Parasitol Res (1990) 76:566–569

Parasitology Research

A new method for purification of *Eimeria tenella* merozoites

M.Q. Xie[1], J.M. Gilbert[2], A.L. Fuller[2], and L.R. McDougald[2]

[1] Veterinary Medicine Institute, Guangdong Academy of Agricultural Science, People's Republic of China
[2] Department of Poultry Science, University of Georgia, Athens, GA 30602, USA

Accepted March 13, 1990

Summary. A rapid and simple method for purifying second generation merozoites of *Eimeria tenella* was developed using a host tissue digestion fluid, containing 0.25% trypsin and 0.5% taurodeoxycholic acid, to liberate merozoites grown in chick embryos or from parasitized ceca. After filtration, the digestion procedure yielded 1.4×10^7 or 8.33×10^7 merozoites per embryo or cecum, respectively. These yields were nine-fold for embryos and three-fold for ceca in comparison to previous reports. Viability of the merozoites was normal as assessed by their ability to reinfect embryos and cell cultures. The new method has advantages in that large numbers of pure, viable merozoites can be obtained quickly and easily, and the procedures require minimal effort and supplies.

Parasite stages such as oocysts, sporozoites, schizonts, and gametocytes have been isolated by various investigators (James 1980; Pugatsch Mencher and Wallach 1989; Schmatz et al. 1984; Wagenbach et al. 1966; Wagenbach 1969) and the host cell-free stages have been helpful in studying reproductive and biochemical aspects of the parasite (Kouwenhoven and Kuil 1976; Laxler et al. 1987; Long 1985; Mencher et al. 1965). However, there have been few attempts to purify the merozoite stage (Doran 1974; Elsner et al. 1974; Stotish and Wang 1975). Pure preparations of viable, infective merozoites would be useful for in vitro immunologic and biochemical studies. Merozoites of *Eimeria tenella* have been successfully purified (Stotish and Wang 1975); however, the procedure was somewhat complicated and the yield of merozoites was low. The average recovery rate was around 54% and the culture contained approximately 5% epithelial cells and red blood cells. In this study, we compared several methods for the purification of merozoites and describe a rapid and simple method for purifying second-generation merozoites of *E. tenella* from chicken embryos and cecal preparations.

Reprint requests to: L.R. McDougald

Materials and methods

Preparation of chorioallantoic membranes (CAM)

Ten thousand to 80000 sporozoites of *E. tenella* were injected into the allantoic cavities of 11-day-old White Leghorn embryos (Long 1965). The embryos were incubated at 41° C for 100–110 h. The CAMs were harvested and then rinsed with PBS (pH 7.2). Afterwards, the CAMs were cut into small pieces with scissors and placed in Hanks' balanced salt solution (HBSS).

Preparation of ceca

Two-week-old broiler chicks were inoculated with 4.6×10^5 sporulated *E. tenella* (Wis) oocysts. Ceca were removed around 110 h postinfection, opened longitudinally, and their contents discarded. The ceca were washed twice with PBS (pH 7.2), the lining was scraped, and the contents were placed in HBSS.

Purification of merozoites

Digestion media were prepared using taurodeoxycholic acid and the enzymes trypsin (EC 3.4.21.4) and hyaluronidase (EC 3.2.1.35) (all from Sigma Chemical Co., St. Louis, MO). These ingredients were dissolved in HBSS in varying combinations and concentrations. The CAM and cecum preparations were placed in flasks containing digestion media and incubated at 40° C for 20–30 min with intermittent shaking. The escape of the merozoites into the media was monitored by light microscopy and incubation was stopped after most of the merozoites appeared to be free. Free merozoites were counted in a hemocytometer prior to further purification or periodically during incubation using the average of duplicate counts. The merozoite-containing liquid was filtered through four layers of cheese cloth to remove coarse tissue debris. The filtered liquid was then centrifuged at 2000 *g* for 10 min and the pellet was resuspended twice in HBSS and centrifuged at 2000 *g* for 10 min. Merozoites in the final suspension were counted (in duplicate) in a hemocytometer.

