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In vitro Model for *Cryptosporidium parvum* Infection

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INTRODUCTION

Cryptosporidium is a genus of protozoan parasites which is a part of the coccidia group, from the phylum Apicomplexa, which also includes *Isospora* and *Cyclospora*.¹ until the mid-1990s, several species of *Cryptosporidium* were described depending on morphology and host specificity. *C. parvum*, *C. muris*, *C. felis* and *C. wrairi* were identified as species that infect mammals. *C. parvum* was the primary species that have been isolated from infected humans^{2,3} and cross-transmission studies indicated that *C. parvum* isolates can be transmitted from humans to animals and between different animals.⁴

C. parvum is an intracellular parasite that infects the microvillous region of epithelial cells in the digestive tract of vertebrates, resulting in watery diarrhea.

In immunosuppressed individuals, the symptoms are particularly severe and can be fatal.^{5,6} The parasite is primarily spread through the fecal-oral route, often through contaminated water.^{7,8} The life cycle of *C. parvum* begins with the ingestion of oocysts by the host. In the intestine, four sporozoites are excysted, which then infect epithelial cells and initiate asexual development. They become internalized and undergo

ABSTRACT

Background: The in vitro culture system of *C. parvum* has been investigated using numerous cell lines that support the growth of different stages of the parasite which is less expensive and more convenient for the study of host-parasite interactions than in vivo models .

Objective of the study: to evaluate in vitro models of *C. parvum* and to establish an in vitro culture system for further research studies .

Material and Methods: Confluent HT-29 Human Colorectal Adenocarcinoma cell monolayers were infected with 4x10⁵ viable *C. parvum* oocysts, suspended in 200 μ l cell culture medium (DMEM). Cells were then incubated for 2 hours at 37°C. Cell monolayers that grow on 13 mm glass coverslips were washed twice with PBS and fixed in methanol for 1 min, stained with 10% Giemsa at different periods (6, 24, 48 and 72 h) and then mounted on glass slides. The parasites were counted, and the obtained data were statistically analyzed .

Results: It was found that the HT-29 cell line supports the in vitro cultivation of *C. parvum*. The maximum numbers of parasites were obtained by 6 h and 24 h post-infection. Only immature stages were observed in the cell line after 6 h of infection. However, there were many immature and mature asexual stages in the cell monolayer 24 and 48 post infection. The maximum numbers of parasites were counted in cell monolayer infected by 4x10⁵ oocysts .

Conclusion: It was concluded that the HT-29 cell line supports the in vitro cultivation of *C. parvum* protozoan parasite .

Keywords: HT-29; Human; Adenocarcinoma cell line, *C. parvum*; Oocysts; In vitro culture.

merogony, in which eight merozoites are produced. Merozoites from this type I meront can either produce another type I or may develop as a type II meront with four merozoites. The four merozoites released from the type II merogony give rise to the sexual developmental stages, the micro and macrogamonts. The release of microgametes, and their union with macrogametes, gives rise to the zygote, which, after two asexual divisions, forms the environmentally resistant oocyst containing four sporozoites while still within the host cell.⁹

Environmentally robust oocysts are shed by infected hosts into the environment. These oocysts can survive the adverse conditions on the environment for months until it is ingested by a new suitable host. In the new host, the life cycle starts again, and multiplication occurs, using resources of the host.⁹

Methods for the in vitro cultivation of the parasite were advanced significantly in the recent years. The parasite can develop in a variety of epithelial cell lines including human enterocyte lines (e.g. Caco 2, HT-29)^{10,11} and a similar murine cell line (CMT-93)¹² Parasite development is limited, but in vitro, growth is enough to support a variety of research studies. Routine methods for parasite preparation and cell culture infection assays for parasite development have been established, however. *C. parvum* oocysts can be generated in rodent animal models¹³ but large-scale production is generally accomplished in neonatal livestock, especially bovine calves and goat kids.¹⁴ Several methods have been described for purification, concentration and surface sterilization of oocysts.¹⁵ Oocysts generated in animals have

limited durability, with infectivity markedly diminished within 6-8 weeks for in vitro and in vivo studies. Moreover, in vitro models enable the amplification of parasitic material for further immunological, biochemical and molecular studies.^{15, 16} Parasite development in cell culture can be assessed microscopically with a variety of histological stains or immunofluorescence¹⁷ and by enzyme immunoassay¹⁸ or PCR assays.¹⁹

The study aims to evaluate and validate in vitro models of *C. parvum* and to establish an in vitro culture system for the parasite for further research studies.

MATERIALS AND METHODS

All plastic consumables (e.g. flasks, pipettes and tissue culture plates) were purchased from Vacsera Vaccination Centers, Cairo, Egypt.

