FOLIA PARASITOLOGICA 45: 93-100, 1998

New species of *Cryptosporidium* (Apicomplexa: Cryptosporidiidae) from lizards

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Key words: Reptilia, Sauria, *Cryptosporidium saurophilum* sp. n., transmission studies

Abstract. A new *Cryptosporidium* species, *C. saurophilum*, is described from Schneider's skinks *Eumeces schneideri* Daudin, 1802. Oocysts were fully sporulated in fresh faeces and measured 5.0 × 4.7 μm (4.4-5.6 × 4.2-5.2 μm). The new species differs from *C. serpentis* Levine, 1980 by having smaller oocysts, developing in a different location of intestine, and by the inability to infect snakes.

Cryptosporidia are coccidian parasites that inhabit the microvilli of epithelial surface of the gastrointestinal and respiratory tracts of a wide variety of vertebrates including humans. Cryptosporidia have also been found in numerous species of reptiles (Upton et al. 1989, O'Donoghue 1995). The first complete account of *Cryptosporidium* species in reptiles was provided by Brownstein et al. (1977) and the name *C. serpentis* was assigned to the snake-derived isolates by Levine (1980). *C. serpentis* Levine, 1980 can cause morbidity and mortality in captive snakes (Cranfield and Graczyk 1994). Developmental stages of *C. serpentis* have been repeatedly detected in the gastric mucosa (Brownstein et al. 1977, Cranfield and Graczyk 1994) or the microvillous borders of the gall bladder and bile ducts in snakes (Cimon et al. 1996).

Examination of captive and wild originated lizards from various localities revealed the presence of a *Cryptosporidium* species, which appeared to differ morphologically from *C. serpentis*. Futher studies revealed this species to be new and the name *Cryptosporidium saurophilum* is proposed. In addition to the description of this coccidium, the prevalence of *C. saurophilum* infections in captive and wild originated lizards, and clinical and histopathological features associated with an outbreak of *Cryptosporidium* infection in lizards raised by a commercial breeder are described.

MATERIALS AND METHODS

Between December 1994 and March 1997, we examined faeces and/or intestinal contents of 220 specimens of wild originated (85) or captive (135) lizards of 67 species belonging to the following families (number of examined species and specimens are reported in parentheses): Agamidae (5/18), Anguidae (1/2), Cordylidae (2/3), Gekkonidae (15/57), Chamaeleonidae (12/42), Iguanidae (14/45), Scincidae (10/35), Teidae (3/5) and Varanidae (5/13).

Fresh faeces or intestinal contents from all animals were placed into 2.5% (w/v) aqueous potassium dichromate solution. All samples were sent without delay to the laboratory where they were screened routinely for parasites by flotation in Sheather's sugar solution (specific gravity 1.30). Faecal specimens containing *Cryptosporidium* oocysts were examined, measured and photographed using Nomarski interference contrast (NIC) microscopy. Measurements are reported in micrometres (μm) as the mean of 30 oocysts, followed by the range in the parentheses.

Clinically affected or moribund lizards were euthanized and necropsied. At necropsy, tissue samples of the stomach, duodenum, small and large intestine, cloaca, heart, lung, liver, gall bladder and kidney were fixed in 10% buffered formalin. Fixed tissues were processed for light microscopy using standard methods. Paraffin sections were stained with haematoxylin and eosin (HE) and Giemsa.

Specimens for scanning electron microscopy (SEM) were fixed in 4% buffered paraformaldehyde at 4°C. Small portions of intestinal mucosa were rinsed several times in distilled water, dehydrated in alcohol and acetone series and desiccated by critical point drying in carbon dioxide. The samples were then gold-coated in spraying device and examined with a JEOL JMS 6300 scanning electron microscope.

For transmission electron microscopy, intestinal specimens were fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1M, pH 7.4) at 4°C and post-fixed in 1% osmium tetroxide in the same buffer. Specimens were washed three times in the same

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buffer, dehydrated in graded alcohols and embedded in Durcupan. Thin sections were stained with uranyl acetate and lead citrate and then examined with a JEOL 1010 transmission electron microscope.