Merozoites from the cecal preparations contained some cellular debris. Merozoites collected from the CAM preparations were not contaminated with bacteria or cellular debris. The cecum merozoites were passed through a 0.2 μm disposable tissue culture filter (Nalgene, Rochester, NY) from which the top filter membrane had been removed. This procedure is the most suitable for obtaining very pure preparations of merozoites. For tissue culture all

the aforementioned steps should be done under sterile conditions. Merozoites in the final suspension were counted (in duplicate) in a hemocytometer.

Determination of merozoite infectivity

The collected merozoites were inoculated into chicken kidney cell cultures or into embryos. Infected monolayers were incubated at 41° C in 5% CO_2 for 24 or 60 h. The monolayers were then examined for parasites by the fluorescent antibody technique utilizing the mouse monoclonal antibody 1A3. Oocysts were harvested from the embryos at 60 h postinfection and their numbers and sporulation rates determined.

Results

Digestion media

Four digestion media were compared to ascertain which of them was most suitable for purifying merozoites from the CAM (Table 1). The yield of merozoites from CAM treated by 0.25% trypsin and 0.75% taurodeoxycholic acid (TDC) was significantly higher than the CAMs treated with 0.1% hyaluronidase, 0.75% TDC, or 0.25% trypsin alone. The trypsin-TDC medium destroyed all contaminating red blood cells and yielded a very pure suspension of merozoites.

Optimization of TDC level

The trypsin-TDC medium was titrated to find the amount of TDC that would preserve merozoite viability (Table 2). TDC at 0.25%, 0.50%, and 0.75% concentrations were tested. Merozoite yields were high for the 0.5% and 0.75% TDC levels; however, there was no significant difference between the two treatments.

Determination of optimum digestion time

CAM digestion time influenced the number of merozoites collected (Fig. 1). Digestion times ranging from 5 to 30 min were compared, and it was found that merozoite yield rose sharply from 5 through 20 min. The recovery rate after 20–30 min incubation did not change significantly.

Determination of inoculum size for embryos

Merozoite yield correlated well with the number of sporozoites in the inoculum (Fig. 2). Increasing the inoculum increased the yield of merozoites and also increased embryo mortality (Fig. 3).

Merozoite viability

The viability of the purified merozoites was tested by inoculating embryos and cell cultures. Embryos yielded unsporulated oocysts at 60 h postinoculation, which later sporulated at a rate of 79% (Table 3). Oocyst yield was dependent on the number of merozoites in the inoculum. Gametocytes and oocysts were observed in cell culture monolayers by the fluorescent antibody technique at 48–60 h postinfection. These tests were not quantitative, thus the percent viability could not be estimated.

Table 1. Effect of digestion medium composition on merozoite yield from CAMs[a, b]

Medium components	Concentration (%)	Merozoites		
		Total No.	No./ embryo	Percent increase
Hyaluronidase	0.1	8.00×10^7	5.71×10^6	–
Taurodeoxycholic acid	0.75	8.25×10^7	5.90×10^6	3.1
Trypsin	0.25	1.35×10^8	9.64×10^6	68.8
Trypsin + taurodeoxycholic acid	0.25 0.75	2.07×10^8	1.48×10^7	158.8

[a] 40000 *E. tenella* (Wis) sporozoites inoculated per embryo
[b] 30-min digestion time
[c] 14 embryos per treatment

Table 2. Effect of different concentrations of taurodeoxycholic acid (TDC) with 0.25% trypsin on merozoite yield from CAMs[a, b]

Concentration of TDC (%)	Merozoites		
	Total no.[c]	No./embryo	Percent increase
0.25	1.58×10^8	9.86×10^6	–
0.50	2.25×10^8	1.41×10^7	42.4
0.75	2.27×10^8	1.42×10^7	43.7

[a] 40000 *E. tenella* (Wis) sporozoites inoculated per embryo
[b] 30-min digestion time
[c] 14 embryos per treatment

