

COMPARISON OF *CRYPTOSPORIDIUM PARVUM* DEVELOPMENT IN VARIOUS CELL LINES FOR SCREENING *IN VITRO* DRUG TESTING

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Abstract. This study describes the development of *Cryptosporidium parvum* in MDCK, MA-104, Hep-2 and Vero cell lines. Differences in susceptibility, infectivity, and the methodology of excystation were determined. Various solutions were considered to determine the factors which enhanced the excystation (eg with and without sodium hypochlorite, trypsin or sodium taurocholate). It was shown that the sporozoites could be excysted in media either with or without trypsin and sodium taurocholate, but the number of sporozoites in the latter solution was less than the former one. Only oocysts digested by sodium hypochlorite and trypsin can enter the culture cells. Numerous meronts and oocysts were demonstrated and persisted for 9 days. Asexual stages were not observed in MA-104. Only few oocysts could be detected 1-3 days post-inoculation. There was a significant difference between the number of oocysts, which invaded MDCK, MA-104, and Hep-2 cells. MDCK gave the highest susceptibility to oocyst invasion among the three cell lines and asexual stages were also found. Among the 25 isolates, which had been cultivated, 23 isolates could infect MDCK and Hep-2. Only 2 isolates could not infect the MDCK cell. These 2 isolates could infect the Vero cell and yielded high numbers of trophozoites. Praziquantel (PZQ), doxycycline, and paromomycin (PRM) were tested on the infecting parasites. The drugs were added either with the inoculum or 24 hours after inoculation. None of them was effective, including PRM, which had been previously reported as effective.

INTRODUCTION

Cryptosporidium parvum is an intracellular but extracytoplasmic protozoan parasite that causes self-limiting diarrhea in immunocompetent individuals (Du pont *et al*, 1995). This parasite causes severe symptoms in the immunocompromised host, especially in AIDS patients (Peterson, 1992). Lack of an effective therapy motivates many workers to search a new target for the treatment of cryptosporidiosis. A few drugs have been found to be effective in animal models

(Theodos *et al*, 1998). Some recent reports stated that some drugs caused a reduction in parasite number and the duration of diarrhea *in-vivo* (Castro-Hermida *et al*, 2001; Rossignol *et al*, 2001).

Several *in vitro* systems for drug testing have been described, using a variety of different procedures and cell lines (McDonald *et al*, 1990; Benbow *et al*, 1998; Perkins *et al*, 1998; Giacomet *et al*, 1999). Most of them describe the detection of parasites in culture cells using either serodiagnosis or radioactive substances, which are complicated and expensive (Arrowood *et al*, 1994; Favennec *et al*, 1994; Wiest *et al*, 1994; Perkins *et al*, 1998; Theodos *et al*, 1998). Due to the observation that both oocysts and sporozoites can invade culture cells, our study was undertaken to demonstrate the difference between the infectiv-

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ity of processed and non-processed oocysts in different cell lines, including in drug applications. The correlation between the invasion of those oocysts in both groups was also shown and the method for screening the effect of antimicrobial agents to *C. parvum* oocysts in MDCK, MA-104, and Hep-2 cells by staining was developed.

MATERIALS AND METHODS

Preparation of culture cells

MDCK (Mardin Darby Canine Kidney), MA-104 (African Green Monkey Kidney Epithelium), Hep-2 (Human Larynx Carcinoma) and Vero (African Green Monkey Kidney Fibroblast) cells were grown in 24-well culture plates for 2 days in a 5% CO₂ incubator. All the culture cells were seeded at a density of 10⁶ per well. MEM (ICN) medium, supplemented with 10% fetal bovine serum, gentamicin and L-glutamine, was used for the cultivation.

Preparation of *C. parvum* oocysts

Diarrheic stools were collected from 25 AIDS patients, who had been diagnosed with active cryptosporidiosis, and stored at 4°C in 2.5% (w/v) potassium dichromate. Stools were then filtered through 10-layer gauze to remove coarse debris. The oocysts were aseptically in 2% sodium hypochlorite solution for 10 minutes at 0°C and washed 3 times at 2,000g for 8 minutes in PBS. The excystation of sporozoites was achieved by incubating oocysts at 37°C for one hour in 0.25% trypsin and 0.75% sodium taurocholate solution at 37°C for one hour. The inoculum was divided into 3 groups; the first, filtered sporozoites, using a sterile 2 µm pore-size filter (Millipore Corp); the second, sporozoites and remaining oocysts or excysted oocysts, directly inoculated into culture cells; and the third, oocysts, without processing or unexcysted oocysts. The number of sporozoites, remaining oocysts, and unexcysted oocysts were counted in duplicate by a hemocytometer chamber under a light microscope (magnification x 400) and the average number was taken.

