavage with 100 000 purified oocysts suspended in 200 μL of distilled water. Fecal samples from each individual were screened daily for the presence of *Cryptosporidium* oocysts using ACMV staining and specific DNA using nested PCR targeting the SSU gene. At least three amplicons of each target gene were sequenced directly in both directions from each infected individual.

All experiments were terminated 30 days post-infection (DPI). Course of infection indicators, including fecal consistency, fecal colour and infection intensity, was examined.

Histopathological and scanning electron microscopy examinations

The gastrointestinal tract of one animal from each group was examined following necropsy at 6 DPI (this time was selected based on preliminary results; data not shown). The entire small and large intestine was divided into 1 cm sections and samples were processed for histology, scanning electron microscopy (SEM) and PCR/sequencing. Specimens for histology were fixed in 4% buffered formalin and processed by the usual paraffin method. Histological sections (5 μm) were stained with haematoxylin and eosin and periodic acid–Schiff stains. The specimens for SEM were fixed overnight at 4 °C in 2.5% glutaraldehyde in 0.1 M phosphate buffer, washed three times for 15 min in the same buffer, post-fixed in 2% osmium tetroxide in 0.1 M phosphate buffer for 2 h at room temperature and finally washed three times for 15 min in the same buffer. After dehydration in a graded acetone series, specimens were dried using the critical point technique, coated with gold and examined using a JEOL JSM-7401F-FE SEM.

Oocyst morphometry

Oocysts of *C. alticolis* sp. n. and *C. microti* sp. n. were examined using differential interference contrast (DIC) microscopy, ACMV staining and fluorescence microscopy (Olympus IX70, Tokyo, Japan) following labelling with genus-specific FITC-conjugated antibodies (*Cryptosporidium* IF Test, Crypto Cell, Medac, Wedel, Germany). Morphometry of oocysts was determined using digital analysis of images (M.I.C. Quick Photo Pro v.3.1 software; Promicra, s.r.o., Praha, Czech Republic) collected using an Olympus Digital Colour Camera DP73. Length and width of 50 oocysts of each isolate were measured under DIC at 1000 \times magnification and the ratio of the length/width of each oocyst was calculated. The mean and standard deviation (S.D.) of length, width and ratio of the length/width of oocysts of each isolate were calculated.

Animal care

Animal caretakers wore disposable coveralls, shoe covers and gloves whenever entering the rooms where animals were housed. All wood-chip bedding, feces and disposable protective clothing were sealed in plastic bags, removed from the buildings and incinerated at the end of the study.

Statistical analysis

Prevalence was calculated by dividing the number of positive individuals by the total number of individuals sampled. Differences in *Cryptosporidium* prevalence were determined by χ^2 analysis using a 5% significance level. The hypothesis tested in the analysis of oocyst morphometry was that two-dimensional mean vectors of measurement are the same in the two populations being compared. Hotelling's T2 test was used to test the null hypothesis. Analyses were performed using program Epi Info (TM) 7.1.1.14 (Centers for Disease Control and Prevention, GA, USA) and R 3.5.0. (<https://www.r-project.org/>).

Results

Prevalence and infection intensity of *Cryptosporidium*

Out of 328 fecal samples from wild-caught common voles, 19 (5.8%) were microscopically positive for the presence of oocysts of *Cryptosporidium* sp. and 74 (22.6%) were positive for the presence of specific DNA of *Cryptosporidium* spp. (Table 1). All microscopically positive samples were also positive using PCR. Positive voles were trapped at 11 out of 16 localities (Table 2). There was no difference ($\chi^2 = 0.0153$; D.F. = 1; $P = 0.9016$) in the prevalence of *Cryptosporidium* spp. in males (22.0%; 41/186) and females (23.2%; 33/142). Similarly, the prevalence did not differ ($\chi^2 = 0.3254$; D.F. = 1; $P = 0.5684$) between juvenile (25.7%; 19/74) and adult voles (21.7%; 55/254; Table 2). Infection intensity, which ranged from 4000 to 42 000 OPG, did not differ ($P = 0.1773$) between males (2000–36 000 with mean 15 000 OPG) and females (4000–42 000 with mean 20 000 OPG). None of the trapped voles had diarrhoea.

Out of 74 voles positive for *Cryptosporidium*, 74,71,33 and 14 were genotyped by sequence analysis of SSU, actin, COWP and HSP70 genes, respectively (Table 2, Fig. 2 and online Supplementary Figs S1–S4). The remaining positive samples yielded sequences of insufficient quality to include in analyses (three actin sequences) or failed to amplify at COWP ($n = 41$) and HSP70 ($n = 60$) loci.

Sequence analysis revealed the presence of eight genotypes of *Cryptosporidium*, of which two are described here as new species (Table 2). ML trees inferred from sequences of SSU, actin, COWP and HSP70 genes individually or SSU, actin and COWP in concatenation formed three major phylogenetic groups (Fig. 2 and online Supplementary Figs S1–S4). Group 1 included *C. microti* sp. n. and *Cryptosporidium* vole genotypes II, III, VI and VII. *Cryptosporidium microti* ($n = 47$) was identical to *Cryptosporidium* sp. isolate 19608-Miar-EU previously recovered from a wild-caught common vole in the Czech Republic [Acc. No. KY657290] and was closely related to *Cryptosporidium* muskrat genotype II [Acc. No. AY737571], *Cryptosporidium* sp. isolate 1857-Mipe-NA from a wild-caught meadow vole

[Acc. No. KY644574] and *Cryptosporidium* sp. isolate 1544-Pero-NA from a wild-caught *Peromyscus* mouse [Acc. No. KY644565] in the USA, sharing 99.2%, 98.8% and 98.6% sequence identity, respectively.

Cryptosporidium vole genotype III ($n = 1$) was identical to *Cryptosporidium* sp. isolate 20062-Miar-EU from a wild-caught common vole in the Czech Republic (Acc. No. KY644593) and clustered with *Cryptosporidium* sp. isolate 10482-Mygl-EU from a wild-caught bank vole (Acc. No. KY644595) and *Cryptosporidium* sp. isolate 2035-Myga-NA from a wild-caught Southern red-backed vole (Acc. No. KY644592) in Slovakia and the USA, respectively, sharing 99.8 and 99.5% sequence identity.

Cryptosporidium vole genotype VI ($n = 5$) was identical to *Cryptosporidium* sp. isolate 24129-Miar-EU from a wild-caught common vole in the Czech Republic (Acc. No. KY644632) and clustered with *Cryptosporidium* vole genotype II ($n = 1$) from the present study (Acc. No. MH145334), sharing 99.1% sequence identity. *Cryptosporidium* vole genotype VII ($n = 5$), a genotype that was first identified in this study, clustered with the *Cryptosporidium* vole genotype (Acc. No. EF641020) and *Cryptosporidium* sp. isolate 1947-Mipe-NA (Acc. No. KY644626), both from wild-caught meadow voles in the USA, sharing 98.9 and 98.5% sequence identity, respectively. *C. alticolis* sp. n. ($n = 7$), the only member of group 2, was identical to *Cryptosporidium* sp. isolate 20065-Miar-EU from a wild-caught common vole in the Czech Republic (Acc. No. KY644657), and clustered with *Cryptosporidium* sp. isolate 2333-Pero-NA from a wild-caught meadow vole in the USA (Acc. No. KY644655) and *Cryptosporidium* sp. isolate Mrb001 from a grey red-backed vole in Japan (Acc. No. AB477098), sharing 97.3 and 97.5% sequence identity, respectively.

Group 3 comprised *Cryptosporidium* vole genotype IV ($n = 3$) and vole genotype V ($n = 5$). *Cryptosporidium* genotype vole V was identical to *Cryptosporidium* sp. isolate 24916-Miar-EU from a wild-caught common vole in the Czech Republic (Acc. No. KY644670) and formed a sister group with muskrat genotype I (Acc. No. EF641013) and *Cryptosporidium* sp. isolate 1962-Mipe-NA from a wild-caught meadow vole (Acc. No. KY644685), both in the USA, sharing 98.1 and 98.0% sequence identity, respectively. *Cryptosporidium* vole genotype IV, which was reported for the first time in this study, clustered outside of this group.