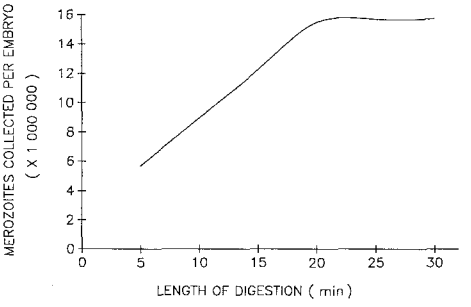

Fig. 1. Effect of different digestion times on the number of merozoites collected

Collection of merozoites from cecal preparations

Pure merozoites were collected from infected ceca about 110 h postinfection by digestion in the 0.25% trypsin

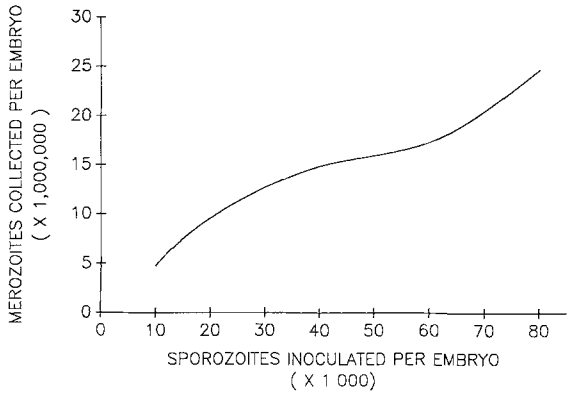

Fig. 2. Effect of inoculum size on the number of merozoites collected

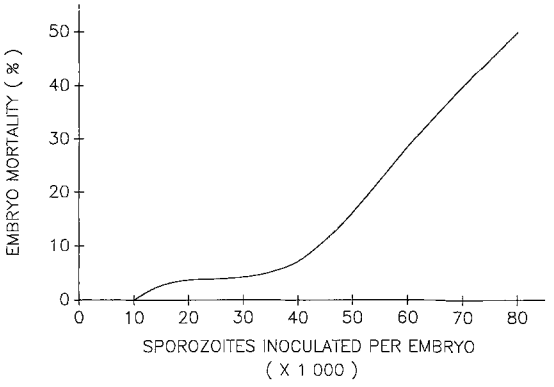

Fig. 3. Effect of inoculum size on embryo mortality

Table 3. Oocyst yields from embryos inoculated with purified merozoites[a]

Inoculum size	Oocysts			
	Total No.[b]	No./embryo	Percent increase	Percent sporulation
1.25×10^6	5.0×10^5	3.33×10^4	–	79
2.50×10^6	$1.0 \varsigma^{106}$	6.67×10^4	100	79
5.00×10^6	4.0×10^6	2.67×10^5	700	79

[a] Oocysts collected 60 h postinfection
[b] 15 embryos per treatment

– 0.50% TDC medium. Pooled ceca from 30 chickens yielded 5.00×10^9 merozoites; the number of merozoites per cecum was 8.33×10^7. This number was six-fold that of the merozoites collected from a chick embryo.

Discussion

There are several published reports on purification of protozoan parasites by DE-52 or glass bead column chromatography or by gradient density centrifugation (Fernando et al. 1984; Hollingdale and Kilejian 1979; Mercado and Katusha 1979; Schmatz and Murray 1981; Schmatz et al. 1984; Wagenbach 1969). Even though pure parasites can be obtained by these methods, the procedures are often complex and recovery rates may be as low as 50%. One successful method for the purification of *E. tenella* merozoites was recommended by Stotish and Wang (1975). It employed hyaluronidase for tissue digestion and 7.5% ficoll and 10% hypaque for removal of the host red blood cells. According to these workers a merozoite yield of 60% or 54% was obtained by density centrifugation or glass bead column chromatography, respectively, with 5% epithelial cell and red blood cell contamination when glass beads were used. We attempted to purify merozoites using DE-52 or glass bead columns, but the yield of merozoites was too low for our needs because many of the parasites were trapped in the columns. Also, some host red blood cells were mixed in the merozoite suspension, and many of the mature schizonts could not be disrupted by digestion with 1 $mg \cdot ml^{-1}$ hyaluronidase.

