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# In Vitro Excystation and Cryoreservation of Ovine and Caprine *Sarcocystis* species

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## ABSTRACT

This paper describes a standard method for in vitro excystation of sporocysts of *S.capracanis*, *S.hircicanis*, *S.ovicanis* and *S.arieticanis*. A standard pretreatment was performed by 20 minutes preincubation of sporocysts in aqueous sodium hypochlorite (NaOCl) solution of 6% or 8% at room temperature. Then, sporocysts were washed 5 times in distilled water and were incubated for one hour at 39$^{O}$C in excystation fluid consisting of RPMI 1640 medium, 10% PCS and 15% bovine bile. Additional sonication of pretreated sporocysts increased excystation rates as compared to not sonicated controls. Excystation rates for 1-9 months old sporocysts were 77% for *S.capracanis*, 77% for *S.hircicanis*, 72% for *S.arieticanis* and 92% for *S.ovicanis*. Sporozoite suspension as obtained by this method proved to be decontaminated and sporozoites were viable and able to invade Vero cells in tissue culture. Sporozoites were also subjected to cryopreservation in RPMI 1640 containing 10% FCS and 7.5% DMSO. After storage in liquid nitrogen, sporozoites proved to be infective for their intermediate hosts after intraperitoneal injection.

## INTRODUCTION

In the heteroxenous life cycle of sarcosporidia, sporozoites are the only infective stages for their intermediate hosts in which they initiate a massive asexual multiplication by schizogony and following cyst formation. Since schizogony may cause disease and even death of animals, more recently the interest in these stages increased markedly. To produce schizonts and merozoites in sufficient numbers for serological, immunological or molecular biological investigations, in vitro excystation of sporozoites is strongly required. Up to date methods for in vitro excystation yielding sufficient excystation rates have been described for *S.bovicanis* (FAYER & LEEK, 1973; CAWTHORN et al., 1986), *S. suicanis* (BERGLER, 1979; STROHLEIN & PRESTWOOD, 1986) and just recently for *S.ovifelis* (MCKENNA & CHARLESTON, 1990) and for *S.capracanis*, *S.ovicanis* and *S.arieticanis* (SANFT, 1990). But only for *S.bovicanis* and *S.suicanis* - and with rather poor excystation rates also for *S.capracanis* and *S.suicanis* - methods have been described to decontaminate sporocysts from bacteria and fungi before excystation of sporozoites (CAWTHORN et al., 1986; STROHLEIN & PRESTWOOD, 1986).

Therefore, the aim of the present study was to establish a simple method for in vitro excystation of sporozoites of different caprine and ovine *Sarcocystis* species that allows the isolation of large numbers of sporozoites free of bacterial and fungal contamination. These sporozoites should be infective for tissue culture and for their specific intermediate hosts directly following in vitro excystation as well as after cryopreservation and storage in liquid nitrogen.

## MATERIALS AND METHODS

*In vitro excystation*.

Sporocysts: Sporocysts of *S.capracanis*, *S.hircicanis*, *S.ovicanis* and *S.arieticanis* were obtained as described previously (HEYDORN et al., 1981) by artificial digestion of intestinal mucosa of dogs who had been fed tissue cysts of the appropriate *Sarcocystis* species. Until use, sporocysts were stored at $4^{O}$C in distilled water containig 20.000 IU/1OOml penicillin, 20.000 µg/1OOml streptomycin and 100 µg/1OOml amphotericin B (mod. after LEEK & FAYER, 1979). They were used within 1-9 months or up to 35 months (longtime study).

*Pretreatment*: Sodium hypochlorite (NaOCl) and sonication.

Sporocysts were washed twice by centrifugation in distilled water at 1.600 x g for ten minutes. Pretreatment was performed at room temperature by resuspending the pellet in 4 ml of NaOCl in the desired concentration. After proper mixing, the suspension was allowed to stand for the desired interval, according to the experimental design. Sporocysts were then washed five times in distilled water. For sonication of sporocysts, we used a Sonifier cell disrupter (W 250, Branson, Conn., USA) with a double step micro tip following the method of SANFT (1990): washed sporocysts were resuspended in 1.5 ml RPMI 1640 cell culture medium (Flow Lab., Irvine, Scotland) and sonicated for 2 minutes on 30% duty cycle and output control 3 in an ice water bath.

*Incubation in excystation fluid*.