Based on evidence that they are genetically and biologically distinct from known *Cryptosporidium* species, we describe *C. alticolis* sp. n. and *C. microti* sp. n. as new species of the genus *Cryptosporidium*. Descriptions of *C. alticolis* sp. n. and *C. microti* sp. n. follow.

***Cryptosporidium alticolis* sp. n.**

Prevalence and infection intensity.—Seven voles (2.1%) from three localities had DNA of *C. alticolis* sp. n. detectable by PCR, of which three had oocysts that were detectable by microscopy with an infection intensity of 2000–8000 OPG (Table 2).

Experimental transmission.—Oocysts of *C. alticolis* sp. n. from naturally infected common voles were infectious for common and meadow voles, but not for yellow-necked mice, SCID mice, BALB/c mice, C57BL/6J mice, brown rats or chickens. The prepatent

period of *C. alticolis* sp. n. in common and meadow voles was 3–4 DPI (Fig. 3). Whereas common voles shed oocysts of *C. alticolis* sp. n. continuously during the patent period (12–15 DPI), meadow voles shed oocysts sporadically up to 12 DPI (Fig. 3). The infection intensity of *C. alticolis* sp. n. in common voles (2000–1000 000 OPG) was higher than in meadow voles (2000–50 000 OPG). No macroscopical changes were observed in the gastrointestinal tract of common or meadow voles infected with *C. alticolis* sp. n. and the surface epithelium remained intact. DNA of *C. alticolis* sp. n. was detected throughout the small and large intestine of common and meadow voles; however, endogenous developmental stages were detected only in the jejunum and ileum by histology and electron microscopy (Figs 4 and 5). *Cryptosporidium alticolis* sp. n. was not detected in the stomach and other organs (liver, pancreas, kidneys, lungs and spleen). None of the experimentally infected common or meadow voles were diarrhoeic. The *lamina propria* in the jejunum and ileum was slightly oedematous with occasional dilatation of lymphatic vessels (data not shown). Sequences of SSU, actin, COWP and HSP70 genes from experimentally infected hosts shared 100% identity with the isolate used in the inoculum.

Taxonomic summary

ZooBank number for species: [urn:lsid:zoobank.org:act:D12C78AA-222E-4E07-A7CE-51AA6A747BC6](https://www.zoobank.org/urn:lsid:zoobank.org:act:D12C78AA-222E-4E07-A7CE-51AA6A747BC6)

Description: Oocysts are shed fully sporulated with four sporozoites and an oocyst residuum. Sporulated oocysts ($n = 50$) measure $4.9\text{--}5.7\ \mu\text{m}$ (mean \pm S.D. = $5.4 \pm 0.2\ \mu\text{m}$) \times $4.6\text{--}5.2\ \mu\text{m}$ (mean \pm S.D. = $4.9 \pm 0.2\ \mu\text{m}$) with a length/width ratio of 1.00–1.20 (mean \pm S.D. = 1.10 ± 0.05) (Fig. 6). Morphology and morphometry of other developmental stages are unknown.

Type host: common vole (*M. arvalis*)

Type locality: Dačice (Czech Republic)

Other localities: Masáková Lhota and Všechnov (Czech Republic)

Site of infection: jejunum and ileum (Figs 4 and 5)

Distribution: Czech Republic

Type material/hapantotype: Tissue samples in 10% formaldehyde and histological sections of infected jejunum (nos. 174/2016, 175/2016, 176/2016 and 177/2016) and ileum (nos. 178/2016 and 179/2016); genomic DNA isolated from fecal samples of naturally (isolation no. 23111) and experimentally (isolation no. 27124) infected *M. arvalis*; genomic DNA isolated from jejunal and ileal tissue of experimentally infected *M. arvalis* (isolation nos. 27035 and 27037, respectively); digital photomicrographs (nos. DIC 1–13/23111, MV 1–11/23111, IF 1–9/23111, HI 1–3/27124 and SEM 1–3/27124) and fecal smear slides with oocysts stained by ACMV staining from experimentally infected *M. arvalis* (nos. 27124/3, 27124/4, 27124/5 and 27124/6). Specimens deposited at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic.

Reference sequences: Partial sequences of SSU, actin, COWP and HSP70 genes were deposited at GenBank under Acc. Nos. MH145330, MH145310, MH145318 and MH145324, respectively.

Etymology: The species name *alticolis* is derived from the Latin noun 'alticola' (meaning a vole).

Differential diagnosis: Oocysts of *C. alticolis* are larger than those of *C. microti* ($P = 0.001$), have similar ACMV staining to other species of *Cryptosporidium* and cross-react with antibodies developed primarily for *C. parvum* (Fig. 6). It can be differentiated genetically from other *Cryptosporidium* spp. based on sequences of SSU, actin, COWP and HSP70 genes. Endogenous development of *C. alticolis* sp. n. takes place in the small intestine, whereas *C. microti* develops in the large intestine.

Cryptosporidium microti sp. n.

Prevalence.

Forty-seven wild-caught common voles (14.3%) from nine localities were positive for *C. microti* sp. n. by PCR, of which 12 had oocysts detectable by microscopy. The infection intensity ranged from 4000 to 42 000 OPG.

Experimental transmission.

Oocysts of *C. microti* sp. n. from naturally infected common voles were infectious for common and meadow voles, but not for yellow-necked mice, SCID mice, BALB/c mice, C57BL/6J mice, brown rats or chickens. Common voles shed *C. microti* sp. n. from 4 to 16 DPI, with oocysts detectable by microscopy throughout this period. The infection intensity ranged from 2000 to 430 000 OPG with maximum shedding at 6–7 DPI (Fig. 7). In meadow voles, DNA of *C. microti* sp. n. was detected from 4 to 14 DPI; however, oocysts were not detectable by microscopy at any time during the patent period.

Sequences of SSU, actin, COWP and HSP70 genes from experimentally infected hosts shared 100% identity with the isolate used in the inoculum. Specific DNA of *C. microti* sp. n. was found exclusively in the caecum and colon of common and meadow voles. Endogenous developmental stages were detected in the caecum and colon of the common vole (Figs 8 and 9), but were not detected in the meadow vole. Infections were not associated with macroscopical or pathological changes in the digestive tract of common or meadow voles and these animals showed no signs of diarrhoea.

Taxonomic summary

ZooBank number for species: [urn:lsid:zoobank.org:act:4FD6136C-3932-4881-BE49-4714A5AB488A](https://www.zoobank.org/urn:lsid:zoobank.org:act:4FD6136C-3932-4881-BE49-4714A5AB488A)

Description: Oocysts are shed fully sporulated with four sporozoites and an oocyst residuum. Sporulated oocysts ($n = 50$) measure $3.9\text{--}4.7\ \mu\text{m}$ (mean \pm S.D. = $4.3 \pm 0.1\ \mu\text{m}$) \times $3.8\text{--}4.4\ \mu\text{m}$ (mean \pm S.D. = $4.1 \pm 0.1\ \mu\text{m}$) with length/width ratio of 1.00–1.06 (mean \pm S.D.

= 1.03 ± 0.02) (Fig. 10). Morphology and morphometry of other developmental stages are unknown.

Type host: common vole (*M. arvalis*)

Type locality: Radimovice (Czech Republic)

Other localities: Da ické, Z atky, Sedle ko, Dolní T ebonin, Pelejovice, Masakova Lhota, Všechnov and Opatovice (Czech Republic)

Site of infection: caecum and colon (Figs 8 and 9)

Distribution: Czech Republic

Type material/haplotypes: Tissue samples in 10% formaldehyde and histological sections of infected caecum (nos. 97/2016 and 98/2016) and colon (nos. 99/2016 and 100/2016), genomic DNA isolated from fecal samples of naturally (isolation no. 24923) and experimentally (isolation no. 28063) infected *M. arvalis*; genomic DNA isolated from caecal and colonic tissue of experimentally infected *M. arvalis* (isolation nos. 29751 and 29753, respectively); digital photomicrographs (nos. DIC 1–11/24923, MV 1–9/24923, IF 1–9/24923, HI 1–3/28063 and SEM 1–3/28063) and fecal smear slides with oocysts stained by ACMV staining from experimentally infected *M. arvalis* (nos. 28063/3, 28063/4, 28063/5 and 28063/6). Specimens deposited at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic.

