FOLIA PARASITOLOGICA 34: 193–197, 1987.

STUDIES ON THE LIFE CYCLE OF CRYPTOSPORIDIUM COCCIDIA IN EXPERIMENTALLY INFECTED CHICKENS

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Abstract. Experimental infections of 7—28-day-old chickens with *Cryptosporidium* oocysts isolated from spontaneously infected chickens demonstrated that the endogenous development of this parasite takes place simultaneously in the organs of digestive, respiratory and excretory systems and in bursa of Fabricius. It was demonstrated for the first time that the oocysts of *Cryptosporidium* are shed through the respiratory tract into the beak cavity. A novel rapid and simple method has been developed for the detection of oocysts. Its principle is the rinsing of the beak cavity. This method enabled to isolate the oocysts from experimentally infected chickens and immediately use them in the dose of 6×10^5 for peroral infection of a 37-day-old chicken. The prepatent period was 8 days, patent period 12 days. On days 4—7 after the first detection of *Cryptosporidium* oocysts in chicken excrements, the oocysts were detected also by the method of beak cavity rinsing. This indicates that the oocysts released from the respiratory tract are infective. This fact is important from the epizootological viewpoint in relation with possible spreading of *Cryptosporidium* infections in chicken farms.

Already 79 years have elapsed since the first Tyzzer's description of *Cryptosporidium* coccidium, *C. muris* Tyzzer, 1907 in gastric gland of laboratory mouse. During this period, another 19 species have been described, most of them on the basis of a supposed host specificity of these protozoans. The life cycle of some of them, with the description of individual developmental stages, has been well documented. However, the number of species has been much reduced in recent studies dealing with experimental cross transfers of oocyst isolates obtained from various host species (Reese and Current 1982, Tzipori 1983, Levine 1984, Upton and Current 1985). The original opinion about *Cryptosporidium* coccidia has been significantly changed by the evidence of their involvement in respiratory diseases of turkeys, chickens and pheasants. In spite of the fact that a lot of information has been obtained about *Cryptosporidium* coccidia, our knowledge of these protozoans is still insufficient.

The first finding of *Cryptosporidium* in chicken was reported by Tyzzer (1929), who identified the parasites as *C. parvum*. However, Levine (1961) determined the coccidia from chickens as an independent species which he named *C. tyzzeri*. Fletcher et al. (1975) found *Cryptosporidium* in the bursa of Fabricius in chickens. Respiratory cryptosporidiosis with the presence of parasites in mucosal epithelium of trachea and in inner surface of mucous glands of chickens was reported by Dhillon et al. (1981). Itakura et al. (1984) described *Cryptosporidium* infection in chicken broilers. In Czechoslovakia, coccidia of the genus *Cryptosporidium* were found in chickens in the mucosal epithelium of sinus infraorbitalis (Pavlásek 1985). Pavlásek and Palkovič (1986) detected oocysts of *Cryptosporidium* in the excrements of spontaneously infected chickens. The parasites morphologically differed from *C. parvum* from calves and therefore the authors considered them to be a new, hitherto undescribed species differing from that infecting the mammals. This species occurs mostly in some domestic birds and sometimes also in wild birds. Pavlásek et al. (1986) described experimental

infection of chickens with this species and its endogenous development. Current et al. (1986) described the life cycle of *C. baileyi* infecting chickens.

The purpose of this paper is to described the course of experimental infection of chickens with *Cryptosporidium*, with the first record of oocysts in the organs of respiratory tract. A description is given of a novel method for the detection of oocysts from respiratory organs in the beak cavity of live infected chickens.

MATERIALS AND METHODS

Isolation of oocysts and preparation of inoculum. Oocysts of *Cryptosporidium* were isolated from the excrements of spontaneously infected chickens (*Gallus gallus* f. *dom.*) obtained from a chicken farm (South Bohemia) in 1985. The isolate was maintained in the laboratory of the Institute of Parasitology, Czechoslovak Academy of Sciences, České Budějovice by repeated experimental infections of chickens. The oocysts were recovered from infected chickens by the method described in a previous paper (Pavlásek 1987) and kept in 2.5 % solution of $K_2Cr_2O_7$ at the temperature of 4 °C. Before the experimental infection (per os) the oocysts were concentrated by saturated sucrose solution and washed in water. The infection dose of 5—6 × 10^5 oocysts/chicken was counted in Bürker's chamber.

