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INFECTIOUS RATES OF HCT-8 CELLS INFECTED WITH *CRYPTOSPORIDIUM PARVUM* SPOROZOITES OBTAINED IN *IN VITRO* DIFFERENT EXCYSTATION CONDITIONS

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ABSTRACT

In this study, HCT-8 cells infected with C. parvum sporozoites which were released in 3 different excystation conditions (TA/cytomixs:RPMI-1640 excystation media (1:1); TA/cytomix:RPMI-1640 media and TA/cytomix media) were counted under fluorescent microscopy by IFA (Immunofluorescent assay method).

Different pulses (pulse 1, pulse 2, pulse 3 and heat inactivated) were applied for C. parvum sporozoities under electroporation conditions of 1000 voltage (kV/cm), 50 capacitance (μF) and 50 recistance (ohm Ω) and cell monolayers were counted under fluorescent microscopy to determine infected cells. As a consequence, the maximum infected cell monolayers were enumerated at pulse 1 under electroporation conditions of 1000V, 50μF, 50Ω

Our results showed that the best condition of excystation was TA/cytomixs:RPMI-1640 excystation media (1:1) with excystation rate of 80% and the other excystation rates were 70% and 60% for TA/cytomix:RPMI-1640 media and TA/cytomix media, respectively.

Keyword: HCT-8 cells culture, electroporation, excystation, *C. parvum*, immunofluorescence assay (IFA)

Introduction

Cryptosporidium parvum oocyst viability can be determined by vital dyes, in vitro excystation, and cell culture; however, neonatal mouse infectivity assays are the reference method. Unfortunately, there have been few efforts to standardize methods for infectivity assays thus casting a veil of uncertainty over the significance and comparability of results. It is important to standardize mouse strain, age of mice at inoculation and necropsy, oocyst, and age of oocysts. However, other factors, including methods used to detect infectivity and to count oocyst doses, appear to be less critical (2).

Electroporation method has been developed into a rapid, simple, and efficient technique for introducing DNA into a wide variety of cells, including bacteria, yeasts, plant cells, and a large number of cultured mammalian cell lines. Three important characteristics of pulse affect the efficiency of electroporation: the length of the pulse, its field-strength, and its shape. The best results are obtained with cell cultures in the mid-log phase of growth that are actively dividing (4).

Excystation, a measure of the ability of the oocyst to release the sporozoites, cannot evaluate infectivity and has limitations regarding the ability to enumerate sporozoites. Until now, the only available infectivity assay requires animal hosts, which is costly and very time-consuming (5).

The aim of the present study was to investigate the best conditions of excystation for *C. parvum* by using electroporation method and IFA. We described here preliminary study which form the basis of future study dealing with *C. parvum* sporozoites since the excystation is used as an indicator of viability.

Materials and Methods

Culture media

Human illeocecal adenocarcinoma cells (HCT-8 cells) (ATCCCCL-244; cat no: 2056312, American Type Culture Collection) were maintained in RPMI 1640 (Sigma; R5886 with 25 mM Hepes, sodyum bi carbonat) supplemented with 10% Horse serum. (Gibco, Kat no: 26050-088), % 1 Penicillin /Streptomicin (Sigma; P0781), 1 mM Sodyum prüvat (sigma; S8636) and, % 1 L-glutamin (Sigma; Kat no: G7513).

The HCT-8 cells were maintained in 75-cm^2 tissue culture flasks in a 5% CO_2 atmosphere at 37°C and 100% humidity and passaged every 2 or 3 days. The cells were passaged by accutase enzyme (catalog: AT04 İnnovative CellTechnologies, Inc.) with phosphatebufferedsaline (PBS)-EDTA. HCT-8 cells required 5- to 10-min incubation in accutase solution at 37°C in the CO_2 atmosphere to assist in the disruption of the monolayer. The cell suspension was pipetted vigorously through a 200-ml pipette tip repetitively until the cells were separated (about 10 times). The cells were pelleted by a 4-min centrifugation (200 x g), resuspended in maintenance medium, and split 1:5 at each passage. 24 well plate were plated with 2.5 x 10^5 cells per well, grown to 70 to 95% confluence (24 to 48 h), and then infected (see below).