Reference sequences: Partial sequences of SSU, actin, COWP and HSP70 genes were deposited at GenBank under Acc. Nos. MH145328, MH145308, MH145316 and MH145323, respectively.

Etymology: The species name *microti* is derived from the Latin noun ‘microtus’ (meaning a vole).

Differential diagnosis: Oocysts of *C. microti* sp. n. are smaller than those of *C. alticolis* sp. n. ($P = 0.001$), have similar ACMV staining to other species of *Cryptosporidium* and cross-react with antibodies developed primarily for *C. parvum* (Fig. 10). It can be differentiated genetically from other *Cryptosporidium* spp. based on sequences of SSU, actin, COWP and HSP70 genes. Endogenous development of *C. microti* sp. n. takes place in the large intestine, whereas *C. alticolis* sp. n. develops in the small intestine.

Discussion

This and other genotyping studies have shown that voles host several *Cryptosporidium* species and genotypes that appear to be host specific and not infectious for humans, but they rarely host *C. parvum* (Feng *et al.*, 2007; Stenger *et al.*, 2018; Ziegler *et al.*, 2007a, 2007b). The finding that oocysts of *C. alticolis* sp. n. and *C. microti* sp. n. are indistinguishable from oocysts of *C. parvum* suggests that earlier detections of *C. parvum*, which were not supported by genotyping data, were misidentifications. Oocyst size is generally only useful

for differentiating intestinal (smaller and rounder) and gastric (larger and more oval) species of *Cryptosporidium* (Ryan and Xiao, 2014).

Cryptosporidium microti sp. n. and *Cryptosporidium* vole genotypes II, III, VI and VII clustered as part of a large heterogeneous group in ML trees. This is generally consistent with the report by Stenger *et al.* (2018) that *Cryptosporidium* genotypes from voles in the Europe and North America formed between three and four phylogenetic groups in ML trees.

Cryptosporidium alticolis sp. n. and *C. microti* sp. n. are genetically distinct from other known species of *Cryptosporidium*. *Cryptosporidium alticolis* sp. n. shares 95.2, 94.7 and 94.3% sequence identity, respectively, with *C. canis*, *C. suis* and *C. parvum* at the SSU locus; 87.9, 90.5 and 89.7%, respectively, at the actin locus; and 84.5, 91.2 and 90.5%, respectively, at the HSP70 locus. At the COWP locus, *C. alticolis* sp. n. shared 88.1 and 89.9% sequence identity, respectively, with *C. canis* and *C. parvum*. *Cryptosporidium microti* sp. n. shared 95.5, 98.8 and 96.4% sequence identity, respectively, with *C. canis*, *C. suis* and *C. parvum* at the SSU locus; 85.6, 91.6 and 90.5%, respectively, at the actin locus; and 84.2, 93.1 and 92.6%, respectively, at the HSP70 locus. At the COWP locus, *C. microti* sp. n. shared 86.7 and 91.5% sequence identity, respectively, with *C. canis* and *C. parvum*. In comparison, *C. hominis* and *C. parvum* share 98–99% identity and *C. muris* and *C. andersoni* share 96–99% identity at these loci.

The prevalence of *Cryptosporidium* in voles ranges from 1 to 100% (Laakkonen *et al.*, 1994; Perz and Le Blancq, 2001; Bajer *et al.*, 2002, 2003; Zhou *et al.*, 2004). The prevalence in wild-caught common voles in the present study (23%) was greater than the 14% reported by Stenger *et al.* (2018) using similar detection methods, and much lower than the 62–73% reported by Bajer *et al.* (2002) and Bajer (2008) using microscopic detection, a method that is less sensitive than PCR. The prevalence of *Cryptosporidium* can be affected by factors such as age, season, population density, location, weather and climate, diet and water consumption (Nichols *et al.*, 2014).

Cryptosporidium microti sp. n. dominated at most locations in this study. Mixed infections were not detected, but they cannot be ruled out because the methods used were not effective at detecting multi-species infections. Microscopy cannot differentiate among species with similar sized oocysts and PCR preferentially amplifies DNA from the dominant species/genotype (Santín and Zarlenga, 2009; Jeníková *et al.*, 2011; Ma *et al.*, 2014; Qi *et al.*, 2015).

Cryptosporidium alticolis sp. n. infects the small intestine, which is similar to most intestinal *Cryptosporidium* spp. of mammals (Ryan and Xiao, 2014). In contrast, *C. microti* is only the third species, after *C. suis* in pigs and *C. oculltus* in rats, reported to infect the colon (Ryan *et al.*, 2004; Vítovec *et al.*, 2006; Kvá *et al.*, 2018). Similar to *C. oculltus* (Kvá *et al.*, 2018), *C. microti* sp. n. localizes to the mucosal surface in the large intestine. In contrast, *C. suis* predominates in the glandular epithelium of the submucosal colonic lymphoglandular complexes in pigs (Vítovec *et al.*, 2006).

Neither *C. alticolis* sp. n. nor *C. microti* sp. n. developed clinical signs in common voles or meadow voles under experimental conditions in the present study. This is consistent with the reports that wild animals rarely display signs of clinical cryptosporidiosis (Sturdee *et al.*,

1999; Hikosaka and Nakai, 2005; Castro-Hermida *et al.*, 2011; N mejc *et al.*, 2012; ondlová *et al.*, 2018).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Ethical standards. The research was conducted under ethical protocols approved by the Institute of Parasitology, Biology Centre and Central Commission for Animal Welfare, Czech Republic (protocol nos. 071/2010 and 114/2013) and Institutional Animal Care and Use Committee North Dakota State University, ND, USA (protocol no. A18014).

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Fig. 1. Sampling locations across the study area in the Czech Republic. Sample site numbers indicate the following: (1) Da ice, (2) Výškovice, (3) Nám š nad Oslavou, (4) Sedle ko u Tábora, (5) Dolní T ebonín, (6) Pelejovice, (7) Radimovice, (8) Budweiss, (9) Bavorovice, (10) Masákova Lhota, (11) Vše chov u Tábora, (12) Opatovice, (13) Love kovice, (14) Sob slav, (15) Dubovice and (16) Zmišovice.

