

J. Parasit., 72(6), 1986, pp. 880–884


# *IN VITRO* EXCYSTATION OF *SARCOCYSTIS CAPRACANIS, SARCOCYSTIS CRUZI* AND *SARCOCYSTIS TENELLA* (APICOMPLEXA)

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ABSTRACT: Improved rates of *in vitro* excystation of sporozoites from sporocysts of *Sarcocystis capracanis, Sarcocystis cruzi,* and *Sarcocystis tenella* were obtained by pretreating sporocysts with an aqueous sodium hypochlorite (NaOCl) solution followed by incubation in excysting fluid (EF). After pretreatment with NaOCl, sporocysts were washed 4 times in Hanks' balanced salt solution and then incubated in various EF (pH 7.4) at 38.5 C in 5% $CO_2$-95% air. Maximum rates of excystation (free sporozoites/(sporozoites in sporocysts + free sporozoites) × 100) for all 3 species of *Sarcocystis* occurred at 4 hr after incubation in EF. These rates were 17% for *S. capracanis* after incubation in EF containing 2% trypsin + 10% caprine bile; 90% for *S. cruzi* in 2% trypsin + 10% bovine bile; and 20% for *S. tenella* in 2% trypsin + 10% caprine bile. Only a 40% excystation rate occurred in sporocysts of *S. cruzi* that had been stored previously for 14 days in aqueous potassium dichromate. Excysted sporozoites of *S. capracanis, S. cruzi,* and *S. tenella* penetrated and developed to mature meronts in bovine pulmonary artery endothelial cells or bovine monocytes.

Now that certain *Sarcocystis* species infecting livestock can be grown from sporozoites to meronts *in vitro* (Speer and Dubey, 1986), there is considerable need to improve the rates of *in vitro* excystation of sporozoites from sporocysts. There are numerous reports on *in vitro* excystation of *Eimeria* and *Isospora* species, but little information exists on excystation of *Sarcocystis* species (see review by Speer, 1983). We report herein the conditions necessary for obtaining better rates of excystation of sporozoites from sporocysts of *S. capracanis, S. cruzi,* and *S. tenella*—3 important pathogens of livestock.

## MATERIALS AND METHODS

### Sporocysts

Sporocysts of all 3 *Sarcocystis* species were collected from the feces or from mucosal scrapings from experimentally infected carnivores as described previously (Dubey, 1980) and stored in Hanks' balanced salt solution, calcium- and magnesium-free, with antibiotics and fungistats (HBSS-PSFM, pH 7.4) (Leek and Fayer, 1979) at 4 C prior to use. Sporocysts of *S. cruzi* were obtained from an experimentally infected dog and were 14 mo old when used in experiments I, II, and IV. Sporocysts of *S. cruzi* that were used in experiment V were obtained from an experimentally infected coyote and stored in 2.5% (w/v) $K_2Cr_2O_7$ at 4 C for 14 days and then in HBSS-PSFM at 4 C for 2 mo. Sporocysts of *S. capracanis* were obtained from an experimentally infected fox and were 38 mo old when used in experiment III. Sporocysts of *S. tenella* were obtained from an experimentally infected coyote and were 1 mo old when used in experiments I, II, and IV.

### Excysting fluid

Excysting fluid (EF) consisted of 2% (w/v) bovine trypsin (1:250, GIBCO, Grand Island, New York) plus 1 of the following: 5% (w/v) bovine sodium taurocholate (Sigma T-0750, St. Louis, Missouri), 5% ovine sodium taurocholate (Difco, Detroit, Michigan) or 10% (v/v) bovine, caprine or ovine bile in HBSS, pH 7.4. Bovine, caprine or ovine bile was collected aseptically and stored at −20 C prior to use. Preliminary tests with various concentrations of trypsin, bile salt and bile showed the above concentrations gave the highest rates of excystation for each of the 3 species of *Sarcocystis.*