Following pretreatment procedures or as untreated controls, sporocysts were resuspended in 1.5 ml of excystation fluid consisting of RPMI 1640, 10% fetal calf serum( FCS) and 15% pooled bovine bile. Incubation was at $39^{O}$C in a water bath for 30 minutes, 1, 2, and 3 hours, respectively. After the desired incubation time, the suspension was checked microscopically for free motile sporozoites and intact sporocysts using a hemocytometer. The excystation rate ( % of excysted sporozoites) was determined as follows: free sporozoites x 100/(sporozoites in sporocysts + free sporozoites). For every individual experiment and after different incubation periods, 3-4 seperate counts of 200 parasites each were made.

*Sporozoite viability test*.
Sporozoites of *S.capracanis* were excysted with 6% and 8% NaOCl /sonication pretreatment, incubated for one hour, then washed twice in RPMI 1640 and were resuspended in cell culture medium (RPMI 1640 containing 10% FCS, 1% glutamin, 1% penicillin/streptomycin). Approximately $10^4$ sporozoites were inoculated into a tissue culture flask (Nunc, Denmark) containing a Vero cell monolayer that had been established 24 hours before.
Cultures were kept at $39^OC$ in 95% air /5% $CO_2$. Supernatant medium containing sporocyst walls and debris was removed 24 hours after parasite inoculation and replaced periodically by fresh medium with constant FCS content.

*Experimental design*:
For every individual experiment, 5-6 repetitions were made using 1-5x$1O^5$ sporocysts each. To show the effects of the excystation fluid and the different pretreatment methods, applied solitary or in combination, we only used sporocysts of *S.capracanis* (5-9 months). Before incubation, controls were either untreated (group 1) or sonicated only (group 2). A ten minute NaOCl pretreatment was used in group 3 (0.8%, 1.2%, 1.6%, 2%, and 2.4% NaOCl, respectively) and group 4 (4%, 6%, 8%, and 10% NaOCl, respectively), a twenty minute pretreatment was used in group 5 (4%, 6%, 8% and 10% NaOCl, respectively). In groups 3S, 4S and 5S, sporocysts were additionally sonicated after the respective NaOCl pretreatment. In group 6, sporocysts underwent a 24 hours NaOCl pretreatment (0.6% and 1.2%, respectively) at $4^OC$.
To elucidate the specific excystation patterns of other *Sarcocystis* species, we also compared sporocysts of *S.hircicanis*, *S.ovicanis* and *S.arieticanis* for excystation rates: sporocysts (1-9 months) underwent a standard one hour incubation following a 20 minutes standard 8% NaOCl pretreatment or sonication, or both or without pretreatment.
A longtime study was performed to reveal changes of excystation patterns according to the age of sporocysts stored in distilled water: up to an age of 35 months, sporocysts of *S.capracanis*, *S. ovicanis* and *S.arieticanis* were excysted at intervals by standard treatment (20 minutes 6% NaOCl /sonication, one hour incubation) and checked for excystation rates.

*Cryopreservation of in vitro excysted sporozoites*:
Sporozoites of *S.capracanis*, *S.ovicanis* and *S.arieticanis* were excysted by standard treatment, washed twice in RPMI 1640 and prepared for cryopreservation as described by SANFT (1990): parasites were resuspended in cryo-medium (RPMI 1640 with 10% FCS and 7.5% DMSO) in portions of 5xl$0^4$ - 5xl$0^5$ live sporozoites /ml and were frozen in a Cryoson automatic deep freezer (Cryoson, Schöllkrippen, Germany). Tubes were stored in liquid nitrogen until use (6-28 days). Prior to animal inoculation, parasites were washed in RPMI 1640. A goat and two sheep were inoculated intraperitoneally with 4x$10^4$ motile sporozoites of the respective *Sarcocystis* species each. General conditions and body temperatures of the animals were monitored daily. At day 112, 121 and 92, respectively, they were killed and the intensity of infection was determined by the number of tissue cysts in the carcasses.

**RESULTS**

*Effects of pretreatments*.

Highest excystation rates, in the following given as mean values, of *S.capracanis* sporozoites occurred after a combined NaOCl /sonication pretreatment of sporocysts (Fig.l). As compared to untreated (group 1: 1%) or only sonicated (group 2: 15%) controls, a ten minutes pretreatment with NaOCl concentrations up to 2.4% followed by sonication raised excystation rates up to 55% (group 3S). As these results were too low compared with results obtained with higher NaOCl concentrations, NaOCl concentrations below 4% were not taken into further consideration.