Faeces or intestinal content containing *Cryptosporidium* oocysts were strained through a graded series of sieves and concentrated by sucrose flotation. To decontaminate the oocysts, the suspension was treated in bleach (SAVO, Bochemie, Bohumín, Czech Republic; 1.25% sodium hypochlorite) for 5 minutes. The concentrated oocysts were stored in 2.5% potassium dichromate at 4°C until used for inoculation. Before inoculation, oocysts were washed 3 times by centrifugation in sterile phosphate-buffered-saline (PBS, pH 7.2) and counted using haemocytometer.

Lizards and snakes used in cross-transmission studies were kept in separate cages under the same ambient conditions (~27°C; 14L/10D photoperiod) and fed laboratory reared crickets with mineral and vitamin supplements or laboratory mice, respectively. Prior to the experimental inoculation, faecal samples of all lizards and snakes were monitored repeatedly (four to five times) for cryptosporidia four to six weeks before the inoculation. Only lizards and snakes that were cryptosporidia-free animals were used for experimental inoculation with 10^3-10^4 *Cryptosporidium* oocysts.

The inoculum of *Cryptosporidium* oocysts originating from Schneider's skinks *Eumeces schneideri* (Daudin, 1802), tree monitor *Varanus prasinus* (Schlegel, 1839) and skinks *Mabuya perrotetii* (Duméril et Bibron, 1839) were used to inoculate different recipient animals (Table 2). Faecal specimens from inoculated and control animals were examined for *Cryptosporidium* oocysts by flotation in Sheather's sugar solution. At different period post-inoculation, inoculated animals were euthanized and necropsied.

RESULTS

Examination of faecal samples collected from wild originated or captive lizards revealed that twenty animals (9.1%) passed cryptosporidial oocysts which differed in size from *Cryptosporidium serpentis*. Below we present the description of this newly found species of *Cryptosporidium*.

Cryptosporidium saurophilum sp. n. Figs. 1-12

Description of oocyst: Oocysts obtained from fresh faeces or intestinal content spherical or ovoidal, 5.0 × 4.7 (4.4-5.6 × 4.2-5.2), shape index 1.09 (1.04-1.12), wall smooth and colourless, composed of a single layer about 0.5 thick. Micropyle and polar granule absent, oocyst residuum present, 2.1 × 2.4 (1.8-2.6 × 1.8-2.6), composed of numerous small granules and one spherical globule 1.8 × 1.6 (1.0-2.0 × 1.0-2.0). Four vermiform sporozoites present within each oocyst, 7.3 × 1.1 (7.8-5.8 × 0.8-1.2) *in situ*, lying parallel along one side of oocyst and tightly enclosing oocyst residuum. Sporozoite refractile bodies absent; nucleus spherical, 0.5 (0.4 × 0.6) located centrally.

T y p e h o s t : Schneider's skink *Eumeces schneideri* Daudin, 1802 (Sauria: Scincidae).

O t h e r h o s t s : *Mabuya perrotetii* (Duméril et Bibron, 1839) (Scincidae), *Eublepharis macularius* (Blyth, 1854) (Gekkonidae), *Varanus griseus* (Daudin, 1803) (Varanidae), *Bradypodion excubitor* (Barbour, 1911), and *B. tavetaneum* (Steindachner, 1891) (Chameleonidae).

T y p e l o c a l i t y : Northern Egypt, detailed locality unknown.

S i t e o f i n f e c t i o n : The histological examination of sections from all five naturally infected *E. schneideri* displayed cryptosporidial infection in the intestine (Fig. 3) and cloaca. No developmental stages were found in other tissues examined.

S p o r u l a t i o n : Endogenous. Oocysts were fully sporulated in fresh faeces.