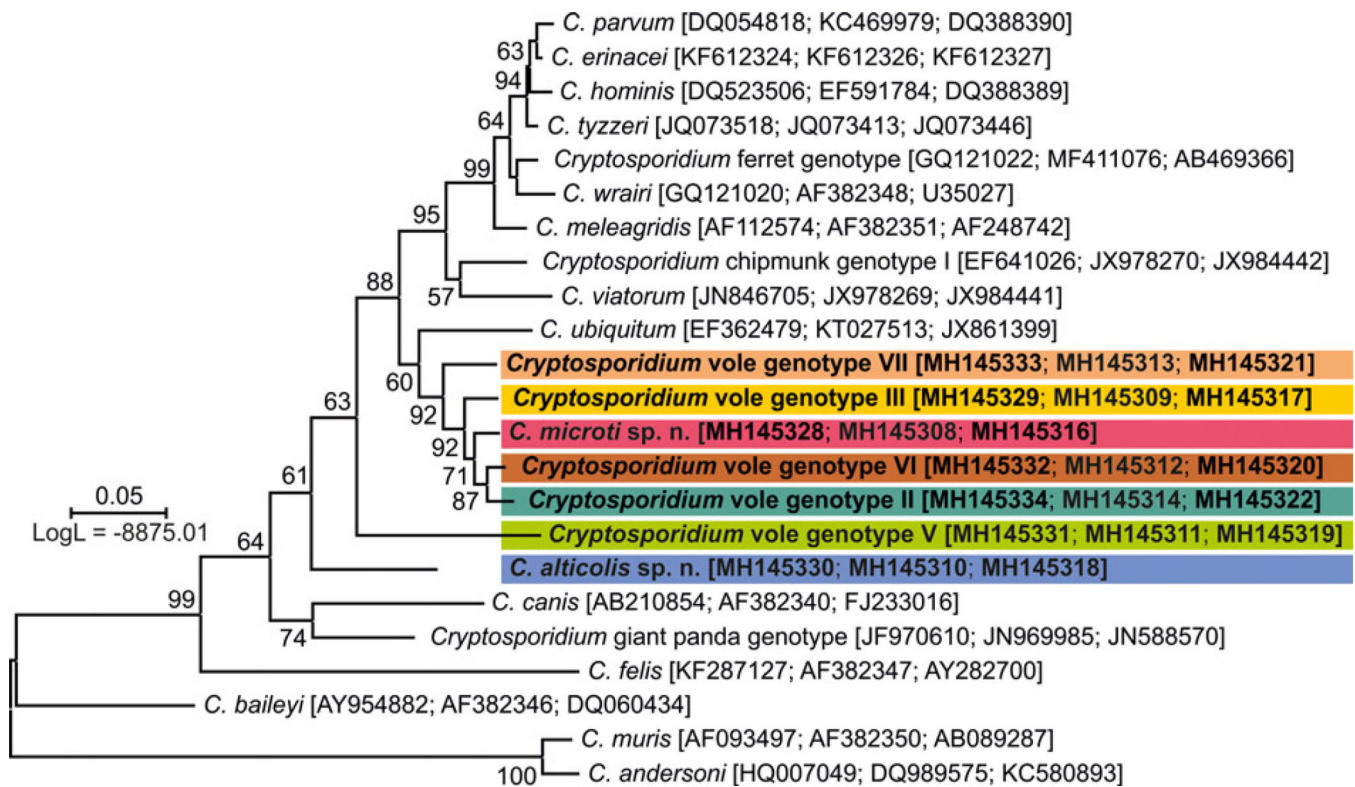


Fig. 2.

A maximum likelihood (ML) tree based on concatenated small subunit rRNA (SSU), actin and *Cryptosporidium* oocyst wall protein (COWP) gene sequences. A representative of each SSU, actin and COWP species/genotype from wild-caught common voles from this study is highlighted in bold. GenBank accession numbers are shown in parenthesis after the isolate identifier. Numbers at the nodes represent the bootstrap values gaining more than 50% support. Branch length scale bar indicates the number of substitutions per site.

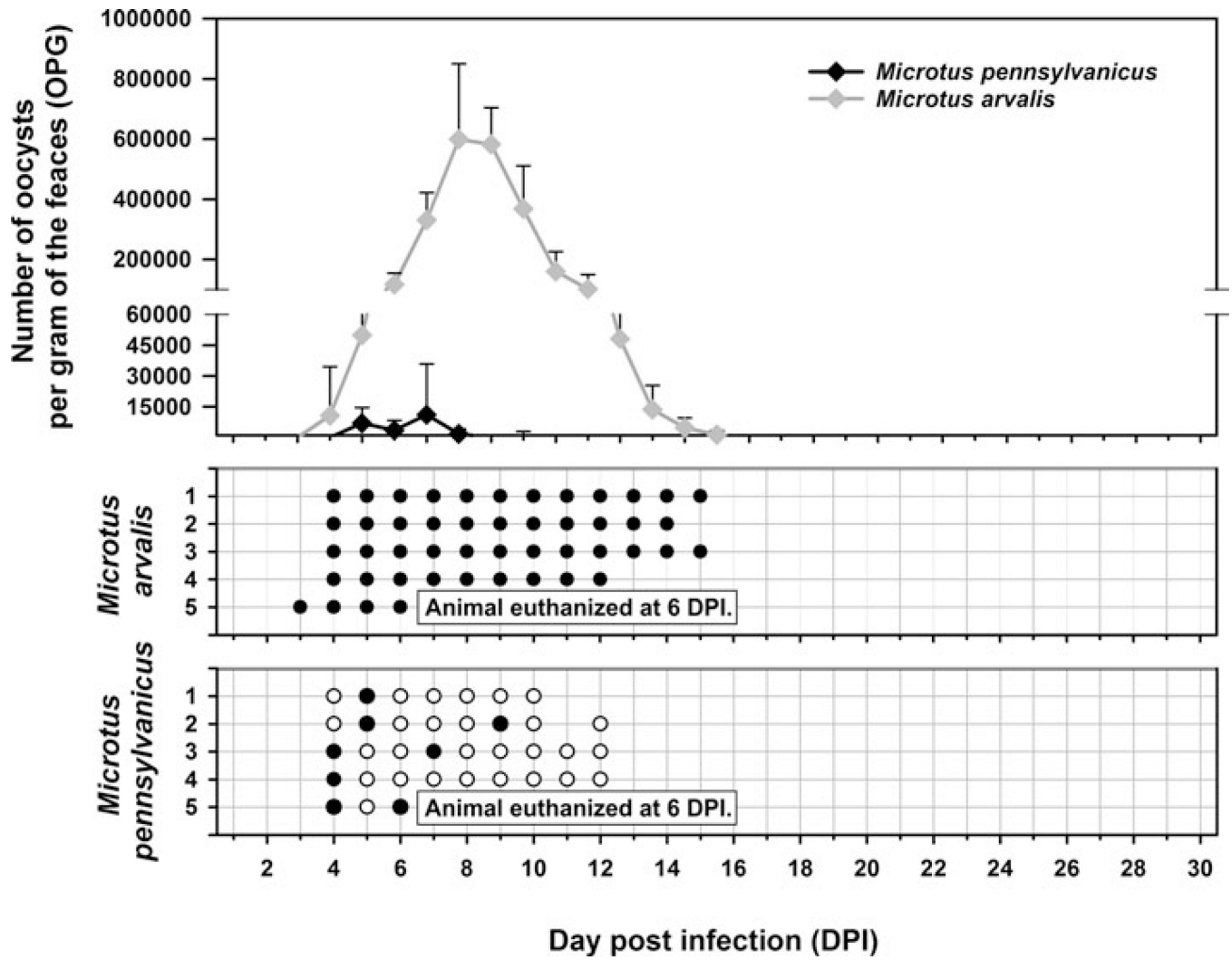


Fig. 3. Course of infection of *Cryptosporidium alticolis* sp. n. in experimentally infected common voles (*Microtus arvalis*) and in meadow voles (*Microtus pennsylvanicus*) based on coprological and molecular examination of feces. Any circles indicate detection of specific DNA, black circle indicates microscopic detection of oocysts.