### Experimental design

*Experiment I:* This experiment was performed to determine the effects of various concentrations of sodium hypochlorite and type and concentration of bile on the rates of excystation of sporozoites of *S. cruzi* and *S. tenella* (Tables I, II). Approximately 5 × $10^5$ sporocysts of *S. cruzi* and *S. tenella* were placed in each of 16 15-ml sterile centrifuge tubes, washed once by centrifugation with HBSS, resuspended in 7 ml cold HBSS (Groups 1–4), 1.3% (Groups 5–8), 2.6% (Groups 9–12), or 5.3% (Groups 13–16) sodium hypochlorite (NaOCl, Purex®) (v/v in distilled water), and incubated for 30 min at 4 C. After incubation, 8 ml cold HBSS were added to each tube, and the sporocysts pelleted by centrifugation. Sporocysts were washed 4× in cold HBSS to remove the NaOCl, and then suspended in 2.5 ml of the appropriate excysting fluid (pH 7.4) that had been filter-sterilized by passage through 0.45 μm Nalge filters (Nalge Company, Rochester, New York), and incubated at 38.5 C in 5% $CO_2$–95% air. At 30 min intervals, the suspension in each tube was

Received 19 December 1985; revised 26 March 1986; accepted 26 March 1986.

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TABLE I. *Effects of pretreatment of sporocysts with sodium hypochlorite and various excysting fluids on the rate of excystation of* Sarcocystis cruzi *sporozoites.*

| Group | Percent NaOCl | Excysting fluid* | Rate of excystation† | | | |
|---|---|---|---|---|---|---|
| | | | 1 hr | 2 hr | 4 hr | 6 hr |
| 1 | 0 | 5-BST | 0[z]‡ | 0.3 ± 0.2[z] | 2.4 ± 0.2[z] | 5.9 ± 1.8[t-u] |
| 2 | 0 | 5-OST | 0[z] | 0[z] | 2.2 ± 1.0[y-w] | 5.4 ± 1.6[t-u] |
| 3 | 0 | 10-BB | 0[z] | 0[z] | 12.6 ± 4.3[p-q] | 20.5 ± 6.2[m-n] |
| 4 | 0 | 10-OB | 0[z] | 0[z] | 0.1 ± 0.1[y-z] | 0.3 ± 0.3[y-z] |
| 5 | 1.3 | 5-BST | 0[z] | 1.4 ± 0.5[w-x] | 7.5 ± 2.2[r-t] | 7.0 ± 1.4[s-t] |
| 6 | 1.3 | 5-OST | 0[z] | 3.9 ± 2.0[u-v] | 11.7 ± 2.5[p-q] | 10.2 ± 2.3[q-s] |
| 7 | 1.3 | 10-BB | 2.4 ± 0.8[v-w] | 6.3 ± 2.5[t-u] | 27.1 ± 4.4[k-l] | 26.9 ± 4.6[k-l] |
| 8 | 1.3 | 10-OB | 1.5 ± 0.8[w-x] | 4.0 ± 0.5[u-v] | 10.9 ± 2.7[q-r] | 11.6 ± 1.9[p-q] |
| 9 | 2.6 | 5-BST | 0.4 ± 0.3[y-z] | 17.6 ± 3.2[n-o] | 70.2 ± 7.2[b-c] | 71.8 ± 10.8[b] |
| 10 | 2.6 | 5-OST | 0.3 ± 0.4 [y-z] | 10.7 ± 2.8[q-r] | 68.5 ± 7.7[b-d] | 60.9 ± 10.9[d-e] |
| 11 | 2.6 | 10-BB | 8.4 ± 2.0[q-t] | 50.3 ± 5.7[f-h] | 89.9 ± 1.7[a] | 88.4 ± 6.0[a] |
| 12 | 2.6 | 10-OB | 0.4 ± 0.3[y-z] | 11.9 ± 1.9[p-q] | 58.3 ± 8.9[e-f] | 68.9 ± 6.1[b-d] |
| 13 | 5.3 | 5-BST | 0.8 ± 0.5[x-y] | 15.2 ± 0.7[p-o] | 43.3 ± 3.0[h-j] | 46.7 ± 6.5[h-i] |
| 14 | 5.3 | 5-OST | 0.6 ± 0.4[x-y] | 5.7 ± 1.2[t-u] | 31.2 ± 6.2[k] | 38.7 ± 8.1[j] |
| 15 | 5.3 | 10-BB | 7.6 ± 3.3[r-t] | 37.9 ± 4.4[j] | 62.0 ± 7.7[c-e] | 54.8 ± 5.6[e-g] |
| 16 | 5.3 | 10-OB | 2.8 ± 0.5[v-w] | 24.2 ± 5.6[l-m] | 38.9 ± 6.8[g-i] | 41.3 ± 2.3[i-j] |

* All excysting fluid contained 2% bovine trypsin plus 5 or 10% bile salt or bile in Hanks' balanced salt solution; abbreviations: BB, bovine bile; BST, bovine sodium taurocholate; OB, ovine bile; OST, ovine sodium taurocholate.