Some oocysts were purified, then antibiotics were applied for cleaning, instead of sodium hypochlorite, to confirm whether the oocysts from this preparation could invade cells or not.

To evaluate the effect of trypsin and sodium taurocholate on the ability of oocysts to invade culture cells, the excysted oocysts were washed 3 times with PBS before inoculation. The number of infected oocysts in this experiment were counted and compared to the excysted oocysts that were directly inoculated into culture cells without washing.

Measurement of drug toxicity in various cell lines

Twenty-four hours after preparing the monolayers, the medium was removed, praziquantel (PZQ) was applied at a 2 fold dilution, between 3,000 to 750 µg/ml. Paromomycin (PRM) was added using the same procedure, from 4,000 to 1,000 µg/ml medium (6.4-1.6 mmol). The monolayers were incubated for 24 to 48 hours at 37°C in a CO₂ incubator to observe for cytotoxicity by examination under light microscope after H&E staining. The cytotoxic level was determined 24 hours after the application of drug. The concentration of drug which showed neither damage to the cell nor abnormal mitotic figures was identified as a safe level.

Infection of cell cultures with *C. parvum*

Sporozoites and oocysts from each group were counted at 5,000/ml and inoculated into monolayers of MDCK, MA-104, and Hep-2 in the presence of selected drugs, except in the control wells. Then the culture plates were incubated at 37°C in a CO₂ incubator for 3 hours. Inoculation was done in duplicates and the experiment was repeated three times.

The coverslips containing *C. parvum*-infected monolayers, control culture cells and infected cells in the presence of the drugs from the MDCK, MA-104, and Hep-2 culture plates were picked up at 30 minutes intervals for 3 hours, to determine the infection rate by staining with Giemsa's and DMSO modified acid-fast stains. After 3 hours, the medium was removed and washed twice with PBS free from calcium and magnesium. The medium containing drugs was replaced and the parasites were cultivated for an additional 48 hours.

Detection of *C. parvum* in cell lines

The coverslips containing infected cells, control cell lines, and also infected cells exposed

to various drugs from all the culture cells were stained by both DMSO modified acid-fast and Giemsa's stains at 24 and 48 hours post-exposure to various drugs. Oocysts were stained by DMSO modified acid-fast while meronts were stained by Giemsa's stain. The number of parasites were counted for 30 fields at 400x magnification and compared with the infected cells alone, infected cells with drugs, and also non-infected cells.

Cell lines were scrapped and fixed in 10% formalin. Then paraffin sections with H&E and Giemsa's staining were performed.

RESULTS

Among the 3 groups of inoculum *ie* sporozoites, excysted oocysts, and unexcysted oocysts, numerous oocysts from the excysted oocyst group were found in MDCK and Hep-2 cells, whereas none of the oocysts from the unexcysted oocysts and also oocysts from antibiotic application group was detected. No development of parasite was found in culture cells from the sporozoite inoculation. Type 2 meronts and oocysts were found during 24-hour post-infection (Figs 1 and 2). The number of oocysts in MDCK at 24 and 48 hours was statistically higher than in Hep-2, which was statistical significant ($p < 0.001$) (Fig 3). Only a few oocysts were detected in MA-104; and they remained in the culture for cells only 1-3 days.

The number of excysted oocysts in MDCK was significantly different from Hep-2 and MA-104 cells ($p < 0.001$) (Fig 4), and there was a correlation with the number of excysted oocysts and unexcysted oocysts in MDCK with the drug application (Fig 5).

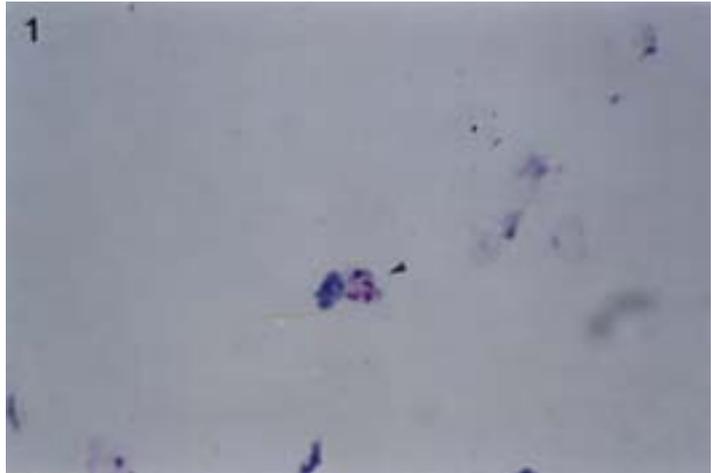


Fig 1—Type 2 meront in a culture medium 24 hours after infection, 1,000x magnification, Giemsa' stain.