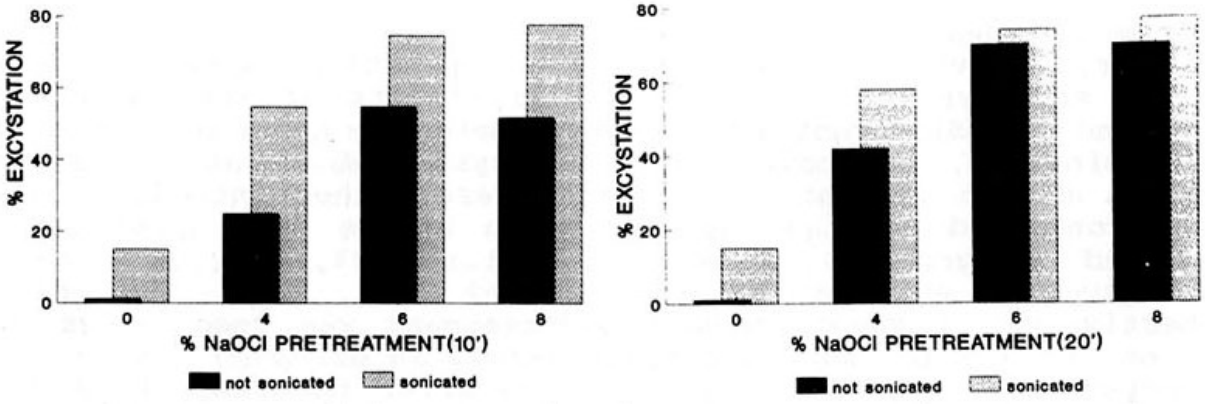



Fig.1.Effect of additional sonication on excystation of NaOCl pretreated *S.capracanis* sporocysts.

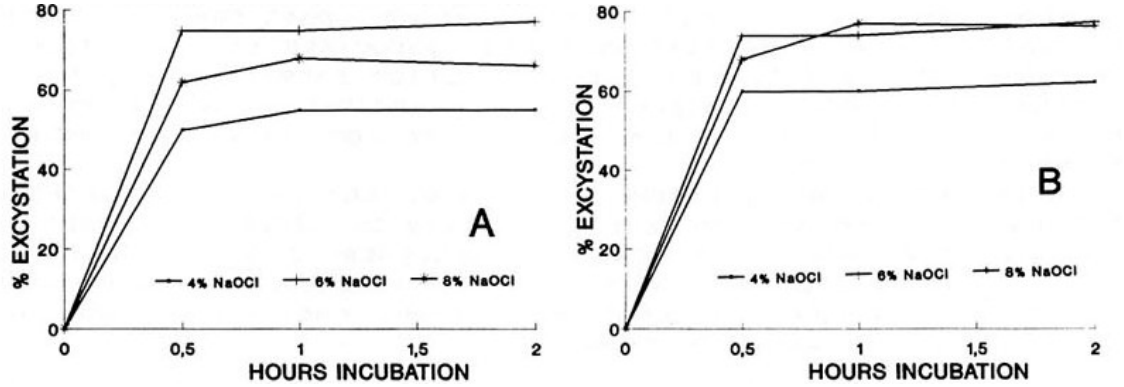



Fig.2.Dynamics of excystation of *S.capracanis* sporozoites:
2A after 10 min NaOCl pretreatment + sonication of sporocysts
2B after 20 min NaOCl pretreatment + sonication of sporocysts

A ten minute pretreatment with 4%, 6%, 8%, and 10% NaOCl (group 4S) followed by sonication led to excystation percentages of 55%, 75%, 68% and 78%, respectively. A twenty minutes pretreatment with 4%, 6%, 8%, and 10% NaOCl (group 5S) and additional sonication led to 60%, 74%, 77% and 81% excystation, respectively. After the use of 10% NaOCl, the excysted sporozoites sometimes showed signs of damage after one hour incubation in excystation fluid.

Therefore, this concentration was not chosen for standard pretreatment although excystation rates exceeded those achieved by 8% NaOCl. If sporocysts were not sonicated after the respective NaOCl pretreatment, the percentage of excysted sporozoites was markedly lower (groups 3, 4, 5; Fig.1).

After a 24 hours pretreatment with 0.8% and 1.2% NaOCl (group 6), excystation rates were 18% and 12%, respectively, and excysted sporozoites showed signs of damage and reduced motility.

Fig.l shows the effect of NaOCl and sonication applied solitary and in combination: in untreated control sporocysts, excystation was raised by 14% after additional sonication. After a 10 minutes NaOCl pretreatment, additional sonication led to 20-30% more excystation whereas after a 20 minutes pretreatment the effect of sonication was markedly lower and the increase of results was only 4-16% as compared to not sonicated sporocysts.

