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PROTOZOOLOGY - SHORT COMMUNICATION

The role of the American cockroach (*Periplaneta americana*) as transport host of *Eimeria tenella* to chickens

Wipaporn Jarujareet [1,2] · Masuko Kobayashi [1] · Kensuke Taira [1] · Hong-Kean Ooi [1]

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Abstract
The role of the American cockroach, *Periplaneta americana* as transport host for *Eimeria tenella* was evaluated. Twenty-four cockroaches were orally fed with sporulated oocysts of *E. tenella.* Their feces and digestive tract were examined for oocysts by sugar centrifugal flotation technique and PCR. Infectivity of the oocysts recovered from the digestive tract of infected cockroaches as well as from their feces was evaluated by orally inoculating them into Boris Brown chickens. *E. tenella* oocysts were found in the digestive tract and feces of infected cockroaches up to day 4 after ingestion of oocysts. Furthermore, oocysts that were recovered from the digestive tract and feces of cockroaches remained infective for 4 and 3 days after ingestion of oocysts, respectively. Presence of oocysts in the feces of chicken that had been inoculated with either digestive tract or feces of *P. americana* demonstrated the infectivity of *E. tenella* oocysts from digestive tract or feces, suggesting that *P. americana* may play a role in the transmission of *E. tenella* among chicken and between chicken flocks.

Keywords Chicken · Coccidiosis · Cockroach · *Periplaneta americana* · *Eimeria tenella* · Transport host

Introduction

Chicken coccidiosis is an intestinal disease caused by the protozoal parasite of the genus *Eimeria*. It has a major economic impact on poultry production worldwide (Gilbert et al. 2011). Chickens are most commonly infected with *Eimeria tenella*, *Eimeria acervulina*, and *Eimeria maxima* (Duffy et al. 2005; Gilbert et al. 2011). The infective stage, the sporulated oocyst, is transmitted via fecal-oral route. Poultry excreta and contaminated litter are recognized as a source of *E. tenella* oocyts contamination (Hafez 2008). Thus, insects associated with poultry excreta and accumulated manure might serve as transport host for *Eimeria*. Darkling beetles and flies have been shown to be able to transport *Eimeria* to chicken (Reyna et al. 1983; Goodwin and Waltman 1996).

Cockroaches, a common insect known to feed on animal excreta, are recognized as premise pest in poultry farms worldwide. Although not common, massive occurrences of cockroaches have been reported in poultry houses (Axtell 1999). Cockroaches not only cause a nuisance, they can also contaminate other objects with disease agents that were attached to their body or in their fecal deposits. Therefore, they have been regarded as potential mechanical vector of foodborne pathogens in poultry production and processing facilities (Kopanic et al. 1994). Additionally, poultry eat a wide range of insects, including cockroaches. Thus, they can be infected with pathogens that are carried by cockroaches. *Oxyspirura mansoni*, a common eyeworm of poultry requires an intermediate host such as a cockroach to facilitate transmission to its chicken definitive host (Almas et al. 2018). Clubb and Frenkel (1992) and Godoy et al. (2009) have demonstrated that psittacine birds can develop sarcocystosis by eating a cockroach that has ingested feces containing *Sarcocystis falcatula* or eating food contaminated by cockroach feces. However, cockroaches have not yet been evaluated for its role in the transmission of *E. tenella*. This study aims to elucidate the role of *P. americana* in the transmission of *E. tenella* oocysts through experimental infection.

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✉ Hong-Kean Ooi
hkooi@azabu-u.ac.jp

[1] Laboratory of Parasitology, School of Veterinary Medicine, Azabu University, 1-17-71, Fuchinobe, Sagamihara, Kanagawa 252-5201, Japan

[2] Faculty of Veterinary Science, Rajamangala University of Technology Srivijaya, 133 Moo 5 Thungyai, Nakhon Si Thammarat 80240, Thailand

Materials and methods

E. tenella oocysts

E. tenella (Taiwan strain) that had been passaged and maintained in our laboratory was used. Species identification and purification of the strain were confirmed by manifestation of gross lesions and PCR (Jarujareet et al. 2018). To obtain the fresh oocysts, 1000 sporulated *E. tenella* oocysts were inoculated directly into the crop of 7-day-old Boris Brown chickens. At 7.5-day post-inoculation, the infected chickens were sacrificed and the cecal contents were collected for oocysts isolation. The oocysts were then suspended in 2.5% potassium dichromate solution to allow for sporulation at 25 °C for 72 h. After confirming the sporulation, the oocysts were stored at 4 °C and used within 1 week for experimental infection of cockroaches. All the chickens used in our experiments were hatched from eggs in the hatchery of our university, specific pathogen-free facility, and being maintained there to prevent any contamination.