Oocyst preparation

C. parvum oocysts (IOWA isolate from a naturally infected calf) were obtained from Bunch Grass farm in Indiana, U.S.A. The oocyst strain had been perpetuated by manually infected C57BL/6 mice. The oocyst age and percent excystation

were recorded for each experiment. The concentration of the oocyst stock suspension was determined by using a direct hemacytometer count, and 10-fold dilutions were made to prepare low concentrations. Negative controls were run concurrently for each experiment and included oocysts that had been heat inactivated by boiling for 10 min. The oocysts were pretreated by suspending 100 ml of stock oocysts (stored in sterile deionized [DI] water) in a 10% bleach solution containing 9 parts sterile ultrapure water (800 ml) and 1 part % 98 sodium hypochlorite (100 ml of 4°C bleach on ice) for 10 min. The preparation was centrifuged at a relative centrifugal force of 8000 rpm for 5 min in a tabletop Microfuge 12 (Beckman Instruments, Palo Alto, Calif.), and the supernatant was aspirated off. The oocysts were then washed twice in sterile water and again centrifuged. The sterilized oocysts were used for inoculation of cell culture with inoculation rate of 1:3 (7.5 x 10^5 oocysts per well were used for which included 2.5 x 10^5 cells in 24 well plate).

Different excsystation conditions

Taurocholic acid: (TA, Sigma T9034), cytomix buffer and RPMI-1640 media were used for excystation solution with 3 different rate. One was Taurocholic acid/cytomix, the second was Taurocholic acid/cytomix: RPMI-1640 media (1:1) and the third was Taurocholic acid/RPMI-1640 media. Same amount of oocyst (10^6/ml) were excysted in 3 different excystation solutions and centrifuged to remove residual then, resuspended in cell culture growth medium warmed to 37°C.

Electroporation

Resuspended oocysts with cell culture were electroporated under electroporation conditions of 1000 voltage (kV/cm), 50 capacitance (μF) and 50 resistance (ohm Ω) in electroporator (ABTXR ECM 630) by giving different pulse (1, 2 and 3 pulses). They were used for cell infection immediately.

Maintenance medium was aspirated from the cell monolayers, and the cells were washed once with sterile 1 x PBS. Oocysts in growth medium were added to the cells and incubated for 90 min in a 5% CO_2 atmosphere at 37°C (to initiate excystation and infection). After cells were ready, electroporated oocysts were added into the cell culture plate and they were incubated at 37°C under 5% CO_2 for 24 to 48h.

Antibody labeling

After incubation, the medium was aspirated and the infected monolayers were washed once with sterile 1x PBS and fixed with 100% methanol (at room temperature, RT) for 10 min then they were washed once again with 1 x PBS. For each well, 1:100 NGS/PBS (Normal goat serum in 1 x PBS) was added and waited for 30 min at RT. The monolayers were labeled with primary polyclonal rat anti-sporozoite (1:1000 was suspended in NGS/PBS) which reacts with all reproductive stages of the organism for 30 min at RT.

The slides were washed with 1x PBS. The secondary antibody was Alexa Fluor 488 anti-rabbit antibody conjugated to fluorescein isothiocyanate (1:1000 was suspended in NGS/PBS) was added to all wells.

The slides were rocked gently for 30 min (under dark conditions) at RT then, they were washed with 1 x PBS and the cells were viewed under fluorescence microscopy. The specificity of the antibody to the reproductive stages was determined by examining the cell monolayer under fluorescent microscopy. The infected cells fluorescence an apple-green color against a relatively dark background of uninfected cells, and the number of foci was determined by averaging whole well counts in triplicate.

TABLE 1

An average number of infected HCT-8 cell monolayers in different excystation conditions

Given pulses	An average number of infected HCT-8 (TA/cytomix)	An average number of infected HCT-8 (TA/cytomix:RPMI-1640 media (1:1)	An average number of infected HCT-8 (TA/cytomix:RPMI-1640 media
No pulse	69	91.3	67.3
Pulse 1	51	73	57
Pulse 2	57	55.6	45
Pulse 3	43	37	25
Heat inctivated oocysts	9	7	9.6

TABLE 2

Standard deviation of infected HCT-8 cells monolayers

Given pulses	SD of Infected HCT-8 cells (TA/cytomix)	SD of Infected HCT-8 cells (TA/cytomix:RPMI-1640 media (1:1)	SD of Infected HCT-8 cells (TA/cytomix:RPMI-1640 media)
No pulse	9.00	40.50103	21.5484
Pulse 1	14.05	14.80	10.44
Pulse 2	5.00	11.06	20.42
Pulse 3	24.02	19.09	11.00
Heat inactivated oocyst	2.00	2.65	3.21

The standard deviation between replicates was determined by Microsoft Excel statistical analysis.