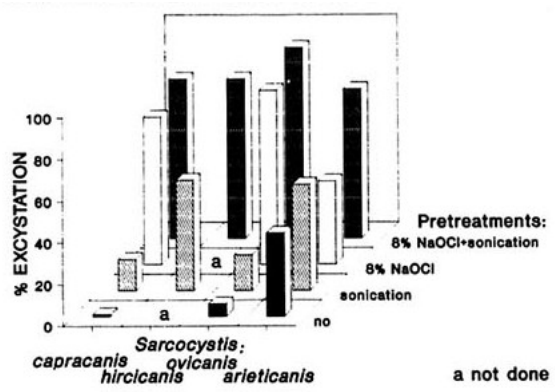
As shown in Fig 2., excystation of sporzoites occurs slightly faster after a 20 minutes NaOCl combined pretreatment but maximum exystation rates do not exceed much those achieved by a 10 minutes pretreatment. Of the maximum achieved results in the respective experiment, 93-100% were reached after a one hour incubation.

After longer incubation, sporozoites began to show signs of damage and lost motility after 2 hours; therefore, one hour was chosen to be standard incubation time for the following experiments.

*In vitro excystation of S.hircicanis, S.ovicanis, S.arieticanis*.

The standard combined sporocyst pretreatment (20' 6-8 % NaOCl /sonication, 1 hour incubation) yielded good excystation rates also for sporocysts of *S.hircicanis* (77%), *S.ovicanis* (92%) and *S.arieticanis* (72%) as shown in Fig.3.

Fig.3. Effect of various pretreatments on the excystation of Sarcocystis sporozoites




*Longtime study.*

The influence of sporocyst age on excystation pattern is shown in Tab.l: after standard pretreatment, sporocysts of *S.capracanis* reach highest excystation rates (91%) at the age of 15-20 months after recovery, whereas younger (1-4, 5-9 months) and older (33-35 months) sporocysts reach markedly lower results (68%, 74% and 58%, means, respectively). A similar pattern was found for fresh and older sporocysts of *S.ovicanis* (48% and 84%, resp.). In contrast, sporocysts of *S.arieticanis* showed highest excystation rates after standard pretreatment when they were 1-4 and 33-35 months of age (72% and 63%). After 9-12 months storage, sporocysts were best excysted without pretreatment (72%) or after sonication alone (68%) whereas NaOCl pretreatment reduced results to 40%.

*Sporozoite viability and infectivity*.
Vero cell cultures inoculated with in vitro excysted sporozoites of *S.capracanis* were kept more than 21 days without contamination; sporozoites were motile and were able to invade cells.
Intraperitoneal inoculation of cryoprserved sporozoites led to building of numerous (*S.capracanis*, *S.ovicanis*) or few (*S.arieticanis*) tissue cysts in the infected animal. None of the animals showed rise in body temperature or other signs of illness in the acute phase of infection.

| sporocyst age in months | Percent excystation[a] | | |
|---|---|---|---|
| | *S.capracanis* | *S.ovicanis* | *S.arieticanis* |
| 1-4 | 68(63-72) | 48(40-55) | 66(42-82) |
| 5-9 | 74(67-81) | - | - |
| 10-12 | -[b] | 84(82-87) | 42(40-45) |
| 15-20 | 91(87-95) | - | - |
| 33-35 | 58(57-59) | - | 63(55-72) |

[a]mean(min-max)
[b]not done

Tab.1. In vitro excystation of *Sarcocystis* sporozoites after prolonged sporocyst storage

## DISCUSSION

As one of the most surprising results of this study, it has been shown that NaOCl may be used in concentrations as high as 6-8% as a pretreatment agent for in vitro excystation and for decontaminating sporocyst suspensions without harming the sporozoites. So far, the highest used NaOCl concentration was 5.3% (CAWTHORN et al., 1986) for in vitro excystation of *S.bovicanis*, *S.capracanis* and *S.ovicanis*. Most authors used NaOCl at $4^{O}$C. We found that at concentrations below 5%, cooling of the NaOCl reduces excystation rates as compared to room temperated disinfectant, whereas at concentrations above 5%, there was no such difference.

NaOCl is reported to remove the outer layer of the oocyst wall of some *Coccidia* spp. (NYBERG & KNAPP; 1970; SPEER et al., 1973; JACKSON, 1964), whereas the lipid-containig inner layer seems not to be affected (RYLEY, 1973). SPEER et al. (1973) have shown that *Isospora canis* sporozoites excyst faster after NaOCl pretreatment of the oocysts indicating that trypsin or proteolytic enzymes and bile salts of the excystation fluid more rapidly evoke alterations of the inner sporocyst layer. We can confirm these findings for the tested Sarcocystis species: after NaOCl pretreatment at high concentrations, 93-100% of the maximum excystation rates were seen after 1 hour incubation (s. Fig.2) whereas low NaOCl concentrations required incubation periods up to 4-6 hours (CAWTHORN et al., 1986; SPEER et al., 1986; MCKENNA & CHARLESTON, 1990). However, NaOCl sporocyst pretreatments of long duration (24 hours) or at concentrations above 8% seem to have an adverse effect on sporozoite viability.

