RESEARCH NOTE

IN VITRO CULTURE OF *TOXOPLASMA GONDII*

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The protozoan parasite *Toxoplasma gondii* is usually maintained in the laboratory through intraperitoneal inoculation of tachyzoites into mice. Many attempts have been made to maintain *T. gondii* tachyzoites *in vitro,* for this can provide valuable information regarding the biology of the parasite (Azab and Beverley, 1974; Scholtyseck and Melhorn, 1970). In this study we examined the suitability of the Raji and NS1 suspension cell lines and Vero, Hela, MK2 and McCoy monolayer cell lines for the *in vitro* culture of the parasite. Parasite yield and viability, percentages of host cell contamination and infected cells were compared.

Confluent monolayers of Hela, MK2, McCoy and Vero cell lines were resuspended in 3 ml of Dulbecco's medium and then digested with about 0.5-1.0 ml 0.25% trypsin in Versene (Gibco) for 5-10 minutes. The contents of the flask were then poured into test-tubes and centrifuged at 3000 rpm for 5-10 minutes. The cell pellet was washed twice with RPMI 1640 (Gibco) containing 100 $\mu g\ ml^{-1}$ of penicillin and 100 $\mu g\ ml^{-1}$ streptomycin (RPMI 1640-SP) and resuspended as a 5% cell suspension with RPMI 1640-SP and 10% fetal calf serum, which was seronegative by IFA of ELISA for *Toxoplasma* antibodies. The cells were cultured at 37°C in an atmosphere of 5% carbon dioxide in six-well plates (Costar, USA) in which coverslips were previously placed.

T. gondii (RH strain) tachyzoites were obtained from Balb/c mice inoculated intraperitoneally with the parasite 4 days previously. Parasites were harvested under sterile conditions by flushing the peritoneal cavity with sterile normal saline. The parasites obtained were washed thrice with RPMI 1640-SP and a suspension of 500 tachyzoites ml^{-1} was made for *in vitro* culture.

When the monolayers of Hela, MK2, McCoy and Vero cells reached 60% confluency, 500 tachyzoites in 1 ml of medium were inoculated into each cell line. At various days during culture, supernatant samples from duplicate wells were taken from the four infected cell lines and the number of tachyzoites was determined using a hemocytometer. The remaining supernatant samples were stored in vials at -20°C until further use.

At various days post-infection, coverslips were removed and the cells were stained with 10% Giemsa in Phosphate buffer at pH 7.2 for 15 minutes and the percentage of infected cells were examined. Duplicate cultures were performed to assess repeatability.

Raji and NS1 cells were grown as suspension cultures in 25 ml flask (Costar). 40,000 tachyzoites were added to 20,000 viable NS1 or Raji cells. At daily intervals, culture supernatant samples from duplicate flasks were taken to assess parasite growth.

The trypan blue exclusion test was performed each time for all six cell lines to assess the viability of the parasite and the host cell. Of the six cell lines employed, Hela and Vero showed the highest rate of parasite increase, all cells grown on coverslip being infected with *Toxoplasma* tachyzoites by day 7 and 12 respectively. The parasite count showed a steady increase in Vero cell cultures and by day 12 post-inoculation had reached 8.38×10^6 tachyzoites ml^{-1} of culture supernatant. There was also a rapid increase in parasite counts in Hela cultures which by day 7 had reached 7.79×10^6 tachyzoites ml^{-1} of culture supernatant.

The supernatants from Hela and Vero cell cultures inoculated a day previously with *T. gondii* tachyzoites did not contain any parasites, probably because they had penetrated the host cells and were undergoing intracellular divisions. Rosette shaped aggregates of parasites, resulting from synchronous multiplication in the cytoplasm of infected cells, were seen as early as 48 hours post-inoculation of the cell cultures. Monolayer cell lines multiplied rapidly and reached 100% confluency by day 4 of culture. Generally, the infected cells degenerated completely four to five days after inoculation with *T. gondii* tachyzoites. Infected Hela and Vero cell lines also produced the lowest percentage of host cell contaminants, these being 7.63% and 3.95% by day 7 and 12 respectively. The other two monolayer cell lines (MK2 and McCoy) contained between 80-100% host cell contaminants during harvesting at any day post-inoculation (Table 1).

MK2 and McCoy monolayer cell lines were not totally infected and produced low yields of tachyzoites at day 12 and day 8 post-inoculation with the highest yield of 2.25×10^4 and 1.91×10^5 tachyzoites ml^{-1} respectively.