Results and Discussion

Infected with *C. parvum* HCT-8 cell monolayers were counted under fluorescent microscopy by IFA and were evaluated by an average of infected HCT-8 cell monolayer and table of standard deviation and graphic of deviation, respectively (as it is shown in **Table 1, Table 2** and **Fig. 1**). In addition, the best condition of excystation was determined by comparing with excystation rates of all different conditions.

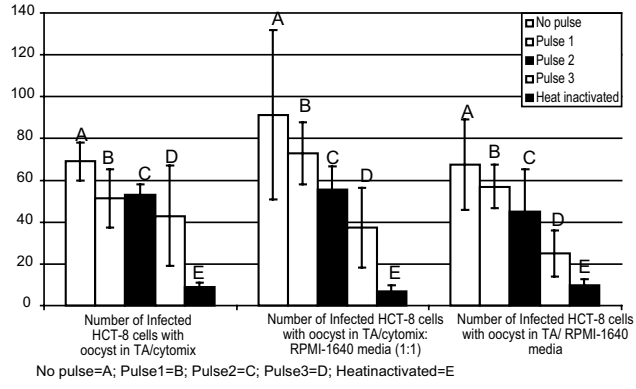


Fig.1. Graphic of Standard deviation for infected HCT-8 cell monolayer

IFA is used for detection of *Cryptosporidium* and *Giardia* species. However, among a variety of limitations, this assay does not directly address viability. Oocysts that contain sporozoites or that are full are considered potentially viable. Viability assays involving vital dyes have been developed but they do not measure infectivity. Excystation, a measure of the ability of the oocyst to release the sporozoites, cannot measure infectivity and has limitations regarding the ability to enumerate sporozoites. Until now, the only available infectivity assay requires animal hosts, which is expensive and very time consuming (5).

However some investigators used the rate of *in vitro* excystation to estimate oocysts viability besides of vital dyes such as 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide. (6).

A viability assay for oocysts of *Cryptosporidium parvum* based on the inclusion or exclusion of two fluorogenic vital dyes, DAPI and propidium iodide, was developed by using several different isolates of oocysts In this research, two similar excystation protocols were utilized, and no significant difference between excystation protocols was detected. Percent excystation of oocyst suspensions could be increased or reduced by inclusion of a preincubation treatment in either excystation protocol, and this alteration was also demonstrated in the viability assay (1).

As negative control in this study heat inactivated oocysts were used. After heat inactivation of *C. parvum* oocysts, they were subjected to electroporation under different pulse types (1, 2, and 3 pulse). Observations showed that heat inactivated oocysts could not infect cell monolayers entirely and infectious rates of cells were highly decreased, since the great number of oocyst were killed by heating.

Ionic components are one of the most important factors for electroporation. Cells in salt buffers have more transfection efficiency than buffers lack of ionic such as sucrose and mannitol (4).

Cytomix buffer (1) was used for all three excystations conditions at the different rates with taurocholic acid and RPMI-1640 media for this study. As it is shown on **Fig. 1**, excystation conditions which included Cytomix buffer in RPMI-1640 media showed the best result for infection of HCT-8 cells.

Infectivity is an important issue that is currently addressed only by using animal models. Cell culture appears to be more cost-effective, reproducible, and practical method. Animal models cannot be used easily to assess single oocyst inoculums or to be used with environmental samples. The FDM can be used with environmental samples for the detection of a single oocyst and can also be used as a viability assay for survival studies and disinfection studies (5).

Conclusions

The observation showed that the oocysts used in this study had excystation rates of 80%, 70% and 60% TA/cytomixs: RPMI-1640 excystation media (1:1), TA/cytomix: RPMI-1640 media, TA/cytomix media respectively. We also determined that the best condition of excystation was TA/cytomixs: RPMI-1640 excystation media (1:1) and Pulse 1 gave the best result when it was compared with all other pulses in electroporation for this study.

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