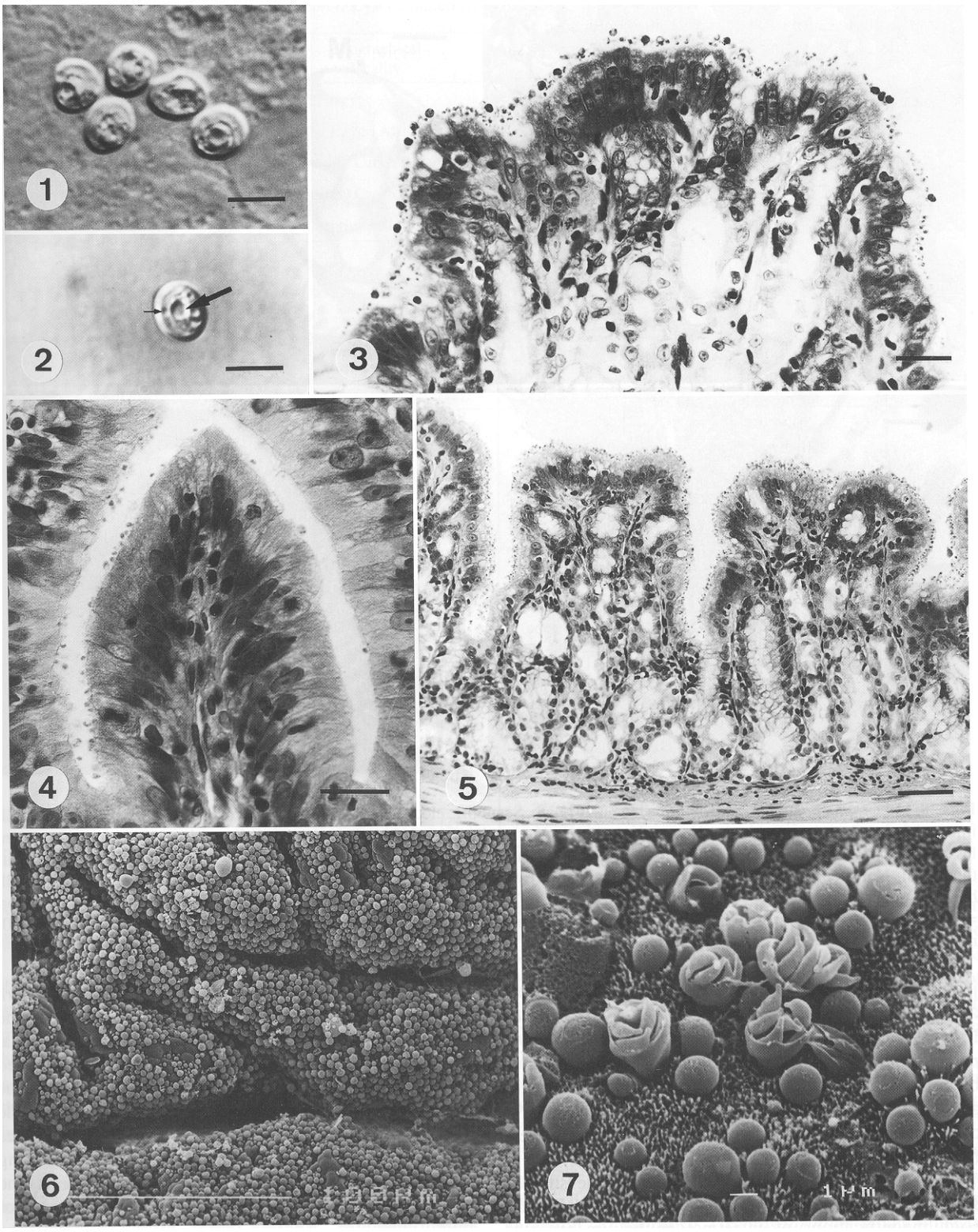
T y p e s p e c i m e a s : Phototypes are deposited in the Institute of Parasitology, Academy of Sciences of the Czech Republic in České Budějovice, Collection Nos. H 170/96 - H 175/96.

E t y m o l o g y : The specific epithet "*saurophilum*" is derived from Greek (*sauros* = a lizard + *phileo* = to love) and reflects the affinity of the described species to lizards.