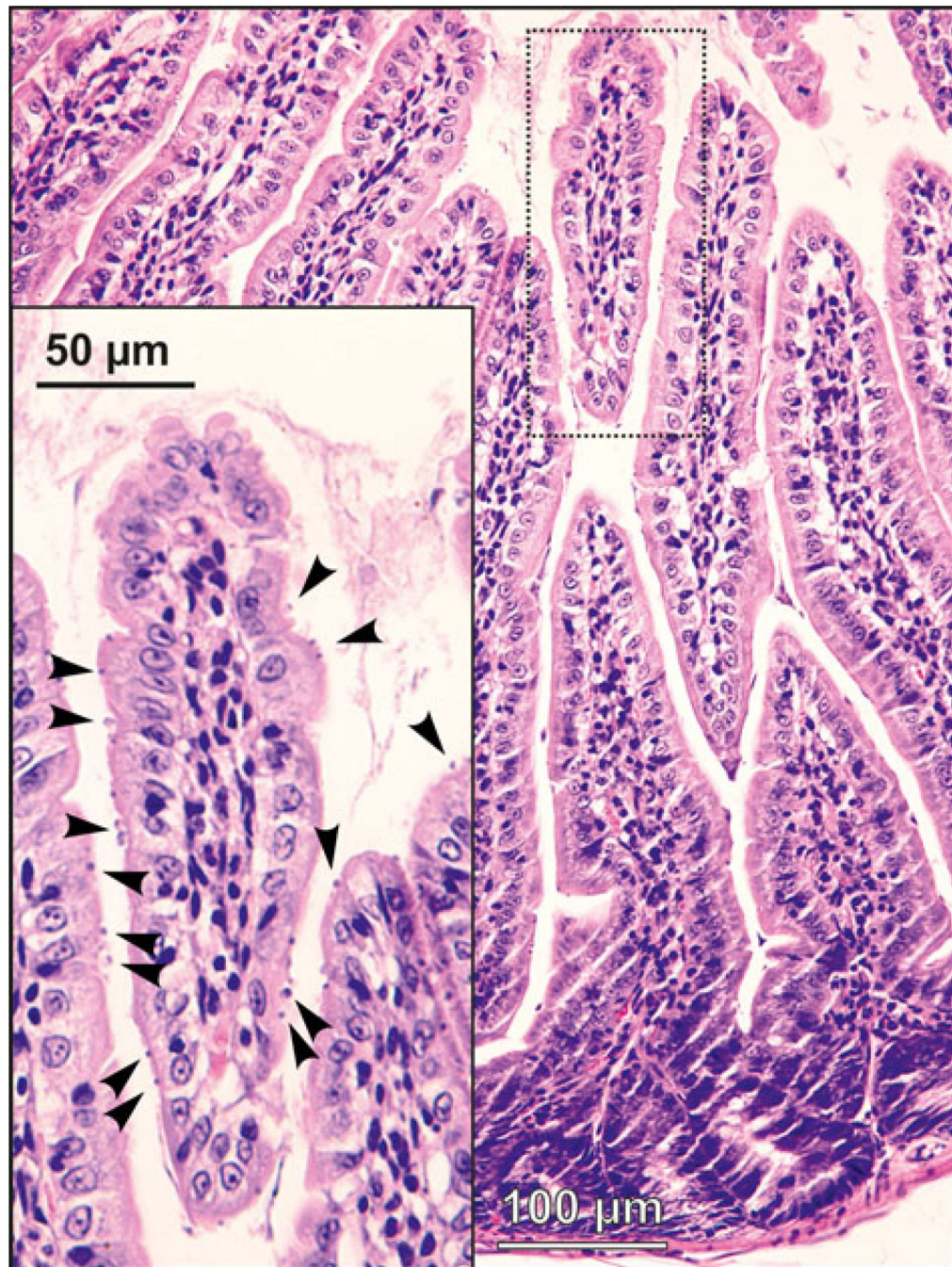


Fig. 4. Developmental stages (arrowheads) of *Cryptosporidium alticolis* sp. n. in mucosal glandular epithelium from the duodenum of an experimentally infected common vole (*Microtus arvalis*). Bar included in each picture.

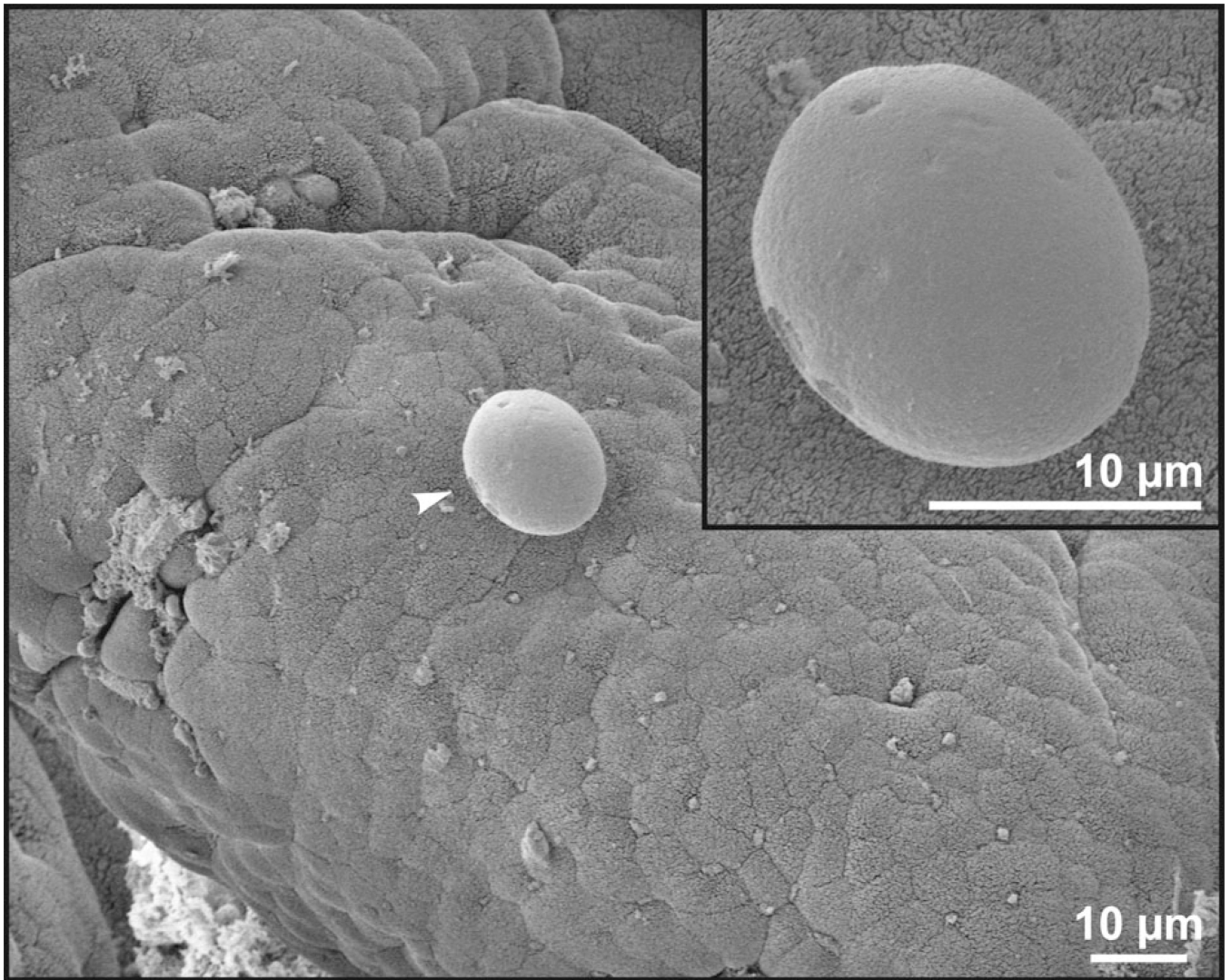


Fig. 5. Scanning electron photomicrograph of the jejunal epithelium of an experimentally infected common vole (*Microtus arvalis*). Attached developmental stage of *Cryptosporidium alticolis* sp. n. (arrowhead; detail in the upper right corner).