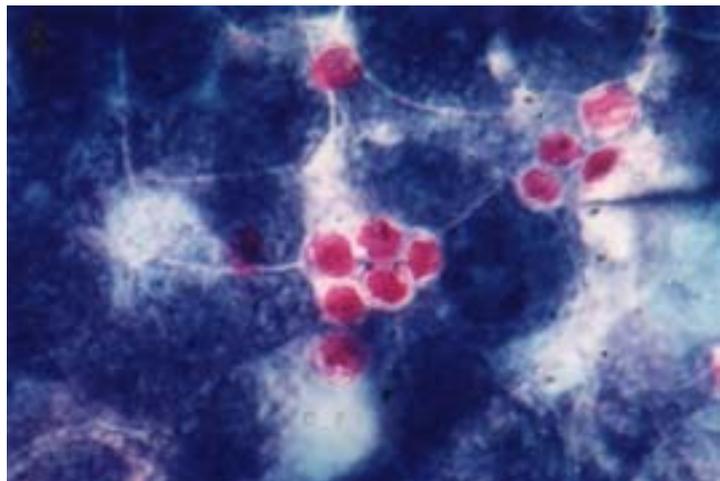


Fig 2—Oocysts in culture cells 24 hours after infection, 1,000x magnification, DMSO modified acid-fast stain.

There was no effect of trypsin and sodium taurocholate on excysted oocysts, which invaded all cell lines (Fig 6).

PRM reduced the number of infected oocysts at the highest concentration (4,000 $\mu\text{g/ml}$) but no abnormal cell lines were detected at this level. In addition, there were no abnormal cell lines detected at a concentration of 750 $\mu\text{g/ml}$ of PZQ, but abundant extracellular parasites were present.

The trophozoites in the culture cells were processed for histopathological sections to investigate whether they could invade the cytoplasm

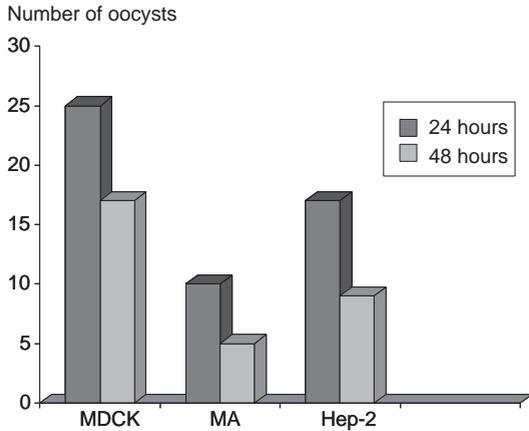


Fig 3–Comparison of the number of oocysts in MDCK, MA-104, and Hep-2 at 24 hours and 48 hours.

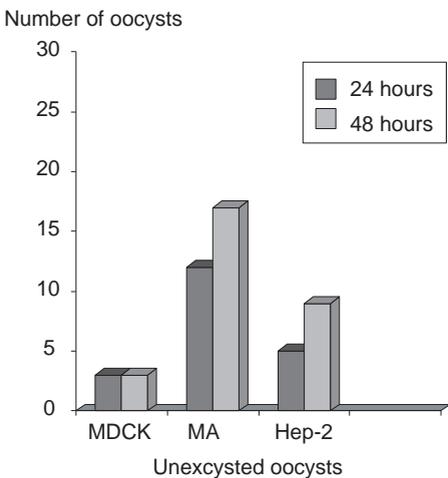
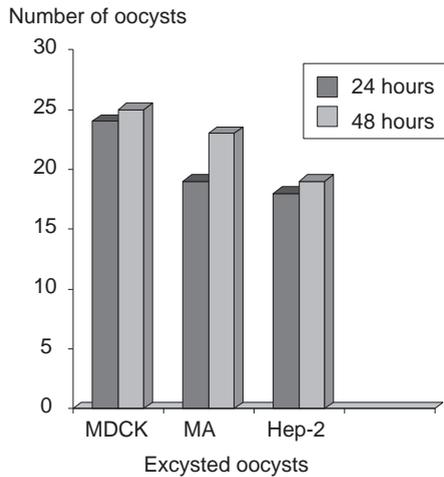


Fig 4–Comparison of the number of oocysts in MDCK, MA-104, and Hep-2 with excysted and unexcysted oocysts at 24 hours and 48 hours.