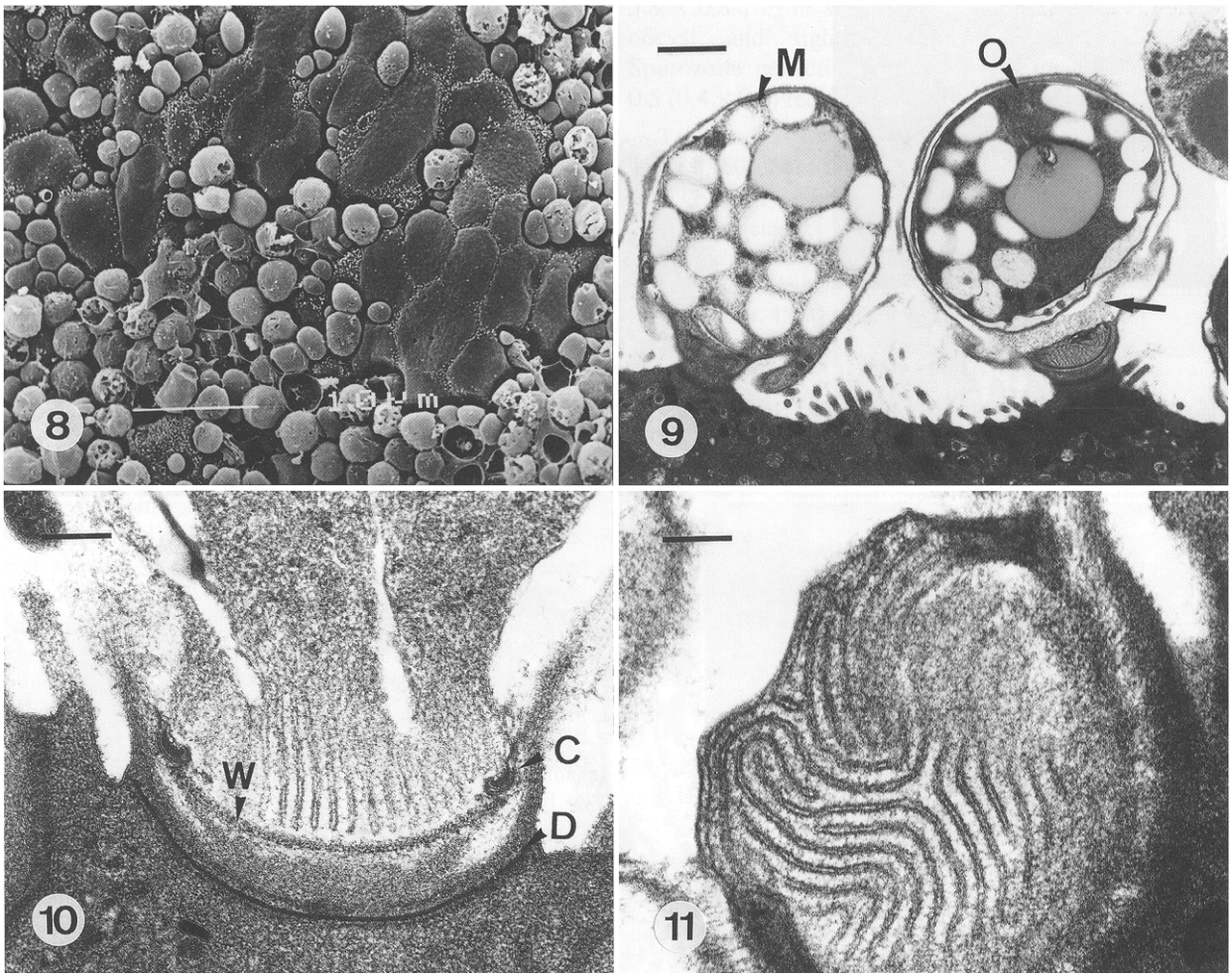
P r e v a l e n c e : Twenty lizards (9.1%), representing seven host species, were infected. Five of eight adult Schneider's skinks *E. schneideri* originating from Egypt; two of ten adult skinks *M. perrotetii* originated from Ghana; eight of twelve captive juvenile leopard geckos *E. macularius* raised in a commercial breeder in the Czech Republic; one of three adult desert monitors *V. griseus* housed in a private reptilian collection in the Slovak Republic; and one of two subadult and one of three adult examined tree monitors *V. prasinus* housed in private collections in the Czech Republic. Table 1 represents measurements of oocysts of five of the isolates. Cryptosporidia were also detected in histological sections from the small intestine of two chameleon species *B. excubitor* and *B. tavetanum* originated from Kenya, but it is unknown if the oocysts represented the species described herein.