Experimental chickens. Chickens of the race Tetra (16 specimens) at the age of 21 days were infected perorally. For the study of the endogenous development of the parasite, always one chicken was sacrificed and dissected on days 1—4, 9, 10, and 11, two chickens on days 5, 6, and 8, and three chickens on day 7.

Chickens of the race Ross (110 specimens) were divided into 5 groups of 22 animals each and infected perorally at the age of 7, 14, 21, 24, and 28 days; 22 chickens served as controls. *Cryptosporidium* oocysts were detected in the chickens at 2—4-day intervals.

Detection of oocysts. *Cryptosporidium* oocysts were detected in the excrements of infected chickens by coprological examination using the methods after Breza (1957) and after Pavlásek (1987). Oocysts from the respiratory tract were detected in the beak cavity by the following method: 1.5 ml and 2 ml of water (37 °C) was gently drived into the beak cavity by means of a syringe, the injection needle of which was replaced by a rubber hose (inner diameter 2 mm, length 5 cm). The fluid was immediately sucked back into the syringe. One to two drops were placed on a slide, covered with a cover glass and the preparation was examined at the magnifications of 400—1,000×. In case of negative finding, a control examination was performed: the fluid was transferred to a tube and water was added to obtain the volume of about 10 ml. The fluid was then centrifuged for 3 min at 2,500 rpm, the supernatant was carefully removed (mucosa often occurs on the surface) and the oocysts were then detected in two ways: 1. some drops of the sediment were examined as a native preparation, or 2. 10 ml of any floatation solution was added to the sediment and further processed using the common floatation and centrifugation procedures. The oocysts were collected by means of a loop (4 mm in diameter) from the superficial membrane, put on a slide and examined in the microscope at the magnifications of 400—1,000×.

Imprints and smears from the surface of mucosal epithelia of the digestive, respiratory and excretory systems were taken from the dissected chickens. The samples were then fixed in methanol and stained after Giemsa. For the detection of *Cryptosporidium* developmental stages, samples for histological methods were fixed in 10% neutral formalin, embedded into paraffin and the sections were stained after Giemsa.

Samples of the bursa of Fabricius were examined by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). For SEM, small pieces of tissue were fixed in 2.5% glutaraldehyde at 4 °C for 2 h, washed in cacodylate buffer, postfixed in 2% OsO_4, washed in redistilled H_2O, dehydrated through ethanol series (30—100%), transferred to acetone and dried by the method of critical point. Then they were coated with gold and examined in TESLA BS 300 scanning electron microscope (produced in Czechoslovakia). For TEM, the tissue was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2 h and postfixed in 1% OsO_4 for 2 h. The sample was then dehydrated through alcohol series and embedded into Epon. Ultrathin sections were cut with Reichert's OM-U2 ultramicrotome, contrasted with 20% uranyl acetate and Reynold's solution of lead acetate, and examined with PHILIPS EM 420 transmission electron microscope.

RESULTS

1. The course of cryptosporidiosis in experimentally infected chickens

Experimentally infected chickens started to shed the oocysts, in relation to their age, on days 4—14 p.i. A large number of developmental stages of *Cryptosporidium* coccidia, particularly of trophozoites and first generation of meronts, were found in the distal parts of small intestine, ileum, but also in caecum, during the first 24 h p.i. Single trophozoites could be detected in the large intestine, cloaca and bursa of Fabricius. While the intensity of infection markedly dropped in the small intestine during 48 h p.i., further development of the parasite, including all hitherto known developmental stages, concentrated particularly into the mucosal epithelium of bursa of Fabricius (Plate I, Figs. 1, 2, 3), cloaca, and large intestine (72—120 h p.i.). In some chickens, developmental stages of *Cryptosporidium* were found in imprints of sinus infraorbitalis since day 5 p.i., in trachea since day 6 p.i. (Plate II, Fig. 4), in bronchi (Plate II, Fig. 5), and lung imprints since day 7 p.i., and in kidneys and ureter on day 8 p.i. Coccidia were found also in smears from the mucosa of conjunctival sac of two chickens on days 7 and 11 p.i., respectively.