As shown in the longtime study, sporocysts of *S.arieticanis* require NaOCl pretreatment when freshly obtained, whereas after 9-12 months of storage in distilled water, NaOCl seems to have a deleterious effect resulting in reduced excystation. Similar findings were made by SANFT (1990) for *S.ovicanis* after formic acid pretreatment.
In contrast, sporocysts of the other three tested *Sarcocystis* species reach excystation maxima after 6-12 months. These findings indicate that distilled water seems to reduce the osmotic stability of the sporocyst walls and therefore in some way intensifies the NaOCl effect. However, sporocysts of *S.arieticanis* seem to have a slightly different biochemical structure which appears to be less resistant to osmotic or mechanical (sonication) influence.
Interestingly, of all the tested *Sarcocystis* species, *S.arieticanis* responded least to NaOCl pretreatment but responded as good as *S.capracanis* and *S.ovicanis* sporocysts to formic acid pretreatment (SANFT, 1990). Following the considerations of MCKENNA & CHARLESTON (1990), this may suggest that the lipid content of *S.arieticanis* sporocyst walls is higher than that of other species for lipid does not dissolve in NaOCl (RYLEY, 1973).

Sonication as a pretreatment procedure has been employed for *Hammondia hammondi* oocysts (SHEFFIELD et al., 1976) and *Sarcocystis* spp. sporocysts (BERGLER, 1979; SANFT, 1990). It has a synergistic effect following NaOCl pretreatment at low concentrations or formic acid (SANFT, 1990) but after NaOCl at high concentrations or prolonged duration (20 minutes), the effect of additional sonication is smaller than expected.
After sonication of sporocysts, excystation of sporozoites seems to occur faster, reducing incubation time to 30-60 minutes. A short incubation time is absolutely desirable because free sporozoites come in direct contact with the chemical agents of the excystation fluid and prolonged incubation leads to damage and lysis of sporozoites, as shown previously (FARR & DORAN, 1962).
In our experiments, we used excystation fluid comprised of RPMI 1640, bovine bile and FCS but without trypsin (SANFT, 1990).
Unlike for *Eimeria* species of birds and ruminants (DORAN & FARR, 1962 JACKSON, 1962; HIBBERT et al., 1969), trypsin is not essential for in vitro excystation of *S.bovicanis* (FAYER & LEEK, 1973) and Sarcocystis species of small ruminants (MCKENNA & CHALESTON, 1990; SANFT, 1990) but has been widely used (BERGLER, 1979; SPEER & DUBEY, 1986; SPEER et al., 1986).
Incubation without trypsin is a considerable step towards gentle sporozoite excystation: WISHER & ROSE (1987) showed that incubation of *Eimeria tenella* sporozoites with bile salts alone led to slight loss of polypeptids from the surface whereas a combined bile /trypsin treatment caused rapid changes in membrane structure, a loss of antigenetic determinants and lysis of cells.
DORAN & FARR (1962) report clumping of *Eimeria acervulina* sporozoites after using bile concentrations exceeding 5%. We never found clumping of sporozoites sooner than after 3 hours incubation.

After NaOCl pretreatment in vitro excystation, *Sarcocystis* sporozoites were capable of invading cultured cells and the sporozoite suspension proved to be decontaminated from bacteria and fungi, which was not the case after formic acid pretreatment (HORN, unpublished).

In our experiments, deep frozen sporozoites proved to be viable and infective after 28 days of storage. Further investigations have to be made determining whether cryopreservation serves as a device for long period storage of infective material. SANFT (1990) found to be no difference in infectivity between freshly excysted and cryopreserved *Sarcocystis* sporozoites after 21 months of storage.

Intermediate hosts showed fewer tissue cysts following intraperitoneal administration of sporozoites than after oral infection with sporocysts (SANFT, 1990) and in our experiments, signs of illness could not be detected. We can only speculate on the reasons: the infective dosage might have been smaller than calculated because a certain percentage of sporozoites may have been viable but not infective? moreover, the actual development of intraperitoneally administered sporozoites may somehow differ from that of orally administered sporocysts and has not yet been investigated.

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