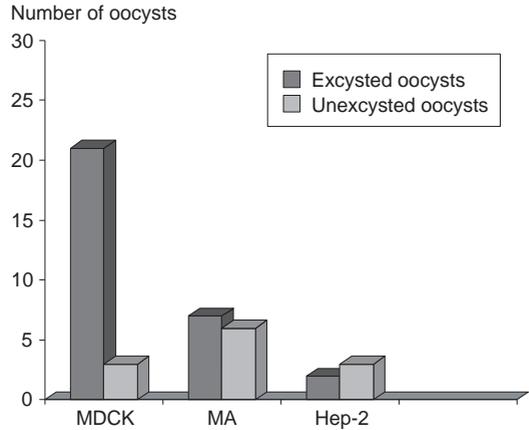


Fig 5–Comparison of the number of oocysts in MDCK, MA-104, and Hep-2 in PZQ application with excysted and unexcysted oocysts at 24 hours.

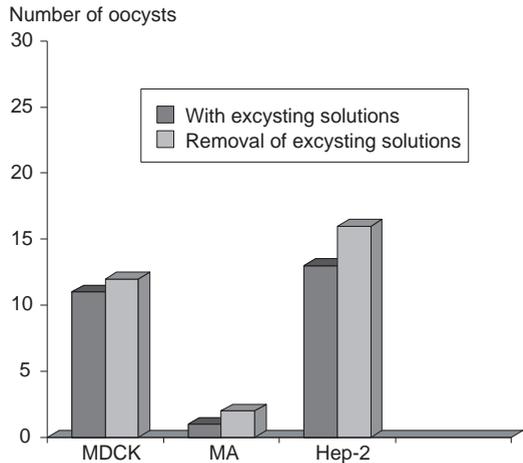


Fig 6–Comparison of the number of oocysts in MDCK, MA-104, and Hep-2 in PZQ application with excysting solution and removal at 24 hours of the excysting solution.

of cells or not. After H&E and Giemsa’s staining, we found that the trophozoites were covered with a thin membrane and narrow spaces were observed between the junction of the membranes and parasites in both H&E and Giemsa’s stains (Figs 7 and 8). Therefore, it can be concluded that the parasites were only beneath the cell membrane.

DISCUSSION

We suggest here a simple method for screening the efficacy of drugs *in vitro*, due to the ob-

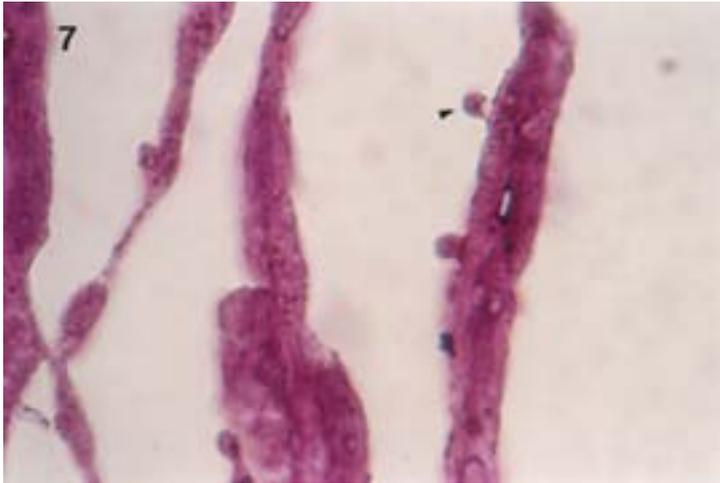


Fig 7—Trophozoites of *Cryptosporidium* in culture cells, paraffin section, H&E stain, 1,000x magnification.

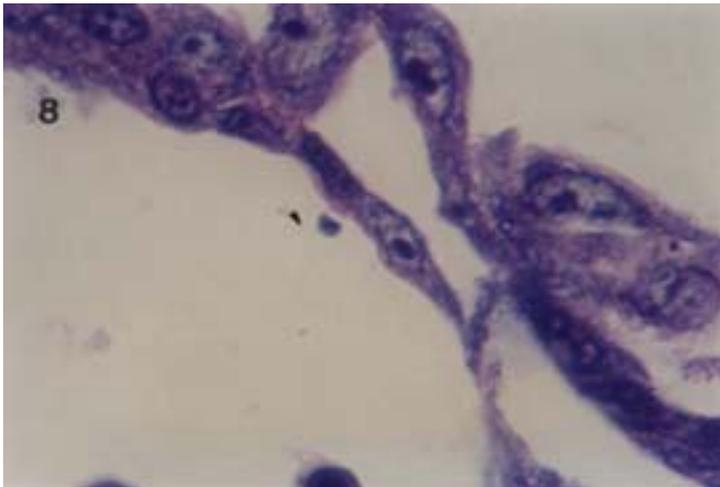


Fig 8—Trophozoites of *Cryptosporidium* in culture cells, paraffin section, Giemsa's stain, 1,000x magnification.