† Rate of excystation (percent) = [(free sporozoites)/(sporozoites in sporocysts + free sporozoites)] × 100.

‡ Mean ± standard deviation. Means having the same superscript letter are not significantly different from one another.

vigorously agitated for 2–3 sec. At 1, 2, 4 and 6 hr of incubation, 0.5 ml of sporocyst suspension was removed from each tube and placed in 0.5 ml of cold Trump's fixative (1% (v/v) glutaraldehyde-4% (v/v) formaldehyde in Millonig's phosphate buffer).

Fixed suspensions were examined by bright-field microscopy with a 40× objective lens and 4 separate counts, of 100 parasites (sporocysts and free sporozoites) each, were made to determine the rate of excystation (free sporozoites/(sporozoites within sporocysts + free sporozoites) × 100) of each experimental group.

*Experiment II:* This experiment was performed to determine whether sporozoites of *S. capracanis, S. cruzi,* and *S. tenella* would excyst after treatment with NaOCl without treatment with EF. Approximately $5 \times 10^5$ sporocysts of each species were placed in each of 4 15-ml sterile centrifuge tubes, washed once with HBSS, centrifuged, resuspended in 7 ml cold HBSS (Group 1), 1.3% (Group 2), 2.6% (Group 3), or 5.3% (Group 4) NaOCl in distilled $H_2O$, and incubated for 30 min at 4 C. Each group of sporocysts was washed 4× with HBSS, suspended in 2.5 ml HBSS and incubated at 38.5 C in 5% $CO_2$–95% air. At 30 min intervals for 4 hr, each suspension was shaken for 2–3 sec. After 4 hr incubation, 0.5 ml of sporocyst suspension was removed from each tube, placed in 0.5 ml cold Trump's fixative, examined by bright-field microscopy, and the rate of excystation determined as in experiment I.

*Experiment III:* Based upon results obtained in experiment I, experiment III was conducted to determine the effects of similar treatments on excystation of *S. capracanis.* Sporocysts of *S. capracanis* were pretreated with 2.6% NaOCl and then incubated in EF consisting of 2% bovine trypsin plus 5% bovine or ovine sodium taurocholate or 10% bovine, caprine or ovine bile.

*Experiment IV:* To determine the effects of storing sporocysts in $K_2Cr_2O_7$ on excystation of sporozoites, $5 \times 10^5$ sporocysts of *S. cruzi,* which had been stored for 2 wk in 2.5% $K_2Cr_2O_7$ at 4 C and then in HBSS-PSFM at 4 C for 2 mo, were subjected to the optimal excysting conditions (i.e., pretreatment in 2.6% NaOCl; 2% bovine trypsin + 10% bovine bile) for *S. cruzi* as determined in experiment I. An additional $5 \times 10^5$ sporocysts were incubated in HBSS without EF. After 4 hr of incubation at 38.5 C, parasite preparations were fixed and counted to determine the rate of excystation as described above.

*Experiment V:* To determine whether caprine bile effected excystation of sporozoites of *S. cruzi* and *S. tenella,* $5 \times 10^5$ sporocysts of each species were prepared as above, pretreated with 2.6% NaOCl, incubated in 10% caprine bile for 4 hr, fixed, and counted as above.