Cockroaches

Twenty-seven laboratory-bred adult American cockroaches, *Periplaneta americana*, were used in this study. The cockroaches were kept in plastic containers that contained folded paper and a stack of wood board for insect refuge. A white paper board was placed on the container floor to facilitate collection of feces of the cockroaches. The containers were placed in a room with temperature maintained at 25 ± 1 °C and a 16/8-h light/dark (16 L/8D) photoperiod. The cockroaches were fed a commercial rodent feed (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and water was provided ad libitum.

Experimental infection of cockroaches with sporulated *E. tenella* oocysts

One week before the beginning of the experiment, feces of cockroaches that dropped onto the white paper board or container floor were collected daily and examined to confirm the absence of any natural *Eimeria* infection by sugar centrifugal flotation technique (Taira et al. 2003). Two days before inoculation, each cockroach was placed in separate 500 ml glass beaker without food and water. Twenty-four cockroaches were orally fed individually with cat liquid snack (Inaba-Petfood Co., Ltd., Shizuoka, Japan) containing 50 μl suspensions of 20,000 sporulated oocysts of *E. tenella* and designated as infected group. Three remaining cockroaches were given an equal volume of cat liquid snack (Inaba-Petfood Co., Ltd., Shizuoka, Japan) mixed with water instead of the oocyst suspension and designated as uninfected control group. After all the food had been ingested, each cockroach was transferred to a new 500-ml glass beaker, provided with water-soaked cotton wads and fed with commercial rodent feed (MF, Oriental Yeast Co., Ltd., Tokyo, Japan). They were kept under the same conditions as described above.

Detection of *E. tenella* oocysts in infected cockroaches

On 1, 2, 3, 4, 5, 7, 10, and 15 days after ingestion of oocysts, three cockroaches from the infected group were sacrificed using diethyl ether followed by decapitation. The digestive tracts of two cockroaches were slit open and washed with 10 ml of saline, followed by homogenization and filtered through a sieve. The filtrate was then transferred to a 15-ml test tube and centrifuged at 2500 rpm (1610×*g*) for 5 min. The supernatant was discarded, and the sediment was re-suspended in 2 ml of distilled water; 1 ml of this suspension was used for inoculation into the chicken, and the remaining 1 ml of the suspension was examined for *E. tenella* oocysts by using sugar centrifugal flotation technique (Taira et al. 2003). Briefly, the remaining 1 ml of the suspension was centrifuged at 2500 rpm (1610×*g*) for 5 min, and the supernatant was discarded. The sediment was re-suspended in 10 ml salt/sugar solution (specific gravity 1.28, prepared as follows: sodium chloride 400 g + water 1000 ml + sugar 500 g). The suspension was stirred and centrifuged again at 2500 rpm (1610×*g*) for 5 min. Salt/sugar solution was added until an upper meniscus was formed, and a coverslip (18 × 18 mm) was placed over the tube. The coverslip was examined 30 min later under a light microscope.

The entire digestive tract of the remaining one cockroach was preserved in 70% alcohol and used for detection of *E. tenella* oocysts by multiplex PCR based on ITS-1 region (You 2014). Primer sequences, amplicon sizes, and specificity are listed in Table 1. The digestive tract of cockroach was processed in the same way as described above until the supernatant was discarded. After discarding the supernatant, 1 ml of distilled water was added to the sediment followed by slight vortexing for re-suspension. The suspension was transferred to a microcentrifuge tube, and oocyst lysis was conducted using the method described by Tang et al. (2018), with minor modification. Briefly, the suspension was centrifuged at 2500 rpm (1610×*g*) for 10 min to sediment the oocysts. The supernatant was discarded completely, and the pellet was re-suspended in 100 μl of sodium hypochlorite and incubated at 4 °C for 1.5 h followed by treatment with 350 μl of saturated salt solution at 55 °C for 1 h. Thereafter, DNA was extracted from the suspension using the InstaGene™ Matrix DNA extraction kit (Bio-Rad Laboratories, Inc., California, United states), according to the manufacturer's instruction. Multiplex PCR was performed in a final volume of 25 μl containing 100 ng DNA template, 0.2 mM of each dNTP, 1 μM of forward primer, and 0.5 μM of each reverse primer, 0.025 U of Ex Tag polymerase (TaKaRa Ex Taq®, Takara Bio, Shiga, Japan), and the manufacturer's supplied reaction

