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Effects of different inoculation routes on the parasitic sites of *Cryptosporidium baileyi* infection in chickens

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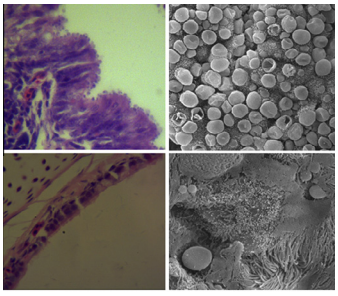
HIGHLIGHTS

- Chickens were successfully infected with *C. baileyi* via the rectum.
- Rectal inoculation leads to parasitization of the BF and cloaca but no other sites.
- Fewer parasites in the trachea of chickens infected with oocysts via crop than in BF.
- *Cryptosporidium* was not transferred through blood.
- Inoculation routes can affect sites of parasitism by *C. baileyi* in chickens.

GRAPHICAL ABSTRACT



More parasites in the bursa of Fabricius than in the trachea

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ABSTRACT

Cryptosporidiosis is prevalent in domesticated, caged, and wild birds. *Cryptosporidium baileyi*, an ascendant species of avian *Cryptosporidium*, is an important pathogen. It causes respiratory disease in chickens, especially chickens younger than 50 days. In this study, SEM, histological, semi-quantitative PCR, and nested PCR techniques were used to explore the impact of different inoculation routes on sites of *C. baileyi* infection in chickens. Results showed that inoculation with sporozoites or oocysts via the rectum was an effective means of causing infection. This may provide an important reference for the development of the transfection system of *C. baileyi* in chickens. Numerous endogenous stages of *C. baileyi* were observed in the bursas of Fabricius (BF) and cloacas of chickens inoculated with sporozoites or oocysts via the rectum, but no parasite was seen in the tracheas of any of these chickens. In chickens infected with oocysts via the crop, the number of parasites in the BF was approximately 23-fold more than in the trachea. All blood samples collected after inoculation were negative for *C. baileyi*. These data show that *C. baileyi* was not transferred by blood circulation between the BF and respiratory tract. Different routes of inoculation were here found to distinctly affect sites of parasitism in chickens. These findings may facilitate further understanding of the biology of *C. baileyi* and efforts to control avian cryptosporidiosis.

1. Introduction

Cryptosporidiosis is one of the most prevalent parasitic infections in domesticated, caged and wild birds, and it has been reported in over 30 avian species worldwide (Amer et al., 2010; Qi et al., 2011; Ryan et al., 2008). Currently, three species of avian

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Cryptosporidium, namely *C. baileyi*, *C. meleagridis*, and *C. galli*, have been recognized. They are distinguished by biological features and genetic analysis (Ryan et al., 2003; van Zeeland et al., 2008; Xiao et al., 2004). *C. baileyi* is the most common *Cryptosporidium* species in chickens. It is especially pathogenic in chickens younger than 50 days old, and it can induce lesions of the respiratory tract, bursa of Fabricius (BF), cloaca, and kidney. Avian cryptosporidiosis has brought considerable economic losses in poultry industry annually (Abbassi et al., 1999; Blagburn et al., 1991; Goodwin and Brown, 1990; Ryan, 2008).

The clarifications of the exact routes of transmission and sites of parasitism of *Cryptosporidium* species in hosts can facilitate understanding of its biological features and control of cryptosporidiosis (Ryan, 2008). Natural infections of *C. baileyi* have been reported at different sites in birds, including the BF, cloaca, large intestine, small intestine, respiratory tract, conjunctiva, kidney, and urinary tract (Abbassi et al., 1999; Goodwin and Waltman, 1994; van Zeeland et al., 2008). After oral inoculation of oocysts, the main parasitic sites in chickens are found to be BF, cloaca, and trachea. Intranasal inoculation of oocysts into chickens can cause extensive parasitization of the respiratory tract by various developing parasites (Lindsay et al., 1986, 1987; Ryan, 2010). Although the BF and cloaca are far from the respiratory tract with respect to space and their structures and functions are very different from each other, they and the respiratory tract are the primary sites of *C. baileyi* in chickens (Goodwin and Brown, 1990; Xiao et al., 2004; Ryan, 2010). The reason for this remains unclear.

Chickens can be successfully infected with *Eimeria tenella*, whose parasitic site (the cecum) is close to the rectum, by inoculating sporozoites via rectal route (Clark et al., 2008; Yan et al., 2009). Because the BF and cloaca are main sites of *C. baileyi* parasitism in chickens and because they are adjacent to the rectum, it has been supposed that chickens become infected with *C. baileyi* through rectal inoculation of sporozoites or oocysts. However, no rectal inoculation of *C. baileyi* sporozoites or oocysts into chickens has yet been reported. In the present study, sporozoites and oocysts of *C. baileyi* were inoculated into 12-day-old chickens via rectal and crop routes to assess the effects of different inoculation routes on the sites of parasitism and the mechanism of transmission of *C. baileyi* in avian hosts.