Pathogenicity. No pathological changes were found in the intestine of adult *Cryptosporidium*-infected lizards (Fig. 4). An outbreak of chronic cryptosporidiosis occurred in a captive colony of *E. macularius*. Juvenile lizards from this colony exhibit progressive weight loss and abdominal swelling, and mortality of juvenile geckoes reached about 50%. At necropsy, juvenile *Cryptosporidium*-infected *E. macularius* had wasting of the dorsal musculature and lack of tail fat bodies. The intestinal and cloacal wall was thick with mucinous material. No other abnormalities were grossly seen. Histopathology of the

(Figs. 1-7-continued) (*Eublepharis macularius*) showing mucosal thickening and numerous cryptosporidia covering mucosal surface. HE. Scale bar = 50 μm. **Fig. 6.** Cloacal mucosa of naturally infected leopard gecko (*E. macularius*) demonstrating massive infection. SEM. **Fig. 7.** Cloacal mucosa of a naturally infected leopard gecko (*E. macularius*) showing numerous *Cryptosporidium* developmental stages. SEM.



Figs. 1-7. *Cryptosporidium saurophilum* sp. n. **Fig. 1.** Nomarski interference contrast (NIC) photograph of oocysts from Schneider's skink (*Eumeces schneideri*). Scale bar = 5 µm. **Fig. 2.** NIC photograph of oocyst from leopard gecko (*Eublepharis macularius*). Note residuum (large arrow) and nucleus in sporozoite (small arrow). Scale bar = 5 µm. **Fig. 3.** Histological section from large intestine of naturally infected Schneider's skink (*E. schneideri*) showing numerous cryptosporidia covering mucosal surface. Giemsa. Scale bar = 30 µm. **Fig. 4.** Histological section from jejunum of naturally infected skink (*Mabuya perrotetii*). HE. Scale bar = 20 µm. **Fig. 5.** Histological section from cloaca of naturally infected leopard gecko (see p. 94)



Figs. 8-11. *Cryptosporidium saurophilum* sp. n. **Fig. 8.** Scanning electron photomicrograph of cloacal mucosa of naturally infected leopard gecko (*Eublepharis macularius*) showing swollen non-infected absorptive cells with rounded appearance and less distinct intercellular borders. **Fig. 9.** Macrogamont (M) with amylopectin granules and young unsporulated oocyst (O) with thick wall. Note separation from attachment organelle (arrow). Scale bar = 1 μm. TEM. **Fig. 10.** Detail of attachment organelle between parasite and host cell. Note electron dense layer (D), web of microfilaments (W) and electron dense collar (C). Scale bar = 300 nm. TEM. **Fig. 11.** Section showing the parasite plasmalemma invaginations of the attachment organelle. Scale bar = 300 nm. TEM.

jejunum and large intestine showed mucosal thickening and numerous developmental stages of *Cryptosporidium* lining the microvillous border surface. The *lamina propria* and submucosa were oedematous and infiltrated by inflammatory cells consisted mainly of heterophils and lymphocytes, but also included plasma cells. No cryptosporidia or associated pathological lesions were found in the stomach or other tissues.

SEM of a mucosal surface of the intestine showed severe infections in all examined *E. macularius* (Fig. 6). High magnifications of intestinal epithelium revealed *Cryptosporidium* developmental stages covering the surface of cloacal mucosa (Fig. 7). Non-infected absorptive cells were swollen, had rounded appearance and were bulbously protuberant with less distinct intercellular borders (Fig. 8).

Transmission electron microscopy. The ultrastructure of developmental stages of *C. saurophilum* was studied in two *E. macularius* and one *E. schneideri*. All *Cryptosporidium* developmental stages were found simultaneously in the posterior portion of the intestinal tract of examined lizards. The parasites were located within parasitophorous vacuoles formed at the microvillous surface of the intestinal mucosa. The developmental stages were attached to the enterocytes and were surrounded by microvilli creating parasitophorous envelope. At the site of contact, the host cell developed an electron dense layer and a web of microfilaments. Above these microfilaments, the parasite formed an attachment (feeder) organelle consisting of a series of parasite plasmalemma invaginations. The exterior membrane of parasite was attached to the interior parasitophorous membrane by the electron dense collar that surrounded its base (Figs. 10 and 11). The attachment organelles remained attached to the residual bodies of meronts and gamonts