Tachyzoites in monolayer cell lines generally showed 100% viability even at day 10 post-inoculation, while the parasites harvested from NS1 and Raji suspension cell lines showed 100% viability of tachyzoites only up to day 4 and day 6 respectively.

The percentage viability of host cells in all six cell lines decreased with age of culture.

Similar patterns of increase in parasite count, percentage of infected cells and percentage of host cell contaminants with culture time were obtained in duplicate experiments showing that the results were repeatable.

Since the speed with which the host cells are destroyed depends on the inoculum size (Kaufman *et al*, 1959) the inoculum size was fixed at 500 tachyzoites ml^{-1} in the present experiment to extend the susceptibility study for a longer duration. Kaufman and Maloney (1962) have observed that the division time of the RH strain parasite was 4.85 hours. The first endodyogenous form of *Toxoplasma* was observed by day 2 in both Hela and Vero cell lines. The results concur with that of Hughes *et al* (1986) who observed the endodyogenous form of *Toxoplasma* in AGMPK cell line after 30 hours post-inoculation.

The lower percentage of host cell contamination during parasite harvest coupled with the fact that Vero host cells multiply at a slower rate make this cell line a more suitable candidate for the long term culture of *Toxoplasma* tachyzoites compared to the Hela cell line. Both MK2 and McCoy cell lines are unsuitable for long term culture of *Toxoplasma* because of the low parasite yield and the very high percentage of host cell contaminants during parasite harvest.

The indirect enzyme-linked immunosorbent assay (ELISA) of Voller *et al* (1976) was used to assess the suitability of excretory-secretory (ES) antigens in cell culture supernatants for use in diagnosis. The wells of the microELISA plates

Table 1

Mean parasite count/ml culture supernatant of Hela, MK2, McCoy, Vero cells (each cell line inoculated with 500 *T. gondii* tachyzoites); NS1 and Raji cells (20,000 cells inoculated with 40,000 tachyzoites).

Cell line	X*	Parasite count/ml Mean ± SD × 10^3	% of host cell contaminants
HeLa	7	7787.50 ± 841.27	7.63
MK2	12	22.50 ± 2.52	97.76
McCoy	10	71.00 ± 10.64	93.34
Vero	10	8375.00 ± 1905.00	3.95
NS1	4	18.00 ± 4.32	60.00
Raji	6	392.00 ± 31.96	51.56

X*- The highest yield of 100% viable parasites harvested from day X.

(Dynatech) were coated with 200 µl of undiluted culture supernatant samples obtained from different days of culture of these cell lines. The plates were incubated overnight at 4°C.

Sera from mice previously immunized with *Toxoplasma* soluble antigens were diluted 1:200 and added into each well. Negative control sera were obtained from uninfected Balb/c mice. The conjugate used was goat anti-mouse IgG (American Qualax), at a concentration of 1:35,000 and the substrate used was orthophenylenediamine. The optical density (OD) values were read using a ELISA reader (Dynatech Inc) at 492 nm.

Relatively high ELISA OD readings (0.328 by day 10 in Vero and 0.223 by day 7 in Hela) were obtained with *T. gondii* ES antigens in the supernatants of Vero and Hela cell cultures. Control sera gave OD values of 0.011 and 0.013 respectively. The culture supernatant from infected Vero cell line gave the highest ELISA values, indicating the production of the largest quantity of *T. gondii* ES antigens secreted into the culture supernatant. These results agree with the findings of Chumpitazi *et al* (1987) who isolated and characterized *Toxoplasma* exo-antigens from *in vitro* culture in MRC5 and Vero cells and showed that exo-antigens produced on Vero cells seemed to be more antigenic than those produced on MRC5 cells.

Further studies (radiolabeling, immunolabeling with monoclonal antibodies) will be necessary to establish the exact type and origin of these exo-antigens.

Both the NS1 and Raji suspension cell lines produced low yields of parasite but with high percentages of host cell contamination, making them unsuitable for long term culture of the parasite.

The Vero cell line appears to be the ideal cell line for the culture of *Toxoplasma gondii in vitro.* Although the Vero cell line produce a high yield of viable *Toxoplasma* tachyzoites, Hughes *et al* (1986) preferred to use AGMPK primary cell line for long term culture of *Toxoplasma.* The disadvantage of such a culture system is that, being a primary cell line, the AGMPK cells have a relatively short life; thus there is a constant need to use cryopreserved stocks.

In conclusion, the higher yields of viable tachyzoites and ES antigens in culture supernatant with low percentage of host cell contaminants make the Vero cell line a suitable candidate not only for the long term maintenance of *Toxoplasma* culture but also for the production of soluble ES antigens.

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