Table 1 Primers based on ITS-1 region used in the multiplex PCR for three *Eimeria* species (You 2014)

Primer	Oligonucleotide sequence (5′-3′)	Amplicon size (bp)	Specify
Forward	GTTGCGTAAATAGAGCCCTCT		*Eimeria* spp.
Reverse	ACCAATGCAGAACGCTCCAG	152 bp	*Eimeria maxima*
	CAAAAGGTGGCAATGATGCT	281 bp	*Eimeria acervulina*
	GTTCCAAGCAGCATGTAACG	554 bp	*Eimeria tenella*

bp = base pairs

buffer. The PCR program consisted of a denaturation step at 95 °C for 10 min followed by 30 cycles of 98 °C for 10 s, 52.5 °C for 30 s and 72 °C for 1 min, and a final extension step at 72 °C for 1 min (You 2014). Then, 5 μl of PCR product was mixed with 1 μl of loading buffer and visualized in a 1.5% agarose gel by electrophoresis. The size of PCR products was determined by comparison with a 0.1–2 kbp gene ladder (Nippon Gene Co., Ltd., Toyama, Japan). Two positive controls were used; DNA extracted from *E. tenella* oocysts that were used for experimentally infecting the cockroaches and the other DNA extracted from a commercial avian coccidiosis trivalent (TAM) live vaccine (Nisseiken Co., Ltd., Tokyo, Japan) comprising of *E. tenella*, *E. acervulina*, and *E. maxima*.

Moreover, on the day that cockroaches were killed, all feces in the beaker were collected and pooled. Half of the feces was homogenized in 2 ml of distilled water followed by inoculation into chicken. The other half was used for examining *E. tenella* oocysts by using sugar centrifugal flotation technique as described above.

Evaluation of infectivity of oocysts in digestive tract contents and feces of cockroaches

The infectivity of the oocysts recovered from the digestive tract contents and feces of the cockroaches was evaluated by orally inoculating the oocysts into naïve (uninfected) chickens. Feces of chickens were tested by sugar centrifugal flotation technique prior to inoculation to confirm that the chickens were free from *Eimeria* infection. Thirty-eight, 7-day-old Boris Brown chickens were divided into 19 groups ($n = 2$). The treatment of each group is shown in Table 3. The chickens, with ad libitum access to water and feed (Pet's One Bird food, Cainz Co., Ltd., Saitama, Japan), were kept and handled according to the rules and regulations laid down by the Institutional Animal Care and Use Committee (IACUC) of Azabu University. Fecal samples from each group of chickens were collected at day 5.5, 6.5, and 7.5 post-inoculation. At day 8 post-inoculation, the chickens were sacrificed for necropsy to confirm the presence of lesion at the predilection site for multiplication of *E. tenella*, that is, the ceca, and to collect cecal content. Fecal samples and cecal contents were examined for the presence of *E. tenella* oocysts by using sugar centrifugal flotation technique.

Results and discussion

The indiscriminate feeding habits of coprophagous insects make them ideal carrier of various microorganisms. Cockroaches, flies, and dung beetle have been known to be transport hosts for a variety of parasites of public health and veterinary importance, such as *Toxoplasma gondii*, *Sarcocystis*, and *Isospora* spp. (Wallace 1972; Smith and Frenkel 1978; Markus 1980; Saitoh and Itagaki 1990). In our study, *E. tenella* oocysts were found in the digestive tract and feces of *P. americana* for four consecutive days after ingestion of the oocysts (Table 2). This indicates that the oocysts are resistant to

Table 2 Detection of *Eimeria tenella* oocysts from digestive tract contents and feces of cockroaches by using sugar centrifugal flotation technique

Group	Days post-ingestion	Digestive tract contents[a]	Feces[b]
Uninfected control	15	0/3	–
Infected	1	2/2	+
	2	2/2	+
	3	1/2	+
	4	1/2	+
	5	0/2	–
	7	0/2	–
	10	0/2	–
	15	0/2	–