Table 1. Measurements of *Cryptosporidium* oocysts isolated from lizards

Host	Geographical locality	Oocyst age	Oocyst size (μm) (range)	Shape index (length/width)
Eumeces schneideri	Egypt, Africa	fresh	4.6-5.6 × 4.2-5.2	1.12
Mabuya perrotetti	Egypt, Africa	fresh	4.8-5.4 × 4.3-5.0	1.09
Eublepharis macularius	captivity Czech Republic	fresh	4.7-5.6 × 4.6-5.2	1.04
Varanus prasinus	captivity Czech Republic	fresh	4.6-5.3 × 4.4-5.1	1.06
Varanus griseus	captivity Slovakia	fresh	4.7-5.6 × 4.2-5.2	1.12

Table 2. Results of experimental cross-transmissions of *Cryptosporidium saurophilum* oocysts obtained from naturally infected lizards

Source of isolate	Recipient host	No. Animals Inoculated (control)	Age of recipient host	Number of Oocysts inoculated	Necropsy of recipient host (days postinfection)	Result +/–
Mabuya perrotetii (Scincidae)	*Chalcides ocellatus* (Scincidae)	2 (1)	8 months	5×10^3	60	+
Mabuya perrotetii (Scincidae)	*Chamaeleo calyptratus* (Chamaeleonidae)	2 (1)	4 months	5×10^3	60	+
Mabuya perrotetii (Scincidae)	*Lacerta viridis* (Lacertidae)	2 (1)	6 months	5×10^3	60	+
Varanus prasinus (Varanidae)	*Chamaeleo calyptratus* (Chamaeleonidae)	2 (2)	4 months	10^3	30	+
Eumeces schneideri (Scincidae)	*Mabuya perrotetii* (Scincidae)	2 (1)	adult	10^4	60	+
Eumeces schneideri (Scincidae)	*Elaphe o. obsoleta* (Colubridae)	2 (1)	4 months	10^4	60	–
Eumeces schneideri (Scincidae)	chickens	3 (1)	8 days	10^4	14	–
Eumeces schneideri (Scincidae)	BALB/c mice	6 (2)	7 days	10^4	4	–
Eumeces schneideri (Scincidae)	SCID mice	4 (2)	10 weeks	10^4	42	–

or were separated from fully formed merozoites or gamonts (Fig. 9).

During merogony, the nucleus divided to form eight daughter nuclei and merozoites formed simultaneously at the surface of the meront. The merozoites became more elongate and remained attached to the residual body when the apical complex, consisted of rhoptries, apical rings and micronemes, developed. The pellicle of the merozoites consisted of the plasmalemma and two inner membranes that formed the pellicular complex.

Microgamonts were relatively rare when compared to other *Cryptosporidium* developmental stages. Immature microgamonts were similar to meronts, however, they were distinguished from meronts by their more numerous and smaller nuclei arranged at the periphery of microgamonts. Microgametes were bullet-shaped and lacked a flagellum and mitochondria. Macrogamonts can be distinguished from other stages by having amylopectin granules (Fig. 9) and two types of wall-forming bodies in the cytoplasm. The first type was electron-dense (WF1) and appeared in large numbers at the periphery of the macrogamonts. The second type (WF2) was composed from granular material and was observed only one per macrogamont. Sporulation of oocysts took place inside the intestine (*in situ* sporulation) and each oocysts contained four sporozoites.

Cross-transmissions of *Cryptosporidium* isolates between hosts. All lizards experimentally inoculated with *Cryptosporidium* oocysts originating from *E. schneideri*, *V. prasinus* and *M. perrotetii* developed infection. None of control animals were infected. None of the lizards showed clinical signs of cryptosporidial infection. At necropsy, *Cryptosporidium* developmental stages have only been detected in association with intestinal mucosa of examined lizards. The histological examination of stomach did not reveal developmental stages of *Cryptosporidium*. Oral inoculations of two juvenile black rat snake *E. obsoleta obsoleta*, one litter of seven-day-old BALB/c mice, four severe combined