During the series of experimental infections of Ross chickens, there appeared panting on days 8—10 p.i. The breathing was then very loud and turned to strong typical hoarseness of all infected chickens on day 14 p.i. (Pavlásek et al., unpublished). On this day, we managed to obtain *Cryptosporidium* oocysts (Plate II, Fig. 7), as products of the endogenous development, from the respiratory organs of a live 35-day-old chicken using the method of beak rinsing. The presence of oocysts and their shedding through the air passages into the beak cavity was evidenced by the dissection of a dead 40-day-old chicken on day 16 p.i. Zygotes and arising oocysts were found in the smears from the mucosal epithelium of trachea and, at a relatively high intensity, also in the larynx (Plate II, Fig. 6). Typical oocysts of this bird species of *Cryptosporidium* (Plate II, Fig. 7), morphologically almost identical with those excreted with the excrements of infected chickens (mean size 6.2 × 4.8 μm), were found before the dissection also in the beak cavity, if the method of beak rinsing was used. The parasites were found also in the bursa of Fabricius and cloaca of the same chicken.

2. Inoculation of chicken with oocysts isolated from beak cavity

Using the method of beak rinsing, a sufficient number of oocysts were obtained from a group of 22 experimentally infected 35-day-old chickens on day 16 p.i. Immediately after isolation, the oocysts at the dose of 6 × 10^5 were used for peroral infection of a 37-day-old chicken from the control group free from *Cryptosporidium* infection (as confirmed by regular examinations during the experiment). First oocysts appeared in the excrements of the infected chicken on day 8 p.i., the patent period was 12 days. The highest number of oocysts were shed on days 4—7 after the first finding of oocysts. At that time, the oocysts could be detected also in the beak cavity. The other control chickens were free from Cryptosporidium infection during the whole experiment.

DISCUSSION

Coccidia of the genus *Cryptosporidium* were found in the respiratory tract of birds (turkeys) probably for the first time by Hoerr et al. (1978). Respiratory cryptosporidiosis in turkeys was described also by Ranck (1979). Dhillon et al. (1981) observed different

developmental stages of *Cryptosporidium*, except the oocysts. According to Itakura et al. (1984), the presence of these protozoans in the respiratory tract causes the clinical symptoms of the disease in chicken farms. The histological changes, particularly in trachea, were so marked that *Cryptosporidium* coccidia were considered by these authors to be the primary cause of the respiratory diseases. Current et al. (1986) assume that *C. baileyi* is responsible for the bursal and respiratory cryptosporidioses in southeastern regions of the U.S.A. Our results and records of coccidia of the genus *Cryptosporidium* in chickens from some of the chicken farms in Czechoslovakia or from experimental infections are consistent with the data published by the above authors. Pavlásek and Palkovič (1986) found in chicken farms oocysts of coccidia morphologically different from *C. parvum* from calves. Since these oocysts were not infective for laboratory mice, the authors deduced that a specific bird species of *Cryptosporidium* was involved. Our studies on the life cycle of this protozoan (Pavlásek et al. 1986), compared to the data about *C. baileyi* published by Current et al. (1986), show that the two species may be identical.

In contrast to Current et al. (1986), in our experiments, after experimental peroral infection the parasites occurred not only in different parts of the digestive system and in bursa of Fabricius, but simultaneously or with a 1—3-day retardation also in the organs of the respiratory system and in some chickens even in the excretory system. If the oocysts were inoculated into rhinal cavities, the parasites appeared not only in the organs of the digestive tract (ileum, caecum, large intestine), bursa of Fabricius, and cloaca, but also in the respiratory tract (Pavlásek, unpublished). The results of our experiments clearly show that there is a correlation between the course of development of cryptosporidial infection and the ability of the parasites to infect the surface of mucosal epithelium in different organs. In our opinion, an important finding is the fact that the endogenous development of *Cryptosporidium* coccidia in the respiratory tract of chickens is terminated, like in the bursa of Fabricius, by the formation of oocysts. They are morphologically identical with those excreted with the excrements of chickens. The new simple method (see Materials and Methods) enables a rapid detection of oocysts in animals suffering from respiratory cryptosporidiosis. Of importance is also the fact that in some chickens the oocysts were found in the beak cavity by 1—2 days earlier than in the excrements.