ervation that the oocysts can invade culture cells after the process of excystation. The experiment was designed to confirm whether the oocysts were superficial to the cells or entered the cells. The infected cells were stained by DMSO modified acid-fast and oocysts with parasitophorous vacuoles were clearly demonstrated. It is possible that the outer part of the oocyst was digested by sodium hypochlorite and trypsin solutions. They then became thin walled oocysts and could infect culture cells as autoinfection *in vivo* (Current and Garcia, 1991). The purpose of using sodium hy-

pochlorite for cleaning *C. parvum* oocysts is useful for excystation and *in vitro* cultivation. Sodium hypochlorite directly kills the microorganisms in the stool and also digests the outer part of the oocysts. From this observation, oocysts could be excysted and some sporozoites released in PBS after cleaning with sodium hypochlorite (unpublished data). Sodium taurocholate also expedited excystation, as described by Gold *et al* (2001). Therefore, both sodium hypochlorite and sodium taurocholate promote the invasion of *C. parvum* oocysts. The character of the oocysts from the inoculum, which presented in MDCK, Hep-2, and Vero cells, staining by DMSO modified acid-fast was different from the oocysts appearing in Hct-8 *ie* without parasitophorous vacuoles. Therefore, the viability of sporozoites in those oocysts and their development in the former group need further studying.

Most cell types in our study except Hep-2 were derived from animals. MDCK, that has been previously reported for its susceptibility to *C. parvum* (McDonald *et al*, 1990; Gut *et al*, 1991; Perkins *et al*, 1998; Gold *et al*, 2001; Hijjawi *et al*, 2001), MA-104, and Hep-2 have never been used for the cultivation of *Cryptosporidium* spp before. However, there was a correlation between the infectivity of MDCK and Hep-2. They could only be infected by excysted oocysts, but not MA-104, which showed no invasion.

No evidence of infection in filtered sporozoites from the first group was observed. This might be due to the mechanical forces during filtration. In addition, the sporozoites are very fra-

gile, and have a short life span (Deng and Cliver, 1998; Tzipori and Griffiths, 1998).

Among 25 isolates, there were two which could not infect MDCK or Hep-2, but they could infect the Vero cell. This might be, if this kind of cell had some properties that enhanced the invasion of parasites, as with many other organisms. Numerous trophozoites, merozoites, and oocysts from the inoculum were observed in the Vero cell and this lasted for a week. These two cases were suspected to be of human genotype, which prefer to infect humans as described by Hijjawi *et al* (2001). However, no new oocyst production was observed. Although both MA-104 and Vero are derived from African monkey kidney, the susceptibility of them is different.

Many anticryptosporidium agents have been tested *in vitro*. None of them were highly effective because the parasites existed by feeding on organelles or electron dense bands which, protected them from the penetration of chemotherapeutic agents (Okhuysen and Chappell, 2001). None of drugs were effective in treating the parasites either *in vivo* or *in vitro*. The same phenomenon was observed in our experiment but the concentration of PRM in our experiment, which demonstrated the reduction of parasites, was higher than in others (Favennec *et al*, 1994; Perkins *et al*, 1998). Although the viability of excysted oocysts which invaded susceptible culture cells was not clearly determined, there was a major difference between the number of excysted and unexcysted oocysts in culture cells, with the application of the drug *ie* PRM. It is possible that the drug may either have interfered with or inhibited the invasion of oocysts. Further studies regarding drug activities are being investigated.

In conclusion, the infectivity of *C. parvum in vitro* probably depends on many factors. Firstly, the type of culture cells alters the different susceptibilities (This may be a signal or chemical agents from host-parasite interactions). Secondly, genotyping of *C. parvum* determines the types of culture cells (human or animal genotype). Thirdly, the viability of the oocysts that are freshly collected effect excystation. Fourthly, the process of excystation *eg* digestion of cyst wall by sodium hypochlorite and trypsin, enhances the invasion of oocysts. Lastly, the degree of infectiv-

ity is different in HIV patients (Xiao *et al*, 2001) or with other unknown factors, which should be further clarified.

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