HT-29: Human Colorectal Adenocarcinoma cell line was obtained from Cell Culture Lab, Faculty of Pharmacy, Al-Azhar University, Cairo. Dulbecco's modified Eagle medium (DMEM), heat-inactivated fetal bovine serum, penicillin-streptomycin, glutamine, nonessential amino acids, phosphate-buffered saline (PBS), trypsin and all other chemicals were obtained from Vacsera Vaccination Centers.

HT-29: Human Colorectal Adenocarcinoma cell lines were grown in DMEM medium supplemented with 10% heat-inactivated fetal calf serum, 4 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% non-essential amino acids in a 5% CO₂ and incubated at 37°C. For experiments, trypsinized cells were seeded into 24-well plastic tissue culture plates with 13mm diameter glass coverslips and grow to confluence over 5 days. All experiments were done in triplicate.²⁰

C. parvum oocysts were obtained from Theodor Bilharz Research Institute (TBRI) Cairo, Egypt. Before use, oocysts will surface sterilized by suspension in 10% commercial bleach solution (0.55% sodium hypochlorite) for 10 minutes and subsequently washed 3 times in DMEM before enumeration in a Neubauer hemocytometer.²¹

Confluent cell monolayers were infected with 4X10⁵ viable *C. parvum* oocysts suspended in 200 µl cell culture medium. Cells were then incubated for 2 hours at 37°C to allow excystation and host cell invasion to occur. Following this period, unattached parasites and oocyst debris were removed by washing twice with the medium.

Wells were subsequently filled with fresh medium and incubated at 37°C for the stated time.²¹

Cell monolayers that grown on 13 mm glass coverslips were washed twice with PBS to remove any traces of medium and fixed in methanol for 1 min, stained with 10% Giemsa (diluted in PBS, pH 7.2) for 2 h, washed in de-ionized water and then mounted on glass slides. Using a Zeiss Axioplan microscope at X1000 magnification with oil-immersion the parasites were counted in 20 fields

across the diameter of the coverslip starting from the periphery.²¹ The obtained data were expressed as means ± SD. Significance analysis was carried out using a two-tailed Student's t-test for unpaired means.

RESULTS

In vitro development of *c. parvum* in intestinal epithelial cells showed that; confluent monolayers of HT-29 to which *C. parvum* oocysts were added, fixed and stained with Giemsa at different periods (6, 24, 48 and 72 hours) after incubation at 37°C. The tested cell line (HT-29) was found to support *C. parvum* growth in vitro. (Figure 1)

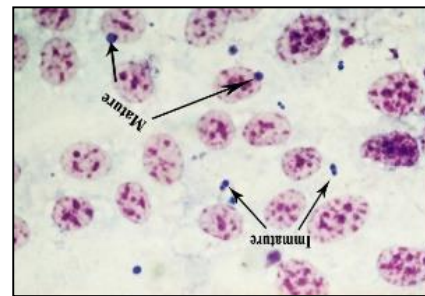


Fig. 1: *C. parvum* development in HT-29 cells. The monolayers were grown on glass coverslips in 24-wells plate, infected with 4x10⁵ oocysts and stained with Giemsa. After 24 h of infection, both immature and mature asexual stages can be identified clearly by microscopy. (X 1000).

The maximal number of intracellular parasites was obtained at 6-24 hs (127 ± 11 and 159 ± 13 parasite/20 fields respectively), but the number of parasites decreased at 48h (91 ± 10 parasite/20 fields) and 72h (39 ± 5 parasite/20 fields) of infection. (Figure 2)

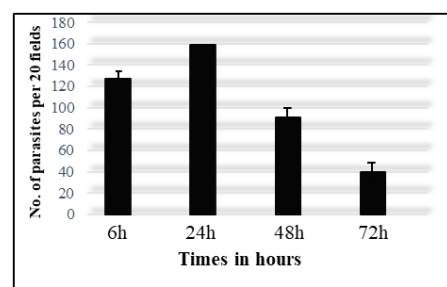


Fig. 2: Pattern of *C. parvum* infection in HT-29 cells. The maximal number of parasites was obtained 6-24 h post-infection with no significant difference in infection observed between 6 and 24h incubation periods ($p > 0.05$). Fewer parasites were obtained at 48h ($p < 0.02$) and 72h ($p < 0.008$) compared with 24h.

Identification of *C. parvum* asexual stages in infected cell monolayers. Study noted that after 6h only immature stages were seen but at 24 h immature and many mature asexual stages could be viewed and by 48 h the number of both immature and asexual mature stages was decreased. (Figure 3 a, b and c)

Infection of cell monolayers by different concentrations of oocysts. The maximal number of parasites was obtained by using 4×10^5 oocysts with no significant difference in infection observed between 4×10^5 and 1×10^5 oocysts ($p > 0.05$). Fewer parasites were obtained when used 2×10^4 compared with 1×10^5 ($p < 0.02$) and 4×10^5 ($p < 0.008$) compared with 24h. (Figure 4)

