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Research paper

Statistical comparison of excystation methods in *Cryptosporidium parvum* oocysts

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ABSTRACT

Excystation of sporozoites of *Cryptosporidium parvum* from oocysts is essential for successful *in vitro* assays. It has also been traditionally used as a measure for oocyst viability and infectivity. Laboratories use various excystation protocols so there is a need to clarify which method is the best. In this study, six different protocols for *in vitro* excystation of *C. parvum* oocysts were compared to find the most efficient excystation method (expressed as percentage excystation). Tested protocols differed in chemical pre-incubation steps, excystation media or time of incubation.

There were significant differences in percentage of excysted oocysts among groups excysted by different methods. There were also significant differences in percentage of excysted oocysts between methods using pre-incubation with sodium hypochlorite and those without. The other variables examined; the presence of trypsin, kind of excystation medium and the incubation time, did not show statistical differences in percentage excystation among groups.

Pre-incubation steps which included sodium hypochlorite, enhancing the permeability of the oocysts were found to increase the excystation ratio and methods using this step were the most effective.

1. Introduction

Cryptosporidium parvum is a protozoan parasite that infects epithelial cells in the microvillus border of the gastrointestinal tract of many mammalian species, including humans. In immunocompetent individuals, cryptosporidiosis can cause a short-term gastrointestinal diarrheal disease. In some populations, like immunocompromised patients, children and the elderly, it can result in significant morbidity and mortality (Thompson et al., 2005; Fayer, 2008). Although a variety of treatments have been tested, no reliably effective and worldwide accessible therapy for cryptosporidiosis is available (Jenkins, 2004; Smith and Corcoran, 2004). The use of an *in vitro* system that enables complete development of *C. parvum*, could support efforts to identify efficacious anticryptosporidial agents prior to testing in animals. *In vitro* excystation protocols of *C. parvum* oocysts imitate host-derived signals: exposure to acid followed by incubation in bile salts, reducing agents and proteases in 37 °C mimic transit through the acidic stomach to the alkaline small intestine. The timed release of sporozoites from oocysts is likely related to the transit time of oocysts through the stomach and into the small intestine where excysting sporozoites can invade epithelial cells. Released sporozoites are considered "short-lived" and have a limited time to successfully invade and proliferate in host cells, thus assuring parasite survival.

Cell culture infectivity assays have several advantages over mouse infectivity assays. We consider them more precise, faster (days instead of weeks), easier to carry out (mice have to be acquired, housed, fed, handled, sacrificed, necropsied *etc.*) and less genetically variable. Furthermore, they are less expensive and materially demanding and not ethically controversial in comparison with *in vivo* assays (O'Donoghue, 1995; Shin et al., 2001; Rasmussen et al., 1993; Smith et al., 2005). Inoculation of cell cultures is preceded by several successive steps including oocyst excystation, cell localization, attachment and invasion (Smith et al., 2005). It follows that excystation of sporozoites from oocysts is essential for successful *in vitro* assays and also for further research.

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Table 1
Summary of excystation methods used.

Method by	Rasmussen et al. (1993)	Petry and Harris (1999)	Mead et al. (1990)	Black et al. (1996)	Gut and Nelson (1999)	Rennecker et al. (1999)
Pre-incubation	2% sodium hypochlorite	0,25% sodium hypochlorite	0,5% sodium hypochlorite, 0,1% sodium thiosulfate		10 mM HCl	
Excystation medium	0,75% NaT and 0,25% trypsin in PBS	0,75% NaT in RPMI medium	0,75% NaT and 0,25% trypsin in PBS	1,5% NaT and 0,5% trypsin in PBS	0,8% NaT in RPMI medium	1,5% NaT and 0,5% trypsin in PBS
Incubation time	45 min (37 °C)	2 h (37 °C)	1 h (37 °C)	2 h (37 °C) 30 min (room temperature)	10 min (37 °C)	1 h (37 °C)

Excystation has been traditionally used as a measure for oocyst viability and infectivity, although, according to some studies, it is not so precise. Neumann et al. (2000) discovered that intact *C. parvum* oocysts that fail to excyst *in vitro* remain infectious to neonatal mice. In spite of this fact, excystation provides a convenient measure of oocyst and sporozoite health and if excystation yields sporozoites at rates higher than 60–70%, the preparation is considered to be suitable for culture (Arrowood, 2008). There are various excystation protocols that are used in different laboratories, so there is a need to clarify which of these methods is the best.