In our investigations, we developed a rapid and simple method for purifying merozoites. The principal contaminants in merozoite purification from CAMs were red blood cells, but addition of 0.5% TDC destroyed these within 10 min. More than 1.4×10^7 merozoites per embryo and 8.33×10^7 merozoites per cecum could be easily harvested. These yields were 9× greater in the CAM and almost 3× in ceca than such yields reported by previous investigators (Stotish and Wang 1975). However, not all of the foregoing differences are necessarily attributable to technique. Some of the merozoites were normal as assessed by embryo inoculation and cell culture techniques, but we did not determine the percent viability. Oocysts obtained from the CAM sporulated normally and were able to infect susceptible chickens.

The new method has the following advantages: 1) Large numbers of merozoites can be obtained quickly and easily. 2) The technique can be performed without expensive supplies or equipment. 3) Merozoites obtained in this way can be freed of contaminating red blood cells. 4) The viability and infectivity can maintained.

References

Doran DJ (1974) *Eimeria tenella*: merozoites produced in cultured cells and attempts to obtain development of culture produced merozoites. Proc Helm Soc Wash 41:169–173

Elsner YY, Roberts WL, Shigenatsu A, Hammond DM (1974) Isolation of schizonts and merozoites of *Eimeria callospermophili* from cultured bovine intestinal cells. J Parasitol 60:531–532

Fernando MA, Al-Attar MA, Bowles GH (1984) Preparation of second generation merozoites of *Eimeria necatrix* free of host tissue. J Parasitol 70:154–155

Hollingdale MR, Kilejian A (1979) Purification of *Plasmodium lophurae* exoerythrocytic merozoites by an ion exchange column. J Protozool 26:616–619

James S (1980) Isolation of second generation schizonts of avian coccidia and their use in biochemical investigations. Parasitology 80:301–312

Kouwenhoven NB, Kuil H (1976) Demonstration of circulating antibodies to *Eimeria tenella* by the indirect immunofluorescent antibody test using sporozoite and second-stage schizonts as antigen. Vet Parasitol 2:283–292

Laxer MA, Healey MC, Youssef NN (1987) Production of monoclonal antibodies specific for *Eimeria tenella* microgametocytes. J Parasitol 73:611–616

Long PL (1965) Development of *Eimeria tenella* in avian embryos. Nature 208:509–510

Mencher D, Pugatsch T, Wallach M (1989) Antigenic proteins of *Eimeria maxima* gametocytes: cell-free translation and detection with recovered chicken serum. Exp Parasitol 68:40–48

Mercado TI, Katusha K (1979) Isolation of *Trypanosoma cruzi* from the blood of infected mice by column chromatography. Preparative Bioch 9:97–106

Patton WH (1965) *Eimeria tenella*: cultivation of the asexual stages in cultured animal cells. Science 150:767–769

Pugatsch T, Mencher D, Wallach M (1989) *Eimeria maxima*: Isolation of gametocytes and their immunogenicity in mice, rabbits and chickens. Exp Parasitol 68:127–134

Schmatz DM, Murray PK (1981) *Trypanosoma cruzi*: Selective isolation of pure trypomastigotes from cultured muscle cells. J Parasitol 67:517–521

Schmatz DM, Crane MSJ, Murray PK (1984) Purification of *Eimeria* sporozoites by DE-52 anion exchange chromatography. J Protozool 31:181–183

Stotish RL, Wang CC (1975) Preparation and purification of merozoites of *Eimeria tenella*. J Parasitol 61:700–703

Wagenbach GE (1969) Purification of *Eimeria tenella* sporozoites with glass bead columns. J Parasitol 55:833–836

Wagenbach GE, Challey JR, Burns WC (1966) A method for purifying coccidian oocysts employing clorox and sulfuric acid-dichromate solution. J Parasitol 52:1222