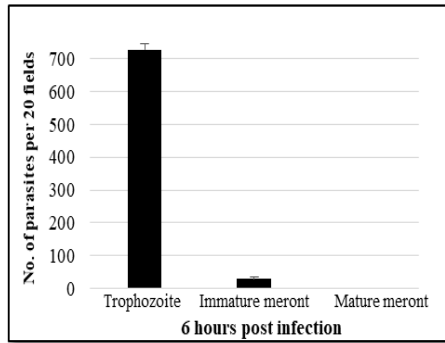


Fig. 3a

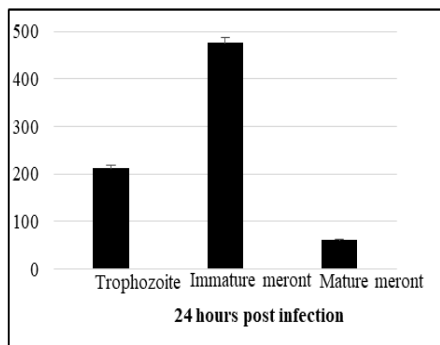


Fig. 3b

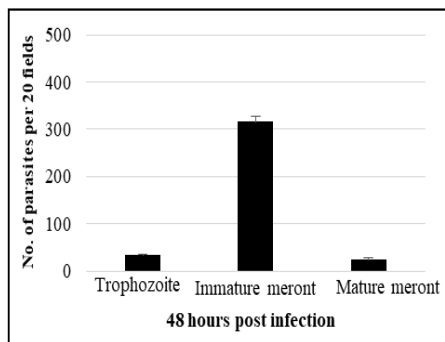


Fig. 3c

Fig. (3 a-c): Identification of immature and mature asexual stages in the cell line. Microscopic examination of Giemsa stained HT-29 monolayer 6h post-infection reveals many immature stages, but no mature parasite could be detected (Fig. 3a). Many immature and mature asexual stages of C-parvum were observed in infected cells 24 hours post-infection (Fig. 3b). After 48 hours of infection, there was significant reduction in the number of immature and asexual mature stages of the parasite (Fig. 3c).

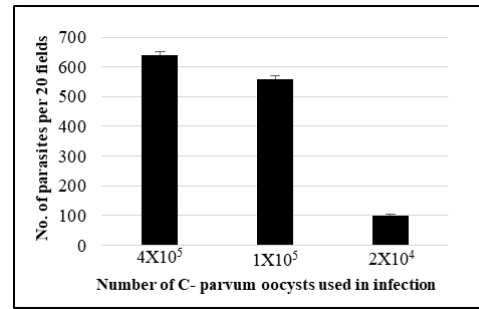


Fig. 4: C. parvum infection in HT-29 cells using variant numbers of viable oocysts. Infection of cell monolayer by using 4×10^5 oocysts produced the highest numbers of parasites with no significant difference in infection observed between 4×10^5 and 1×10^5 oocysts ($p > 0.05$). Fewer parasites were obtained when used 2×10^4 compared with 1×10^5 ($p < 0.02$) and 4×10^5 ($p < 0.008$).

DISCUSSION

A key goal of this study was to confirm the development of C. parvum in the intestinal epithelial cell line. The present study was shown that C. parvum can invade and develop within HT-29. However, only asexual development was predominantly observed with gametes being observed only occasionally. Complete development of C. parvum in vitro has been previously described 1, 5 and parasite growth was maintained for up to 25 days by multiple sub-culturing. It appears that adjustment of pH (7.2 -7.6) by the regular change of culturing media at 2-3 days intervals and adding HEPES buffer may be the key to successful long-term in vitro cultivation of C. parvum.¹⁵ Also, thin-walled oocysts are required to maintain parasite growth by releasing sporozoites that infect new cells (autoinfection) and it was reported that thin-walled oocysts occurred in vitro after the passage of parasite for 12 days.¹⁵ Lack of thick-walled oocysts may reflect the absence of certain enzymes and hormones that are present in vivo or a lack of effective host immune response that may induce the parasite to produce this type of oocysts.^{15, 22} For my thesis research, this long term cultivation was not essential.

In the present study, the maximal number of parasites was obtained at 6-24h and development had decreased by 48-72h of infection. Only immature stages were showed after 6h of infection, but many immature and asexual mature stages were observed in the cells 24 and 48h post-infection as shown in fig. (3a- c). C. parvum developmental stages were easily identified by Giemsa staining in HT-29 cells. Interestingly, infection of cell monolayers by different concentrations of C. parvum oocyst reveals that the maximal number of parasites was obtained by using 4×10^5 oocysts with no significant difference in infection observed between 4×10^5 and 1×10^5 oocysts. Taken together, these results recommend the use of HT-29 cells to support the study of C. parvum development in vitro.

Having demonstrated that *C. parvum* grows in the HT-29 cell line, further investigation of parasite interactions with host cells was possible using these in vitro models of infections.

CONCLUSION

It was concluded that the HT-29 cell line supports the in vitro cultivation of *C. parvum* protozoan parasite.

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