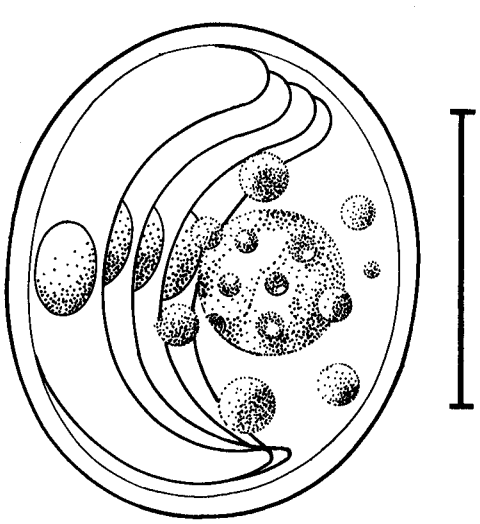


Fig. 12. Composite line drawings of sporulated oocyst of *Cryptosporidium saurophilum* sp. n. Scale bar = 3 µm.

immunodeficient mice and three chickens with *Cryptosporidium* oocysts originating from *E. schneideri* did not result in transmission. Also, histological sections did not confirm developmental stages of *Cryptosporidium* in any inoculated snakes, mice or chickens. The results of cross-transmission studies with *C. saurophilum* isolates are summarised in Table 2.

Remarks. To date, only *Cryptosporidium serpentis,* has been reported from reptilian hosts. *C. saurophilum* is structurally similar to *C. serpentis*, but has smaller oocysts, differs in site of infection and spectrum of susceptible hosts. These data lead to the conclusion that the organisms described herein is a new species.

DISCUSSION

Eight species of *Cryptosporidium* are currently considered to be valid on the basis of oocyst morphology, differences in site of infection and host specificity (Fayer et al. 1997). The size of oocyst of *Cryptosporidium serpentis* was observed by several authors. Tilley et al. (1990) reported the mean size of *C. serpentis* oocysts, isolated from naturally infected yellow rat snake *Elaphe obsoleta quadrivittata*, as 6.2 × 5.3 (5.6-6.6 × 4.8-5.6) µm. Cranfield and Graczyk (1994) found snake-originated *C. serpentis* isolates to measure 6.1 × 5.3 (5.7-6.5 × 4.8-5.5) µm. Upton et al. (1989) examined nine *Cryptosporidium* isolates from reptiles and suggested, based on differences in size of oocysts, that more than one species of *Cryptosporidium* probably occurs in reptiles.

In this study, we examined five *C. saurophilum* isolates from lizards and mean size of oocysts was 5.0 × 4.7 (4.4-5.6 × 4.2-5.2) µm. Although many factors affecting *Cryptosporidium* oocyst morphology were recorded, and the dimensions of *Cryptosporidium* oocysts found in reptiles overlap, the mean size of *C. saurophilum* oocysts recovered from lizards in this study is smaller than that of all known isolates from snakes (Upton et al. 1989, Tilley et al. 1990, Cranfield and Graczyk 1994).

Developmental stages of *C. serpentis* have been detected in association with the gastric mucosa in snakes (Brownstein et al. 1977, Cranfield and Graczyk 1994) and the microvillous borders of the gall bladder and bile ducts (Cimon et al. 1996). The first complete report of the cryptosporidiosis in lizards was made by Dilleyhay et al. (1986). In that report, a subadult Senegal chameleon *C. senegalensis* exhibited developed clinical cryptosporidiosis and histological examination revealed numerous parasites lining the gastric mucosa. As demonstrated in this study, *C. saurophilum* developmental stages have only been detected in association with intestinal and cloacal mucosa of examined saurian hosts. No developmental stages were found in the stomach of any examined animals. Similar site of *Cryptosporidium* infection was also found in Madagascar giant day gecko *Phelsuma madagascariensis grandis*, starred lizard *Agama stellio*, chameleon *Bradypodion pumilum* and skink *Mabuya striata* (Upton and Barnad 1987, Ostrowska and Paperna 1990, Dollahon et al. 1993, Pavlásek 1997). These results strongly indicate that *Cryptosporidium* species infecting intestinal and cloacal regions in lizards differs from *C. serpentis* from snakes and furnish additional support for the designation of *C. saurophilum* species.