Our experiments demonstrated that in case of respiratory cryptosporidiosis the oocysts are shed into the beak cavity of infected birds and are immediately infective for sensitive chickens. These data supplement the present knowledge of the biology and epizootology of protozoans of the genus *Cryptosporidium* and contribute to the elucidation of the ways of spreading of these coccidia.

ИЗУЧЕНИЕ ЦИКЛА РАЗВИТИЯ КОКЦИДИЙ РОДА *CRYPTOSPORIDIUM* У ЭКСПЕРИМЕНТАЛЬНО ЗАРАЖЕННЫХ КУР

И. Павласек

Резюме. С помощью экспериментального заражения 7—28-дневных кур ооцистами *Cryptosporidium*, изолированными из спонтанно зараженных кур, было доказано, что эндогенное развитие этого паразита протекает одновременно в органах пищеварительной, дыхательной и экскреторной системы и в сумке Фабрициа. Впервые было доказано, что ооцисты *Cryptosporidium* выделяются через дыхательный путь до полости клюва. Описан новый, быстрый и несложный метод для обнаружения ооцист, принцип которого заключается в промывании полости клюва. С помощью этого метода были выделены ооцисты из экспериментально зараженных кур и в дозе 6 . 10^5 непосредственно использованы для орального заражения 37-дневной куры. Препатентный период был 8 дней, патентный период — 12 дней. Через 4—7 дней после первого обнаружения ооцист *Cryptosporidium* в экскрементах кур ооцисты были обнаружены также с помощью метода промывания полости клюва. Эти результаты показывают, что ооцисты, выделенные из дыхательной системы, инфекционные. Этот факт имеет большое значение с точки зрения эпизоотологии в соответствии с возможностью распространения ооцист *Cryptosporidium* в птицефармах.

