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Short communication

An improved *in vitro* infection model for viability testing of *Cryptosporidium parvum* oocysts

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Abstract

An *in vitro* infection model for the protozoan parasite *Cryptosporidium parvum* was evaluated for its suitability to determine the viability status of oocysts. Adherent HCT-8 cells were used as host cells and confluent monolayers were inoculated with oocyst suspensions in the presence of 0.4% sodium taurocholate which proved not to be cytotoxic. For a semi-quantitative detection of the infection a PCR-based assay was developed. The influence of physical (elevated temperature) and chemical (chlorocresole) inactivation methods on oocyst viability were evaluated. A minimum of 1000 untreated oocysts was necessary to establish a reproducibly detectable infection of the cells. With 10 and 100 oocysts, 30 and 78% of cell cultures, respectively, could be diagnosed as infected. For thermal inactivation two different temperature levels were used (38 and 55 °C). 55 °C, irrespective of incubation time, was sufficient to inactivate the oocysts to a degree below the detection limit. An elevation of temperature to 38 °C, in contrast, had no appreciable effect on oocyst infectivity in cell culture. Neopredisan® efficacy against the parasite was tested at 0.25, 1 and 4% concentration. 0.25 and 1% had no discernible inhibiting effect on the developmental potential of the oocysts, while 4% Neopredisan® resulted in a significant inhibition of *Cryptosporidium* development which was, however, not as prominent as heating to 55 °C, and not all oocysts could be inactivated.

Keywords: *Cryptosporidium parvum*; Infectivity; *In vitro* cultivation; PCR; Inactivation; Chlorocresole

1. Introduction

Due to the growing public attention the cryptosporidia, especially *Cryptosporidium parvum,* are recently receiving (Fayer, 2004; Olson et al., 2004; Carey et al., 2006) the demand for robust and reproducible viability tests for oocysts as infectious stages in the environment is increasing. Besides other epidemiologically important features the oocysts have also been found to exhibit a considerable tenacity and resistance to most disinfection procedures effective against bacterial and viral pathogens (Barbee et al., 1999). Finally, the minimal amount of oocysts required to induce infection is rather low, in the range of 10–1000, depending on the isolate examined (DuPont et al., 1995; Chappell et al., 1999). However, due to their morphological peculiarities evaluation of oocyst viability is difficult. In recent years, cell culture systems have been reported to be suitable for this purpose as well, their sensitivity equalling that of *in vivo* assays (Rochelle et al., 2002, 2003; Jenkins et al., 2003; Joachim et al., 2003). In this study, a previously described cell culture assay coupled with PCR verification of infection (Joachim et al., 2003) was optimised for semiquantitative evaluation to test the viability of *C. parvum* oocysts after chemical or thermal disinfection *in vitro*.

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2. Materials and methods

A German field isolate of *Cryptosporidium* determined as *C. parvum* bovine genotype by sequence analysis (Morgan et al., 1999; Sturbaum et al., 2003) was passaged *in vivo* in calves and subsequently collected and purified essentially as described (Joachim et al., 2003) except that the oocysts were stored in phosphate buffered saline (PBS) supplemented with penicillin/streptomycin/amphotericin B at 4 °C for a maximum of 6 months.

Permanent cultures of HCT-8 cells (ECACC, European Collection of Cell Cultures, Cat. No. 90032006) were maintained as before (Joachim et al., 2003). For infection with oocysts cells were seeded into 24-well cell culture plates at concentrations of 2×10^5 cells per well and grown to 70% confluence (approximately 48 h); then growth medium was replaced by excystation medium (i. e. growth medium supplemented with 0.4% sodium taurocholate, TCh, Sigma–Aldrich, Taufkirchen, Germany) and titration series of oocysts (10–100,000 per well) added to the cavities. After 1 h excystation medium was diluted 1:8 with fresh growth medium and the cultures were incubated overnight. Twenty-four hours later the medium containing unexcysted oocysts and oocyst walls was removed, cells were washed twice with (PBS) and fresh medium was added for incubation for another 24 h.

Excystation rates of oocysts at different TCh concentrations (0.4, 0.8 and 2.0%) were determined by incubation at 37 °C for 2 h. Aliquots were examined under the microscope (phase contrast) and the proportion of excysted oocysts calculated. Also, various TCh concentrations in medium (0.1, 0.2, 0.4, and 0.8%) were applied on confluent cell monolayers and the effects (cell detachment) was observed.

Cell monolayers infected with untreated oocysts were compared to those inoculated with potentially inactivated parasites. To estimate the influence of non-viable or inert, unexcysted oocysts potentially remaining on cell monolayers throughout the experiments on the detection assay, titration series of heat-inactivated (70 °C for 15 min) oocysts were added to cell cultures as above.

To evaluate the assay and inactivation procedures, oocysts were treated with the disinfectant Neopredisan® (25% chlorocresole, Menno Chemie, Norderstedt, Germany). Three different concentrations (0.25, 1 and 4%) were prepared from a commercially obtained stock solution defined as 100%. The procedure described by Joachim et al., 2003 was followed with incubation times of 60–120 min.

To assess the efficacy of a physical disinfection procedure oocysts were also exposed to elevated temperatures: 38 °C for 1, 4 and 24 h (“mesophilic” treatment), and 55 °C for 4, 12 and 24 h (“thermophilic” treatment) in a thermomixer (Model 5436, Eppendorf, Hamburg, Germany). The oocysts were then resuspended in cell culture medium and inoculated on cell monolayers as described above.

In contrast to the previously developed assay, Accutase® (PAA, Cölbe, Germany) was used to detach the cells from the well bottoms. DNA extraction was done using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. In the last step the DNA was eluted from the column with 50 μl of elution buffer and stored at 4 °C for a maximum of 1 week until PCR amplification. The final PCR mixture contained 3.5 mM magnesium chloride, 0.5 mM each of dATP, dCTP, dGTP and dTTP, 10 pmol of each primer (CP 3.4–3′ and CP 3.4–5′; Petry et al., 1998; Joachim et al., 2003) and 1 U of Taq polymerase (Promega, Mannheim, Germany) in the supplied buffer. 2.5 μl of DNA was added to 22.5 μl of the master mix. The PCR program consisted of an initial denaturation step of 94 °C for 3 min followed by 35 cycles of 94 °C for 45 s, 57 °C for 45 s, 72 °C for 1 min, and a final extension step of 72 °C for 10 min. After the run was completed the products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and photographed using a digital camera. Presence or absence of the expected DNA band (650 base pairs in size) was recorded. As an additional parameter, band intensities were assessed and roughly graded into three classes, strong, medium, and weak. Negative controls (no-infection-control, NIC from uninoculated monolayers; no-template-control, NTC) were included in each trial.

The data were analyzed using the SPSS statistical software package version 11.5.1 (SPSS Inc., Chicago, USA). χ^2-test, Fisher–Yates test, and Mann–Whitney *U*-test were applied. *P* values of 0.05 or lower were considered significant.

3. Results and discussion

The aim of the present study was to optimise a previously published assay and to apply the new protocol to both chemically and thermally inactivated oocysts. One crucial step is minimising the manipulations of the pre-treated oocysts, while optimising the excystation rates *in vitro*. Most oocysts excysted on cell monolayers within 1 h after inoculation. As described before (Kato et al., 2001; Hou et al., 2004) the