*Experiment VI:* To determine whether the optimum excystation rates could be replicated, sporocysts of each of the 3 *Sarcocystis* spp. were incubated as above under optimum excystation conditions (2% trypsin + 10% caprine bile for *S. capracanis* and *S. tenella*; 2% trypsin + 10% bovine bile for *S. cruzi*). Sporocysts of each species were stored in HBSS-PSFM at 4 C before use. This experiment was conducted over a 4 mo period during which *S. capracanis* was excysted 3 times with 1 to 2.5 mo old sporocysts; *S. cruzi* 3 times with 3 to 5 mo old sporocysts; and *S. tenella* 4 times with 3 to 4 mo old sporocysts. Experiment VI was also used to compare the excystation rates of 1 to 2.5 mo old sporocysts with that of 38 mo old sporocysts of *S. capracanis.*

### Statistical analysis

Data on rates of excystation were arcsine of the square root-transformed to ensure homogeneity of variances (Zar, 1974). Means for each experimental group were tested for significant differences, using either one-way analysis of variance and Duncan's multiple range test ($\alpha = 0.05$) for multiple sample comparisons, or Student's *t*-test ($\alpha = 0.05$) for 2 sample comparisons.

TABLE II. *Effects of pretreatment of sporocysts with sodium hypochlorite and various excysting fluids on the rate of excystation of* Sarcocystis tenella *sporozoites.*

| Group | Percent NaOCl | Excysting fluid* | Rate of excystation† | | | |
|---|---|---|---|---|---|---|
| | | | 1 hr | 2 hr | 4 hr | 6 hr |
| 1 | 0 | 5-BST | $0^{p}$‡ | $0^{p}$ | $0^{p}$ | $0^{p}$ |
| 2 | 0 | 5-OST | $0^{p}$ | $0^{p}$ | $0^{p}$ | $0^{p}$ |
| 3 | 0 | 10-BB | $0^{p}$ | $0^{p}$ | $0.1 \pm 0.1^{n-p}$ | $0.1 \pm 0.1^{n-p}$ |
| 4 | 0 | 10-OB | $0^{p}$ | $0^{p}$ | $0^{p}$ | $0.2 \pm 0.2^{m-p}$ |
| 5 | 1.3 | 5-BST | $0^{p}$ | $0^{p}$ | $0.3 \pm 0.3^{k-p}$ | $0.1 \pm 0.1^{o,p}$ |
| 6 | 1.3 | 5-OST | $0^{p}$ | $0^{p}$ | $0.1 \pm 0.1^{n-p}$ | $0.1 \pm 0.1^{o,p}$ |
| 7 | 1.3 | 10-BB | $0.1 \pm 0.1^{n-p}$ | $0.1 \pm 0.1^{o,p}$ | $0.3 \pm 0.4^{m-p}$ | $0.3 \pm 0.1^{k-o}$ |
| 8 | 1.3 | 10-OB | $0^{p}$ | $0.3 \pm 0.3^{k-o}$ | $0.3 \pm 0.4^{l-p}$ | $0.3 \pm 0.2^{k-p}$ |
| 9 | 2.6 | 5-BST | $0^{p}$ | $0.1 \pm 0.1^{n-p}$ | $2.4 \pm 0.4^{d-g}$ | $2.1 \pm 0.3^{d-g}$ |
| 10 | 2.6 | 5-OST | $0.2 \pm 0.2^{m-p}$ | $0.6 \pm 0.6^{j-n}$ | $2.2 \pm 1.2^{e-g}$ | $2.1 \pm 0.9^{e-g}$ |
| 11 | 2.6 | 10-BB | $1.3 \pm 0.5^{g-i}$ | $3.3 \pm 1.3^{c-e}$ | $7.2 \pm 1.6^{a}$ | $6.9 \pm 1.7^{a}$ |
| 12 | 2.6 | 10-OB | $0.8 \pm 0.5^{h-k}$ | $3.0 \pm 2.0^{d-f}$ | $6.7 \pm 0.7^{a}$ | $5.1 \pm 0.7^{a,b}$ |
| 13 | 5.3 | 5-BST | $0.1 \pm 0.1^{n-p}$ | $0.5 \pm 0.6^{k-o}$ | $1.1 \pm 0.7^{h-j}$ | $0.9 \pm 0.8^{i-l}$ |
| 14 | 5.3 | 5-OST | $0.1 \pm 0.3^{o,p}$ | $0.6 \pm 0.2^{i-m}$ | $0.5 \pm 0.6^{j-o}$ | $1.8 \pm 0.7^{f-h}$ |
| 15 | 5.3 | 10-BB | $1.1 \pm 0.6^{h-j}$ | $2.6 \pm 0.5^{d-f}$ | $3.5 \pm 0.9^{b-e}$ | $3.7 \pm 1.5^{b-d}$ |
| 16 | 5.3 | 10-OB | $0.6 \pm 0.5^{j-n}$ | $3.2 \pm 0.5^{c-e}$ | $4.7 \pm 1.2^{b-c}$ | $4.4 \pm 1.0^{b,c}$ |

*, †, ‡ Refer to Table I for details.