2. Materials and methods

2.1. Preparation of oocysts and sporozoites

C. baileyi oocysts (isolated from a chicken farm in Luoyang of Henan province and here referred to as LY isolate) were passaged in chickens, purified using Sheather's sugar floatation, and stored in 2.5% potassium dichromate solution at 4 °C. Oocysts used for infection were treated in 1% NaClO solution on ice for 10 min to kill other microbes, rinsed three times with deionized water to remove residual NaClO, and finally resuspended with distilled water (Eckert, 1995; Huang et al., 2011). Oocysts were counted by a blood cell counting plate before inoculation. The viable oocysts used in this study were stored for no more than 2 weeks after reproduction (Surl et al., 2003).

Sporozoites were extracted with an extraction buffer described previously (Yan et al., 2009). Then 5 ml purified oocyst suspension (10^6/ml) was washed three times with deionized water to remove potassium dichromate. The oocysts were treated with 1% NaClO solution to kill other microbes. The oocyst precipitate was resuspended in the extraction buffer that had been preheated to 40 °C. The mixture was incubated at 40 °C for 40–60 min (mixed every 5 min). The excystation rate of sporozoites was evaluated under a light microscope 20, 30, 40, 50 and 60 min into incubation. The incubation of oocyst excystation was not expected to cease before the excystation rate reached 100%.

2.2. Experimental design

First, 120 highland chickens (1 day old) were purchased from Modern Poultry Company in Luoyang, China, and reared in a cryptosporidia-free environment. At the age of 12 days, they were randomly divided into 5 groups of 24 birds each as follows: 5.0×10^5 oocysts per bird via crop, 2.0×10^6 sporozoites per bird via rectum, 5.0×10^5 oocysts per bird via rectum, and 2.0×10^6 sporozoites per bird via crop. The remaining chickens, in group 5, served as negative controls (Table 1).

Each set of 24 chickens was reared in individual wire-bottomed cage placed on fecal trays to collect feces conveniently. All the containers of food and water were treated in hot-air sterilizer. The breeding room, cages, and other facilities were disinfected by gasoline blast burner before use. Fecal samples from all chickens were examined for oocysts of *Cryptosporidium* before inoculation to ensure that none of the experimental chickens were already infected with *C. baileyi*. Starting on day 3 post inoculation, fresh fecal samples were collected daily from each group, mixed, and 10 g of the mixed feces from each group was examined using the Sheather's sugar floatation method and a blood cell counting plate to determine the dynamics of oocyst shedding (Eckert, 1995).

Tissue samples from the BF, cloaca, large intestine, small intestine, proventriculus, trachea, and lung were collected from 6 experimental chickens per group at the time of peak oocyst shedding. The experiment was consistent with guidelines for animal experimentation and with pertinent laws and regulations of China on the treatment and use of laboratory animals. Each tissue sample was divided into four portions, fresh subsamples were prepared for mucosal shaving examined using modified acid fast staining. The second set of subsamples were fixed in 10% formalin for histopathological observation, and the third were cut into 3–4 mm in size and fixed in 2.5% (v/v) glutaraldehyde in 0.1 M PBS (pH 7.2) for more than 4 h at 4 °C for scanning electron microscopy (SEM). For semi-quantitative PCR analysis, small pieces of samples were preserved in 80% ethanol alcohol until further use.

Table 1
Shedding of *C. baileyi* oocysts in chickens inoculated with oocysts or sporozoites via the crop or rectum.

Group[A]	Route of infection	Parasite	Inoculation dose[B]	Prepatent period	Patent period	Peak time of oocyst shedding	OPG value each day at the peak period of oocyst shedding ($\times 10^5$)
1	Crop	Oocysts	5.0×10^5/bird	Day 4 PI	13 days	Days 10–12 PI	5.89 ± 0.17^{ac}
2	Rectum	Sporozoites	2.0×10^6/bird	Day 5 PI	14 days	Days 9–12 PI	20.7 ± 0.11^{b}
3	Rectum	Oocysts	5.0×10^5/bird	Day 7 PI	12 days	Days 10–13 PI	$5.08 \pm 0.22^{c,a}$
4	Crop	Sporozoites	2.0×10^6/bird	N/A	N/A	N/A	$0^{d,e}$
5	Crop	Sterilized PBS	N/A	N/A	N/A	N/A	$0^{e,d}$

Notes: [A] There were 24 twelve-day-old chickens per group; [B] Inoculation doses of oocysts (IDO) were determined using a blood cell counting plate. Inoculation doses of sporozoites (IDS) were calculated according to the following formula: IDS = 4 × IDO (4 sporozoites per oocyst); OPG: oocysts per gram of chicken feces; N/A: not applicable because no oocysts were seen in feces of chickens from group 4 or 5 during the test. Chickens from group 5 served as negative controls and were not inoculated with any parasites. [a–e] Different lowercase superscript letters indicate significant differences ($P < 0.05$). PI: post-inoculation.