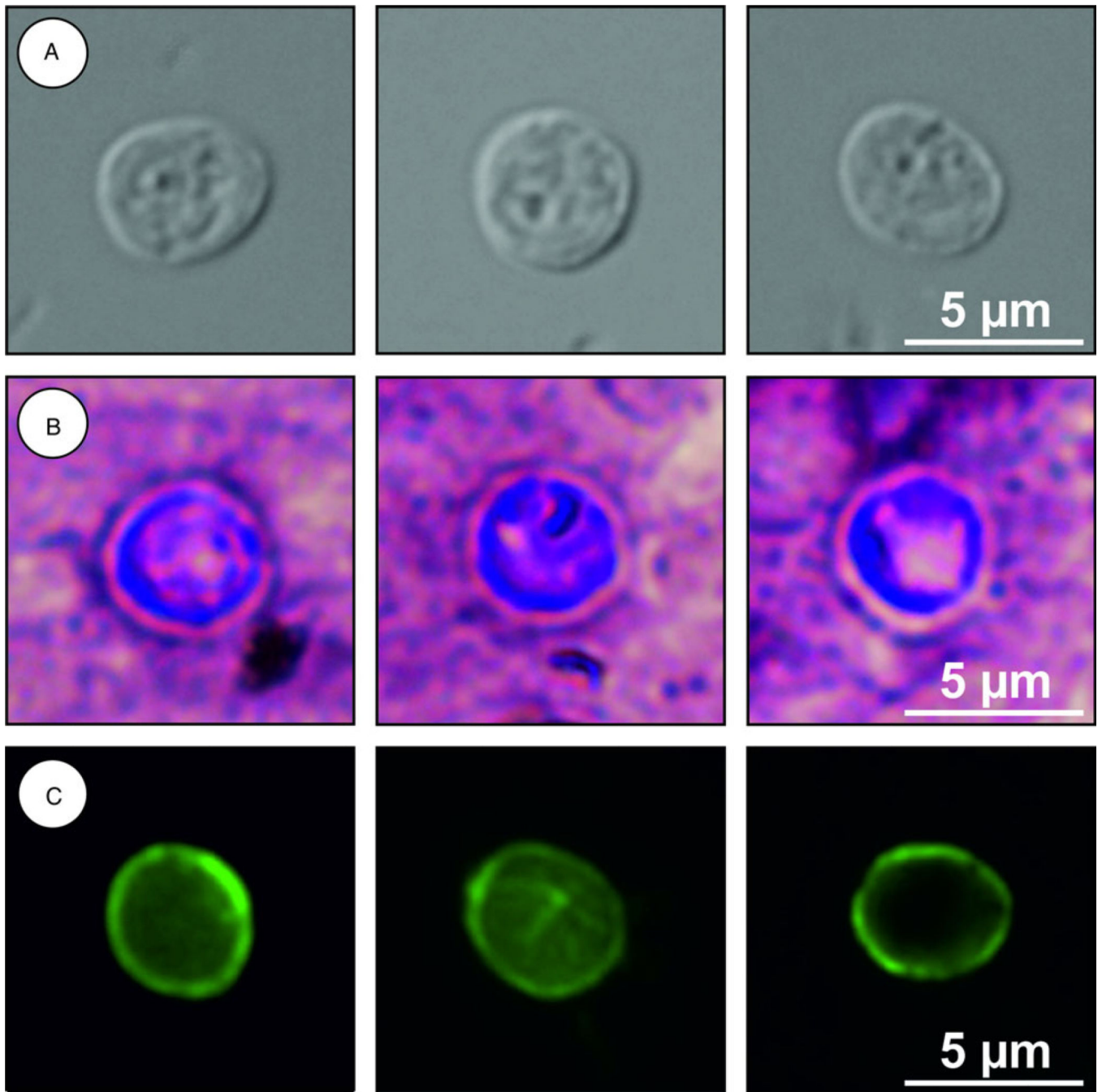


Fig. 6. *Cryptosporidium alticolis* sp. n. oocysts visualized in various preparations: (A) differential interference contrast microscopy and stained by (B) aniline-carbol-methyl violet and (C) anti-*Cryptosporidium* FITC-conjugated antibody. Bar included in each picture.

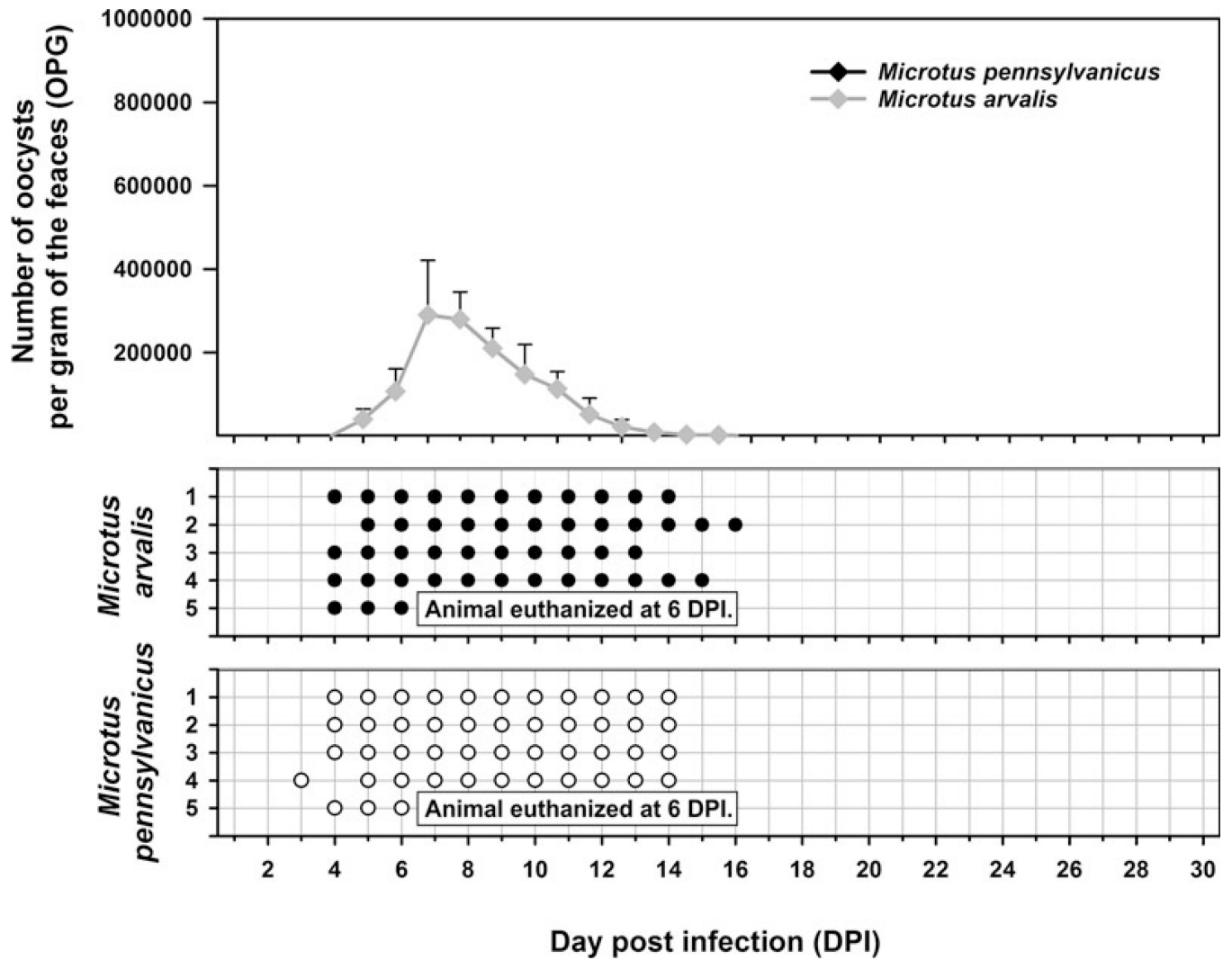


Fig. 7. Course of infection of *Cryptosporidium microti* sp. n. in experimentally infected common voles (*Microtus arvalis*) and in meadow voles (*Microtus pennsylvanicus*) based on coprological and molecular examination of feces. Any circles indicate detection of specific DNA, black circle indicates microscopic detection of oocysts.