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Received 30 October 1986

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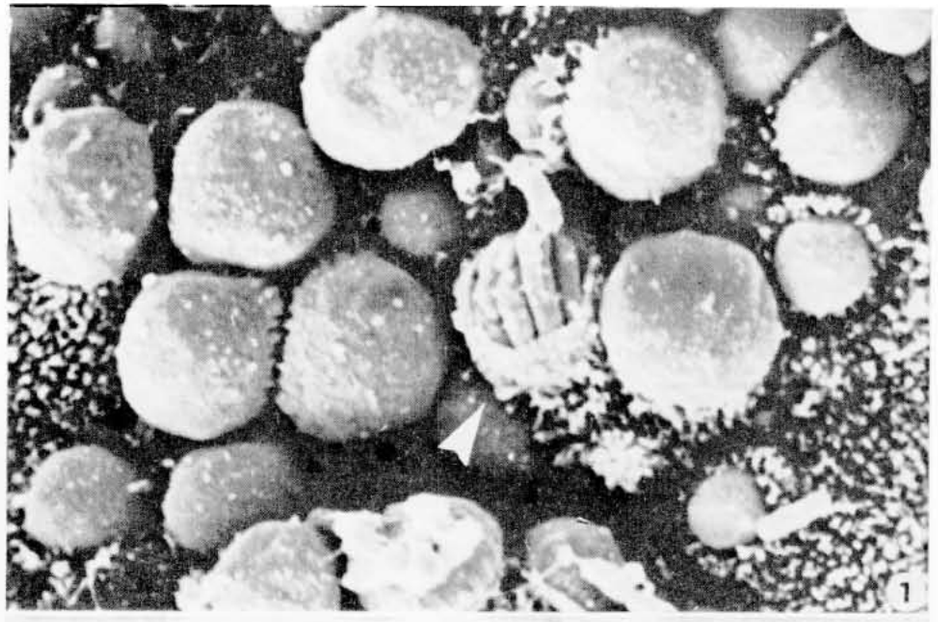
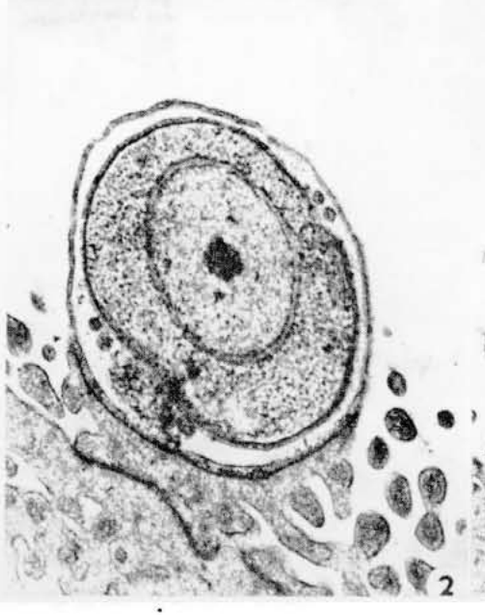
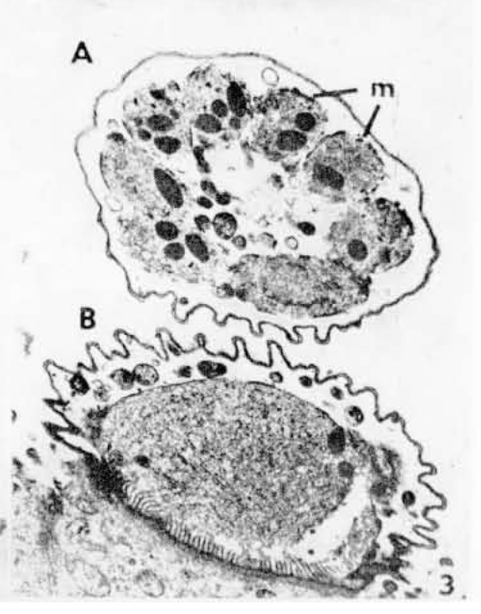
FOLIA PARASITOLOGICA 34: 197—198, 1987.

FINDING OF COCCIDIA OF THE GENUS CRYPTOSPORIDIUM IN THE ORGANS OF CALF EXCRETORY SYSTEM

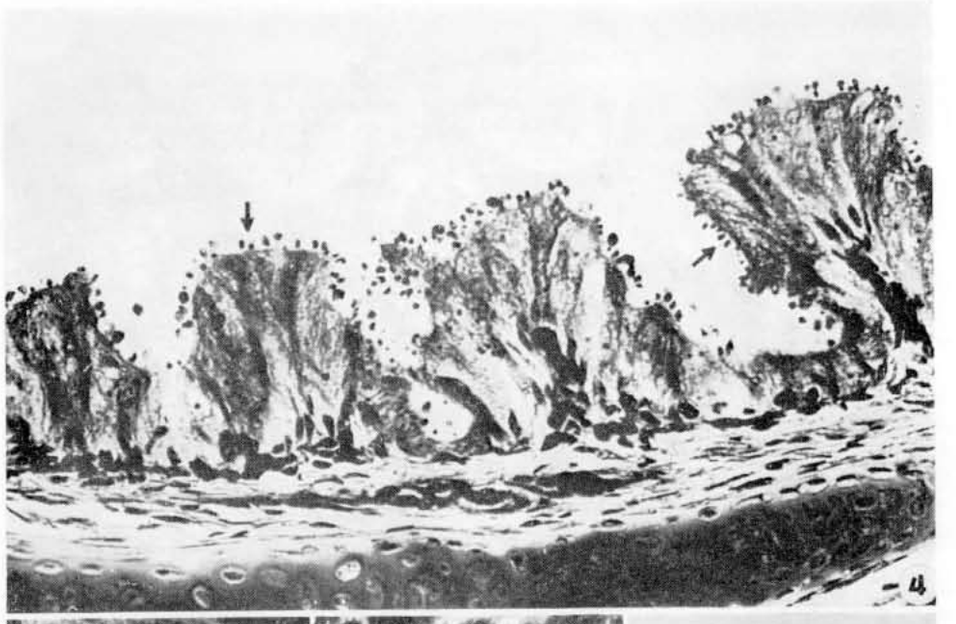
Coccidia of the genus *Cryptosporidium* have been increasingly dealt with in the literature during the last years, particularly after the year 1982. New methods of the diagnostics enabled to discover *Cryptosporidium* in various animal species and man and the host spectrum is still increasing. Tyzzer's original and excellent scheme of the life cycle of *Cryptosporidium parvum* published in 1912 (Tyzzer E. E., 1912: Arch. Protistenk. 26: 394—412), which is valid also for other described species of this genus, was later supplemented with further data. For example, Iseki (Iseki M., 1979: Jap. J. Parasit. 28: 285—307) studied the ultrastructure of *C. felis*, Current and Long (Current W. L., Long. P. L., 1983: J. Infect. Dis. 148: 1108—1113), described the course and characterized individual stages of the life cycle of *Cryptosporidium* coccidia on the basis of experiments performed with chicken embrya. According to these authors, the ability of autoinfection is ascribed to the oocysts not leaving their host and since they do not differ in their morphology from the oocysts excreted