In this study, we compared six of the most well known *in vitro* excystation protocols differing in pre-incubation steps (pre-treatment with sodium hypochlorite/HCl/no pre-treatment), excystation media (PBS/RPMI and trypsin/no trypsin) or incubation time. All of these variables were tested in the past (Woodmansee, 1987; Kar et al., 2011) but their combination can bring different results in the excystation success rate.

2. Materials and methods

2.1. Cryptosporidium parvum *oocysts*

Cryptosporidium parvum oocysts were obtained from the faeces of naturally infected bovine calves. All of the experimental procedures were conducted in accordance with applicable laws of the Czech Republic on the use of experimental animals and the safe use of pathogenic agents. The study was conducted under protocol approved by the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences and Central Commission for Animal Welfare, Czech Republic (protocol no. 073/2010).

2.2. Stool collection and purification of oocysts prior to excystation test

After collection, faeces were stored unpreserved at 4 °C for one week before use. The stool sample was defatted with organic solvent diethyl ether before further processing for a higher quality of oocyst purity (ratio of stool to solvent 2:1). This stool was passed through a sieve (standard tea sieve) and processed using the microscale cesium chloride (CsCl) gradient technique (Arrowood and Donaldson, 1996) as follows: the sieved stool sample was diluted with phosphate-buffered saline (PBS) and passed through a finer, nylon sieve (40 μm mesh size). The sieved sample was centrifuged (800 × *g* for 20 min at 4 °C), supernatant discarded, pellet resuspended in PBS repeatedly, until the supernatant was clear. The sediment was resuspended in a small amount of PBS to get a liquid suspension. RTA tubes (2.0 ml) were filled with 1 ml of cesium chloride suspension (21.07 g of CsCl in 100 ml of deionized H_2O) and 500 μl of liquid sieved stool solution was carefully layered over the CsCl solution. Tubes were centrifuged at 12100 × *g* for 3 min at 20 °C. Oocysts were collected from the middle layer, transferred to microcentrifuge tubes, diluted with PBS and centrifuged (750 × *g* for 3 min at 20 °C) three times so that cesium chloride was washed out. Pellets from all tubes were pooled, centrifuged again and stored at 4 °C.

2.3. Test of viability

Viability of the oocysts was tested with propidium iodide staining: 10 μl of the oocyst suspension was diluted with 100 μl of PBS and 30 μl of stock solution of propidium iodide was added. This suspension was incubated for 3 min at room temperature in the dark and then examined using a fluorescence microscope with an excitation wavelength 535 nm.

2.4. Excystation methods

In each of the following six methods, three 1.5 ml microcentrifuge tubes were used; each contained 0.5×10^6 oocysts in 1.5 ml of PBS (Table 1).

2.4.1. Method by Rasmussen et al. (1993)

Oocysts were washed three times in PBS (centrifugation at 12,100 × *g* for 3 min). McCoy's medium, used in the original paper, was replaced with PBS. Oocysts were then suspended in 2% sodium hypochlorite and incubated for 10 min. After that, oocysts were washed five times in PBS, suspended in an excystation medium consisting of 0.75% taurocholic acid (sodium taurocholate, NaT) and 0.25% trypsin in PBS and incubated at 37 °C for 45 min.

2.4.2. Method by Petry and Harris (1999)

Oocysts were centrifuged (12,100 × *g* for 3 min), supernatant discarded and the pellet with oocysts was re-suspended in 1:20 diluted commercial bleach (0.25% sodium hypochlorite) and incubated for 10 min on ice. Afterwards, oocysts were washed three times in water (12100 × *g* for 3 min), suspended in excystation solution (0.75% sodium taurocholate in RPMI 1640 medium) and incubated at 37 °C for 2 h.

2.4.3. Method by Mead et al. (1990)

Oocysts were centrifuged (12,100 × *g* for 3 min) in order to remove PBS, then suspended in 1 ml of 0.5% sodium hypochlorite, incubated for 5 min in 4 °C and centrifuged (6800 × *g* for 1 min). The oocyst sediment was neutralized by suspending in 0.1% sodium thiosulfate (diluted in PBS), centrifuged (2450 × *g* for 3 min) and washed twice with PBS. Subsequently, oocyst suspensions were suspended in excystation solution (consisting of 0.75% sodium taurocholate and 0.25% trypsin diluted in PBS) and incubated for 1 h at 37 °C. Afterwards, the oocyst/sporozoite suspensions were washed twice in PBS.

2.4.4. Method by Black et al. (1996)

Oocysts were suspended in 500 μl of PBS, added to 500 μl of excystation medium (150 mg of sodium taurocholate and 50 mg