[a] Number positive/number tested

[b] Pooled feces from three cockroaches

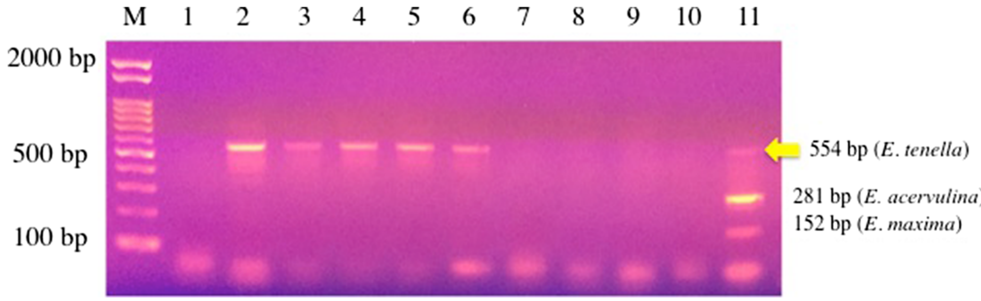

Fig. 1 Detection of *Eimeria tenella* in cockroaches' digestive tract using multiplex PCR. Lanes 1, negative control; 2, positive control; 3–6, samples from digestive tract contents of 1, 2, 3, 4-day post-ingestion (DPI) cockroaches, 7–10, samples from digestive tract contents of 5, 7, 10, 15 DPI cockroaches; 11. sample from a commercial attenuated live vaccine containing oocysts of *E. tenella*, *E. acervulina*, and *E. maxima*); M, molecular size marker

enzymes and microflora in the digestive tract of cockroaches, and can be carried in *P. americana*'s digestive tract and dispersed through their fecal materials. However, *E. tenella* DNA could not be detected in the digestive tract contents of experimentally infected cockroaches from day 5 after ingestion of oocysts (Fig. 1). Although this suggest that *E. tenella* oocysts could not be retained for a long time in the cockroach, continuous exposure to *E. tenella* oocysts-contaminated food source may lead them to become a persistent carrier. Moreover, since *P. americana* can move up to 38 m, such mobility can result in the dissemination of oocysts over a significantly wide area (Smith and Frenkel 1978).

Previous studies have reported that *Eimeria* oocysts could be detected in chicken farms that practice the "all in and all out" method, and thoroughly cleaned out the house and supplied fresh litter before arrival of a new flock. It is likely that the infection might have originated from oocysts that had survived from the preceding flock. Transmission from one flock to the next depends on the survival of a few oocysts in or around the poultry house, for instance in dust or in transport hosts such as insects (Reyna et al. 1983; Chapman et al. 2016). In our study, the presence of *E. tenella* oocysts in the feces of chicken that had been inoculated with either digestive tract contents or feces of infected *P. americana* (Table 3)

Table 3 Evaluation of infectivity of *Eimeria tenella* oocysts recovered from digestive tract or feces of cockroaches by inoculation into chickens

Chicken groups (*n* = 2)	Inoculation with	Oocyst in chicken feces (Day post-inoculation)			Oocyst in cecal contents of chicken on the day of necropsy
		5.5	6.5	7.5	
1 (Positive control)	Sporulated oocysts of *E. tenella*	+	+	+	+
2 (Negative control)	DC of uninfected- cockroach	–	–	–	–
3	DC of 1 DPI-C	–	+	+	+
4	DC of 2 DPI-C	–	+	+	+
5	DC of 3 DPI-C	–	+	+	+
6	DC of 4 DPI-C	–	–	+	+
7	DC of 5 DPI-C	–	–	–	–
8	DC of 7 DPI-C	–	–	–	–
9	DC of 10 DPI-C	–	–	–	–
10	DC of 15 DPI-C	–	–	–	–
11 (Negative control)	Feces of uninfected- cockroach	–	–	–	–
12	Feces of 1 DPI-C	–	+	+	+
13	Feces of 2 DPI-C	–	+	+	+
14	Feces of 3 DPI-C	–	–	+	+
15	Feces of 4 DPI-C	–	–	–	–
16	Feces of 5 DPI-C	–	–	–	–
17	Feces of 7 DPI-C	–	–	–	–
18	Feces of 10 DPI-C	–	–	–	–
19	Feces of 15 DPI-C	–	–	–	–

DC, digestive tract contents; *DPI-C*, day post-infected cockroaches demonstrated the infectivity of *E. tenella* oocysts from the cockroach digestive tract or feces. This suggests that chicken can be infected through direct ingestion of infected cockroaches or through ingestion of their feces.

In conclusion, our study demonstrated that *P. americana* may play a role in the transmission of *E. tenella* among chickens and between chicken flocks. The presence of cockroaches can be associated with a risk for transmission of *Eimeria*. And that more severe infestation may also have a more significant impact on *Eimeria* transmission. Thus, a proper poultry management system to control cockroach and other insect infestation is essential to reduce the risk of *Eimeria* transmission.

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