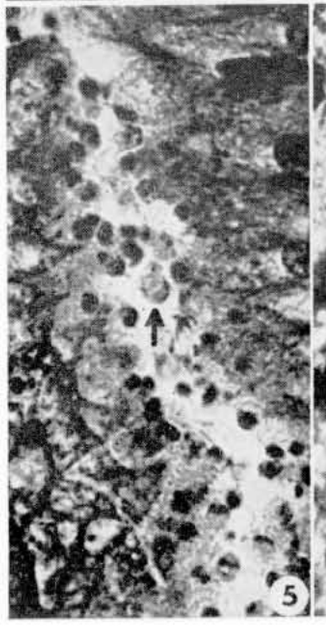


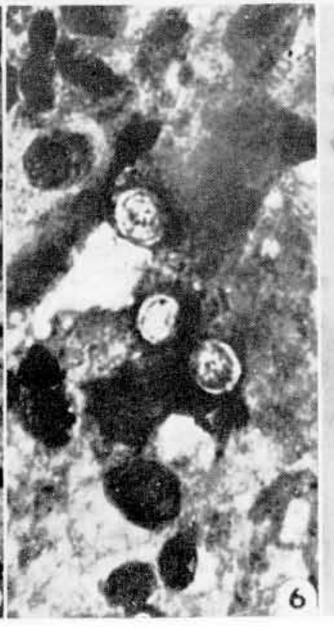




Figs. 1—3. Developmental stages of *Cryptosporidium* coccidia in the mucosa of bursa of Fabricius from experimentally infected chicken. **Fig. 1.** SEM of developmental stages, meront and merozoites — arrow (× 5.000). **Fig. 2.** TEM of the trophozoite (× 18.500). **Fig. 3.** TEM: A — meront with merozoites (m), B — unidentified developmental stage (× 10.500).







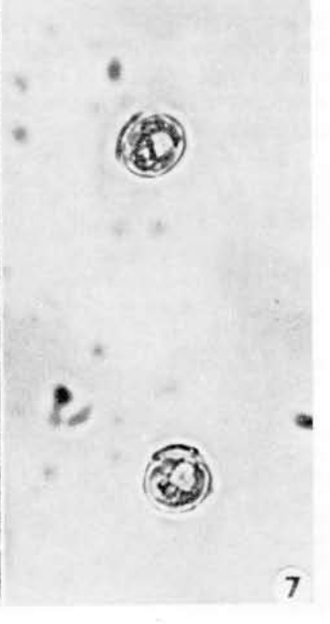


Fig. 4. Trachea of a 12-day-old experimentally infected chicken. Cryptosporidia on the surface of epithelial cells (arrows). Giemsa stain (× 300). **Fig. 5.** Section through bronchus with numerous cryptosporidians (arrows). Giemsa stain (× 1.000). **Fig. 6.** Zygotes and arising oocysts, smear from the larynx. Giemsa stain (× 1.400). **Fig. 7.** *Cryptosporidium* oocysts isolated from the beak cavity of chicken by the new method (× 1.600).