### Sporozoite viability test

Sporozoites of *S. capracanis, S. cruzi,* and *S. tenella,* excysted by pretreatment of sporocysts with 2.6% NaOCl and incubated in EF that induced the maximum rate of excystation, were each inoculated into cultured bovine endothelial cells or bovine monocytes as described (Speer and Dubey, 1986). Each day for 30 days after sporozoite inoculation, cultures were examined with phase-contrast microscopy, and the extent of parasite development was recorded.

## RESULTS

### *Sarcocystis cruzi*

The greatest rate of excystation (90%) of sporozoites of *S. cruzi* occurred when sporocysts were treated with 2.6% NaOCl and then incubated for 4 or 6 hr in EF containing bovine bile (Table I). There were no significant differences in rates of excystation between those parasites incubated for 4 or 6 hr. When compared to incubation in EF containing bovine bile, significantly less excystation occurred in those groups of sporocysts that were incubated in EF containing either ovine bile or bovine sodium taurocholate (61–72%: Table I, Groups 9, 10 and 12).

TABLE III. *Effects of type of bile on the rate of excystation of* Sarcocystis capracanis *sporozoites.*

| Excysting fluid* | Rate of excystation at 4 hr† |
|---|---|
| 5-BST | $5.8 \pm 3.2^{c}$‡ |
| 5-OST | $7.1 \pm 1.6^{c}$ |
| 10-BB | $16.5 \pm 3.4^{d}$ |
| 10-OB | $13.2 \pm 2.2^{d}$ |
| 10-OB | $17.3 \pm 2.1^{d}$ |

*, †, ‡ Refer to Table I for details.

Significantly greater excystation occurred in sporocysts that were pretreated with 1.3, 2.6 or 5.3% NaOCl than nonpretreated ones (Table I). The excystation rate of those pretreated with 2.6% NaOCl was significantly greater than those pretreated with 1.3 or 5.3% NaOCl. Significantly greater excystation occurred in those pretreated with 5.3% NaOCl than in those pretreated with 1.3% NaOCl.

Upon exposure to EF containing bovine bile, significantly fewer sporozoites excysted (40%) from sporocysts that had been stored in 2.5% $K_2Cr_2O_7$ than excysted (90%) from those stored in HBSS-PSFM. No excystation occurred in sporocysts that were stored in $K_2Cr_2O_7$ or HBSS-PSFM or pretreated with NaOCl and incubated without EF. Less than 1% excystation occurred in EF containing caprine bile.

Replicate experiments that involved incubation of 3 to 5 mo old sporocysts under optimum excysting conditions (2% trypsin + 10% bovine bile) resulted in a mean excystation rate of 88.8% (87.2% to 91.3%).

### *Sarcocystis tenella*

The factors influencing excystation of *S. tenella* sporozoites were similar to those for *S. cruzi* (Table II). For instance, significantly more excystation occurred in sporocysts pretreated with 2.6% and 5.3% NaOCl than in those pretreated with 1.3% NaOCl or HBSS. Also, significantly more excystation occurred in EF containing bovine or ovine bile than bovine or ovine sodium taurocholate at 1, 2, 4, and 6 hr incubation. Spo-

rozoites of *S. tenella,* however, underwent significantly more excystation in EF containing caprine bile (20%) than in EF containing ovine or bovine bile (5–7%).

A mean excystation rate of 20.2% (19.3% to 21.1%) was obtained in replicate experiments involving 3 to 4 mo old sporocysts incubated in optimum excystation conditions (2% trypsin + 10% caprine bile).