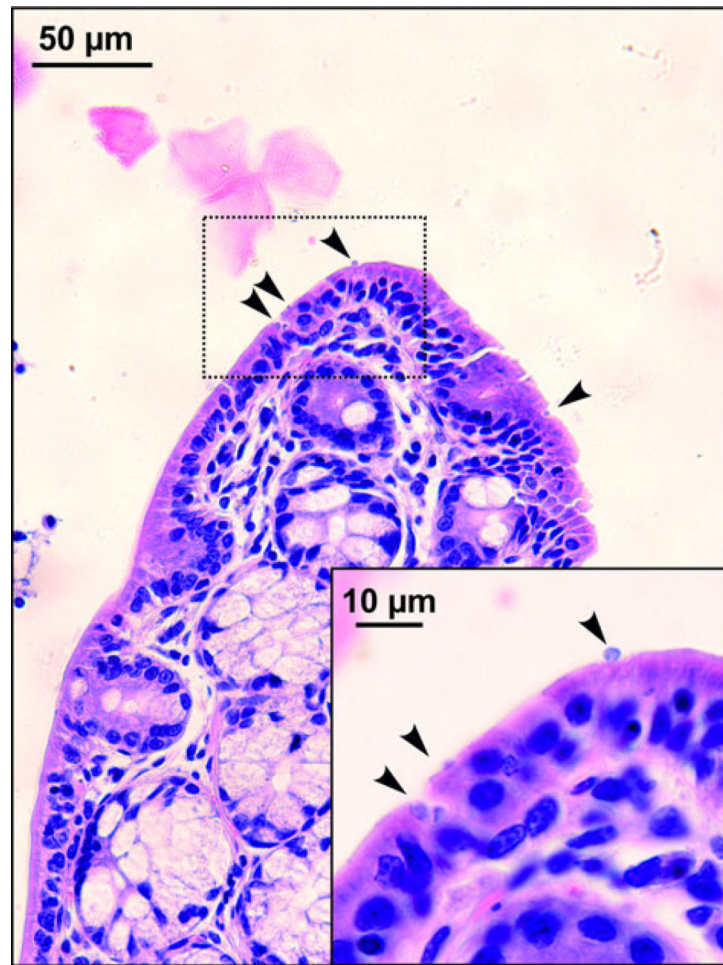


Fig. 8. Developmental stages (arrowheads) of *Cryptosporidium microti* sp. n. in mucosal glandular epithelium from the colon of an experimentally infected common vole (*Microtus arvalis*). Bar included in each picture.

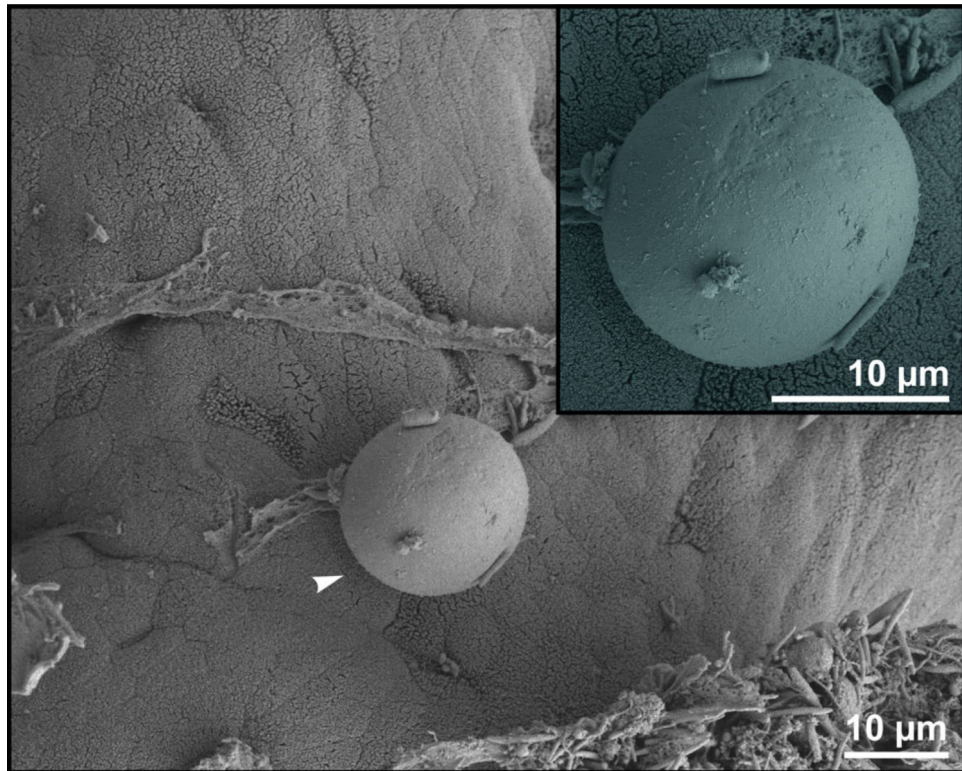


Fig. 9. Scanning electron photomicrograph of the colon epithelium of a common vole (*Microtus arvalis*). Attached developmental stage of *Cryptosporidium microti* sp. n. (arrowhead; detail in the upper right corner).

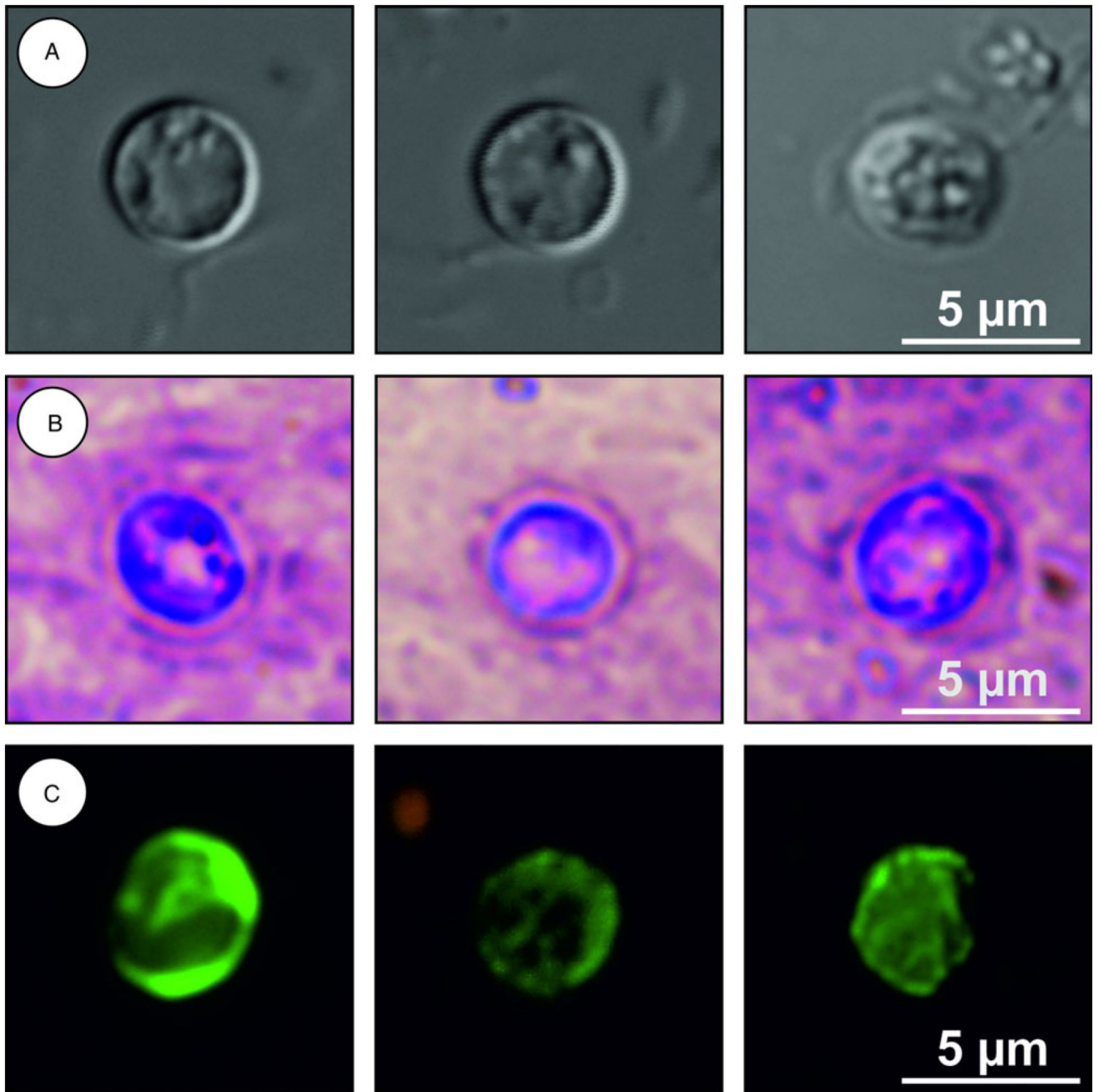


Fig. 10. *Cryptosporidium alticolis* sp. n. oocysts visualized in various preparations: (A) differential interference contrast microscopy and stained by (B) aniline-carbol-methyl violet and (C) anti-*Cryptosporidium* FITC-conjugated antibody. Bar included in each picture.