In snakes, cryptosporidial infections are associated with chronic gastric disease (Upton 1990, Cranfield and Graczyk 1994). Clinical gastric cryptosporidiosis has also been reported in lizards. Dillehay et al. (1986) and Frost et al. (1994) described the clinical and histopathological features associated with gastric cryptosporidiosis in a subadult chameleon *C. senegalensis* and ocellated lacerta *Lacerta lepida,* respectively. In the present study, an outbreak of cryptosporidiosis in juvenile *Eublepharis macularius* was observed. The clinical features were characterised by the progressive weight loss, abdominal swelling and mortality reached 50% in juvenile lizards. Microscopically, the jejunum, large intestine and cloaca were thickened, and numerous cryptosporidia were present on the mucosal surface. The *lamina propria* and submucosa were oedematous and infiltrated by inflammatory cells. Generally, the characteristics of clinical signs and pathological lesions were different from those found in snakes infected with *C. serpentis* (Brownstein et al. 1977, Dillehay et al. 1986, Carmel and Groves 1993).

In lizards, the intestinal location of *Cryptosporidium* developmental stages was confirmed by scanning electron microscopy. High magnifications of intestinal epithelium with cryptosporidia revealed ultrastructural changes on the surface of intestinal mucosa. These

ultrastructural changes were similar to those described previously in experimental cryptosporidiosis in calves (Heine et al. 1984), piglets (Vítovec and Koudela 1992), and neonatal mice (Vítovec and Koudela 1988). The ultrastructure of *Cryptosporidium* developmental stages from *E. macularius* and *E. schneideri* was identical to that described in starred lizard *A. stellio* and conformed in detail to that of *Cryptosporidium* isolates found in the intestine of mammal and birds (Fayer et al. 1997).

Only few cross-transmission studies have been conducted with reptilian *Cryptosporidium* isolates to date. Cranfield and Graczyk (1994) succeeded to inoculate captive black rat snake *Elaphe obsoleta obsoleta*, yellow rat snake *E. obsoleta quadrivittata*, and corn snakes *E. guttata guttata* with *C. serpentis* oocysts originating from trans-pecos rat snake *E. subocularis*.

No experiment was carried out in which *Cryptosporidium* oocyst isolates originating from lizards have been transmitted to other lizards. As demonstrated in this study, the infections developed in all lizards inoculated with *Cryptosporidium* isolates of saurian origin infections. Moreover, the results of the present study also showed that *Cryptosporidium* isolate originating from skink *E. schneideri* was not transmissible to black rat snakes *E. obsoleta obsoleta*, whereas these snakes were, in studies of Cranfield and Graczyk (1994), 100% susceptible to *C. serpentis*. The results of our transmission experiments indicate that *C. saurophilum* is not identical species with *C. serpentis*.

As was shown by Tilley et al. (1990), *C. serpentis*oocysts derived from a spontaneously infected yellow rat snake *E. obsoleta quadrivittata* are unable to establish infection in neonatal BALB/c mice. Oocysts recovered from a savannah monitor *Varanus exanthematicus* were used for inoculation of five-day-old ICR outbred mice and none became infected (Upton 1990). More recently, Fayer et al. (1995) inoculated five-day-old inbred BALB/c mice with different isolates of *C. serpentis*. Histological sections of stomach and intestine 4 days post inoculation did not contain *Cryptosporidium* developmental stages. These data correspond well with results of our experiments, which also failed to infect seven-day-old BALB/c mice, severe combined immunodeficient mice (SCID) and chickens with skink-derived *Cryptosporidium* oocysts.

Cryptosporidium species infecting intestinal and cloacal regions in lizards differs from *C. serpentis* from snakes in all examined aspects and provide support for the designation of *C. saurophilum*. However, the results of the present study do not exclude an existence of other *Cryptosporidium* species infecting stomach in lizards (Dilleyhay et al. 1986, Frost et al. 1994).

Acknowledgments. This study was supported by the Grant Agency of the Czech Republic (Grant No. 508/95/0273). We thank Marie Váchová, Jan Šlapeta and Jiří Volf for care of experimental animals, Jan Šlapeta kindly made line drawings. We also thank Veronika Schacherlová and Petra Eliašová for preparing tissue samples for histology and electron microscopy.

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Received 10 December 1997

Accepted 3 March 1998