#### *Sarcocystis capracanis*

Sporozoites of *S. capracanis* excysted (13–17%) equally well in EF containing bovine, caprine or ovine bile, whereas significantly less excystation occurred in EF containing bovine or ovine sodium taurocholate (Table III).

Replicate experiments with 1 to 2.5 mo old sporocysts incubated under optimum excysting conditions (2% trypsin + 10% caprine bile) resulted in a mean excystation rate of 18.1% (17.3% to 19%).

#### Viability of sporozoites

Sporozoites of *S. capracanis, S. cruzi,* and *S. tenella* that excysted from sporocysts pretreated with 2.6% NaOCl and then EF penetrated and developed to mature meronts in bovine pulmonary artery endothelial cells or bovine monocytes.

## DISCUSSION

Sporozoites of *Sarcocystis capracanis, S. cruzi,* and *S. tenella* excysted in EF (trypsin + bile or bile salt) only, without pretreatment with NaOCl. However, pretreatment of sporocysts with NaOCl markedly increased the rates of excystation for all 3 species. Significantly greater excystation resulted when sporocysts were pretreated with 2.6% NaOCl than with 1.3% or 5.3% NaOCl. Sodium hypochlorite has been found to remove the outer layer of the oocyst wall of *Eimeria, Isospora* and *Cryptosporidium* spp. (Knapp and Nyberg, 1970; Roberts et al., 1970; Speer et al., 1973; Reduker et al., 1985), but the ultrastructural changes in sporocysts of *Sarcocystis* spp. caused by NaOCl treatment have not yet been determined. In oocysts pretreated with NaOCl, sporozoites of *Cryptosporidium parvum* will spontaneously excyst at 38 C in the absence of excysting fluid (Reduker and Speer, 1985). In contrast, even after pretreatment of sporocysts with NaOCl, sporozoites of *S. capracanis, S. cruzi,* and *S. tenella* still required EF to excyst.

Excysting fluid comprised of bile was more effective than bile salts in inducing excystation of sporozoites of all 3 species of *Sarcocystis.* Sporozoites of *S. capracanis* excysted equally well in all 3 bile types. More sporozoites of *S. cruzi* excysted in bovine and ovine bile than in caprine bile. Surprisingly, *S. tenella* had a significantly greater excystation rate in caprine than in ovine or bovine bile.

We found, as did Leek and Fayer (1979), that storage of sporocysts of *S. cruzi* in $K_2Cr_2O_7$ has a deleterious effect on excystation of *S. cruzi.* The rate of excystation of sporozoites from sporocysts of *S. cruzi* that had been stored for 2 wk in $K_2Cr_2O_7$ was only 40% compared to 90% for those stored in HBSS-PSFM.

Based on the findings in the present study, optimum conditions for excystation of *S. cruzi* sporozoites from sporocysts that have been stored in HBSS-PSFM are pretreatment in 2.6% NaOCl at 4 C for 30 min, 4 washes in HBSS, incubation in EF consisting of 2% bovine trypsin plus 10% bovine bile in 5% $CO_2$, 95% air at 38.5 C for 4 hr. Fayer and Leek (1973) obtained an excystation rate (based on empty sporocysts) comparable to that of the present study (90%) by pretreating sporocysts of *S. cruzi* with cysteine hydrochloride for 18 hr, followed by exposure to trypsin + bovine bile for 1.5 hr. Their sporocysts were obtained from feces; however, in the present study sporocysts obtained from the gut wall had a similar high rate of excystation. Cysteine hydrochloride and NaOCl evidently have similar beneficial effects on excystation of *S. cruzi* sporozoites. However, NaOCl acts more rapidly than cysteine hydrochloride, requiring only a 30 min pretreatment. Further study is required to determine the optimum conditions for excystation of sporozoites of *S. capracanis* and *S. tenella.* It is noteworthy that no difference was found in rate of sporozoite excystation between 1 to 2.5 mo old and 38 mo old sporocysts of *S. capracanis.*

## ACKNOWLEDGMENTS

This study was supported in part by funds from the Montana State University Agricultural Experiment Station (MSUAES) and by an International Collaborative Research Grant from the Natural Sciences and Engineering Research Council of Canada to R.J.C. This study was published as MSUAES Journal Series No. 1778.

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