Table 1.Number of wild-caught common voles positive for *Cryptosporidium* by PCR and microscopy, by sex and age

Sex	Age	<i>n</i>	PCR positive	Microscopically positive
Female	J	29	9	3
	A	113	24	3
Male	J	45	10	3
	A	141	31	10
Total		328	74	19

J, juvenile; A, adult.

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Table 2.

Cryptosporidium spp. in wild common voles (*Microtus arvalis*)

Isolate ID	Location (number of screened samples/positive)	Microscopical positivity (OPG)	Genotyping at the gene loci (GenBank Acc. No. used in the phylogenetic trees)			
			SSU	Actin	COWP	HSP70
19608 ^a	Da ice (97/25)	Yes (4000)	<i>C. microti</i>	<i>C. microti</i>		
19612 ^a		No	<i>C. microti</i>	<i>C. microti</i>		
19615		No	<i>C. microti</i>	<i>C. microti</i>		
19618 ^a		No	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>	
20055 ^a		No	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>	
20057		Yes (4000)	<i>C. microti</i>	<i>C. microti</i>		
20059		Yes (18 000)	vole VII	vole VII	vole VII	
20063		No	vole V	vole V	vole V	
20065 ^a		Yes (6000)	<i>C. alticolis</i> (KY 644657)	<i>C. alticolis</i>	<i>C. alticolis</i>	<i>C. alticolis</i>
23407		No	<i>C. microti</i>	<i>C. microti</i>		
23408		No	<i>C. microti</i>	<i>C. microti</i>		
23409 ^b		No	vole V (MH145331)	vole V (MH145311)	vole V (MH145319)	vole V (MH145325)
23410		No	<i>C. microti</i>	<i>C. microti</i>		
23390		No	vole V	vole V		
22731		Yes (8000)	<i>C. alticolis</i>	<i>C. alticolis</i>	<i>C. alticolis</i>	
23392 ^b		No	vole VII (MH145333)	vole VII (MH145313)	vole VII (MH145321)	vole VII (MH145327)
23393		No	<i>C. microti</i>	<i>C. microti</i>		
23250		No	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>	
23251		No	<i>C. microti</i>	<i>C. microti</i>		
23231		No	<i>C. microti</i>	<i>C. microti</i>		
23111 ^b		Yes (2000)	<i>C. alticolis</i> (MH145330)	<i>C. alticolis</i> (MH145310)	<i>C. alticolis</i> (MH145318)	<i>C. alticolis</i> (MH145324)
23112		No	<i>C. alticolis</i>	<i>C. alticolis</i>	<i>C. alticolis</i>	

Isolate ID	Location (number of screened samples/positive)	Microscopical positivity (OPG)	Genotyping at the gene loci (GenBank Acc. No. used in the phylogenetic trees)			
			SSU	Actin	COWP	HSP70
23746 ^a		Yes (30 000)	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>
23747		No	<i>C. alticolis</i>	<i>C. alticolis</i>		
23748 ^a		No	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>
20062 ^{a,b}	Vyškovice (3/1)	No	vole III (MH145329, KY644593)	vole III (MH145309)	vole III (MH145317)	
23750 ^a	Nám š : nad Oslavou (40/8)	No	<i>C. microti</i>	<i>C. microti</i>		
23400 ^{a,b}		No	vole VI (MH 145332)	vole VI (MH 145312)	vole VI (MH 145320)	vole VI (MH 145326)
23405 ^a		No	vole VI	vole VI		
28082 ^b		Yes (16 000)	vole IV (MH145335)	vole IV (MH145315)		
30906		No	vole IV	vole IV		
30908		No	vole IV	vole IV		
30909 ^b		No	vole II (MH145334)	vole II (MH145314)	vole II (MH145322)	
30928		No	<i>C. microti</i>	<i>C. microti</i>		
22339	Sedle ko u Tábora (35/2)	No	<i>C. microti</i>	<i>C. microti</i>		
22336		No	<i>C. microti</i>	<i>C. microti</i>		
21146	Dolní T ebonín (32/8)	Yes (36 000)	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>	
22352		No	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>	
23115 ^a		No	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>	
23236 ^a		No	<i>C. microti</i> (KY644604)	<i>C. microti</i> (KY657294)	<i>C. microti</i>	
23743 ^a		No	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>	
24128 ^a		Yes (24 000)	vole VI	vole VI	vole VI	
24129 ^a		No	vole VI (KY644632)	vole VI	vole VI	
25643 ^a		No	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>	
24514	Pelejovice (37/2)	No	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>
24916 ^a		No	vole V (KY644670)	vole V		

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			SSU	Actin	COWP	HSP70	
24919 ^a	Radimovice (18/7)	No	<i>C. microti</i>	<i>C. microti</i>			
24922 ^a		No	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>	
24923 ^b		No	<i>C. microti</i> (MH 145328)	<i>C. microti</i> (MH145308)	<i>C. microti</i> (MH145316)	<i>C. microti</i> (MH145323)	
24924		No	<i>C. microti</i>	<i>C. microti</i>			
24926 ^a		No	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>	
25163 ^a		No	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>	
25164 ^a		No	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>	
28061		eské Bud jovice (2/1)	No	vole V			
28315		Masáková Lhota (18/7)	No	<i>C. alticolis</i>	<i>C. alticolis</i>		<i>C. alticolis</i>
28317	Yes (10 000)		<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>		
28566	Yes (4000)		<i>C. microti</i>	<i>C. microti</i>			
28567	No		vole VI	vole VI			
28665	No		<i>C. microti</i>	<i>C. microti</i>			
28667	No		<i>C. microti</i>	<i>C. microti</i>			
29936	No		<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>		
28422	Všechov u Tábora (30/12)		No	vole VII	vole VII		
28423			Yes (42 000)	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>	
28425			No	<i>C. alticolis</i>	<i>C. alticolis</i>		
28428			No	vole VII	vole VII		
28429			Yes (8000)	<i>C. microti</i>	<i>C. microti</i>		
28539			Yes (14 000)	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>	
28540			Yes (32 000)	<i>C. microti</i>	<i>C. microti</i>		
28541		Yes (18 000)	vole VII	vole VII			
28543		No	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>		
28545		Yes (6000)	<i>C. microti</i>	<i>C. microti</i>			
28546	No	<i>C. microti</i>	<i>C. microti</i>				

Isolate ID	Location (number of screened samples/positive)	Microscopical positivity (OPG)	Genotyping at the gene loci (GenBank Acc. No. used in the phylogenetic trees)		
			SSU	Actin	COWP
28549		Yes (8000)	<i>C. microti</i>	<i>C. microti</i>	HSP70
30904	Opatovice (4/1)	No	<i>C. microti</i>	<i>C. microti</i>	<i>C. microti</i>

Isolates were characterized by microscopy, including infection intensity expressed as number of oocyst per gram of feces (OPG), and PCR analysis of the small ribosomal subunit rRNA (SSU), actin, *Cryptosporidium* oocyst wall protein (COWP) and 70 kDa heat shock protein (HSP70) genes. Only localities where *Cryptosporidium-positive* animals were trapped are shown.

^a Sequences of SSU and actin previously obtained in the study of Stenger *et al.* (2018).

^b Sequence of isolates used in phylogeny trees.