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Research Note

Optimization of *Toxoplasma gondii* cultivation in VERO cell line

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Abstract. *In vitro* culture of *Toxoplasma gondii* can provide tachyzoites which are active, viable and with desirable purity. Thus the aim of this study was to optimize the cell culture method for *T. gondii* propagation to obtain a consistent source of parasites with maximum yield and viability, but minimum host cell contamination for use in production of excretory-scretory antigen. Tachyzoites with seed counts of 1×10^6, 1×10^7 and 1×10^8 harvested from infected mice were added to VERO cells of different degrees of confluence, namely 50%, 85% and 100%, and examined periodically using an inverted microscope. When the maximum release of the tachyzoites was observed from the host cells, the culture supernatant was removed and the tachyzoites harvested. Using a Neubauer chamber, the percentages of viable tachyzoites and host cell contamination were determined using trypan blue stain. Parameters that gave the best yield and purity of viable tachyzoites were found to be as follows: VERO cells at 85% confluence in DMEM medium and inoculum comprising 1×10^7 tachyzoites. After about 3 days post infection, the tachyzoites multiplied 78×, with a yield of ~7.8×10^8 per flask, 99% viability and 3% host cell contamination. This study has successfully optimized the method of propagation of *T. gondii* tachyzoites in VERO cells which produce parasites with high yield, purity and viability.

Toxoplasma gondii is an obligate intracellular protozoan parasite. The RH strain can be easily grown *in vivo* and *in vitro*, and a culture system which can reliably provide tachyzoites of consistent high quality would greatly benefit studies on the live organism (Chatterton *et al.*, 2002).

The Sabin-Feldman dye test is the gold standard among serological tests for detection of *T. gondii* infection, it is also the most difficult test to maintain because of its requirement for viable tachyzoites, and this has been made possible by *in vitro* culture of *T. gondii* (Evans *et al.*, 1999). Production of tachyzoites is also important for studies on experimental models (Roos *et al.*, 1994; Chatterton *et al.*, 2002), therapeutics (Bunetel *et al.*, 1995), genetic (Sibley & Boothroyd, 1992), biochemical pathways (Fichera *et al.*, 1995), isolation (Derouin *et al.*, 1987; Calico *et al.*, 1991) and for development of serological tests (Ashburn *et al.*, 2000).

Toxoplasma gondii RH strain has the most active intracellular multiplication rate among all *Toxoplasma* strains. Antigen production via passage in mice is considered as an expensive method. The inoculated mice survive infection for only 4–6 days and the tachyzoites are often contaminated with peritoneal cells. Propagation of tachyzoites in cell culture is more economical (Diab & El-Bahy, 2008). The tachyzoites replicate intracellularly with a generation time of 6–9 hours *in vitro*. After increasing in number to 64–128 parasites per cell, the host cell

ruptures, and they infect the neighbouring cells (Radke & White, 1998). *In vitro* culture systems for *T. gondii* are advantageous for studies that require a system which is free of attack by immune effector mechanisms (Chatterton *et al.*, 2002). With the requirement for an inexpensive source of parasites for diagnostic use and the interest in secreted antigen for both diagnostic and research use, there is an obvious need for culture conditions to be standardized (Hughes *et al.*, 1986).

A variety of cell lines and culture methods have been used for *in vitro* culture of *T. gondii* (De Meerschman *et al.*, 2002). In the present study African green monkey kidney cells (VERO) were used to optimize the cell culture method (Costa-Silva *et al.*, 2008) for *T. gondii* propagation in order to harvest a consistent source of good quality parasites, with minimal contamination from host cells, for use in production of *Toxoplasma* excretory-secretory antigen.

Two growth media namely DMEM (containing 4.5 g/l D-glucose, 584 mg/l L-glutamine and 3.7 g/l sodium bicarbonate, Gibco BRL, USA) and RPMI-1640 (containing 2.0 g/L D-glucose, 0.3 g/l L-glutamine, 2.0 g/l sodium bicarbonate, 1.0 mM sodium pyruvate, Gibco BRL) were used. Both media were supplemented with 10% fetal bovine serum (Gibco BRL) and 1% penicillin/ streptomycin (Gibco BRL), at pH 7.2.

Six weeks old female healthy Swiss albino mice were infected with virulent RH strain of *T. gondii*. Tachyzoites at seed count of 1×10^3 were suspended in 0.2 ml RPMI-1640 medium, and intraperitoneally injected into the mice. The mice were sacrificed after 3-4 days by ether inhalation. The parasites were then harvested from the peritoneal cavity by lavage with 5 ml RPMI-1640 medium.

The VERO cell line used as host cell was maintained with either DMEM or RPMI-1640, in 75-cm^2 cell culture flasks (Nunclon, Roskilde, Denmark) and was incubated at 37°C in a 5% CO_2 atmosphere. Before the host cells were seeded with tachyzoites, they were divided into three groups (each in triplicates); one group was grown to 100% confluence while two other groups were grown to 85% and 50% confluences, respectively. It took each about 6 hours for 50% host cell confluence to reach 85% confluence, and for the latter to reach 100% confluence. When the desired monolayer of host cells confluence was obtained, the old medium was removed and fresh medium was added. Different seed counts of tachyzoites i.e. 1×10^6, 1×10^7 and 1×10^8, were then inoculated into the each group of flasks.

They were examined periodically using an inverted microscope. When the maximum release of tachyzoites was observed (2-6 days), the culture supernatant was removed and the tachyzoites were harvested. The percentages of tachyzoites viability were then examined microscopically with trypan blue staining whereas the yield of fresh tachyzoites as well as the host cells contamination were determined using a Neubauer chamber. The experiment was repeated three times. The VERO cells were routinely subcultured every 3-5 days by trypsinisation of confluent monolayers. Statistical analysis using multivariate general linear model was used to perform the various comparisons of the results; p value of less than 0.05 was considered as statistically significant.

As shown in Table 1, using RPMI-1640 as the growth medium, the multiplication of harvested tachyzoites was highest (62×) using a parasite inoculum of $1x10^7$ and 85% confluent VERO cells. In comparison, seeding with ten times more tachyzoites (1×10^8), resulted in ten times less multiplication of the tachyzoites. Furthermore, by seeding at 1×10^7 tachyzoites, the host cell contamination was 1% lower (or purity was 1% higher) and the viability of tachyzoites harvested was 1% higher than obtained with the higher inoculum. Meanwhile the lowest multiplication of harvested tachyzoites was obtained using 50% confluent VERO cell and 1×10^8 tachyzoite inoculum, despite achieving 100% tachyzoite viability. Using an initial 100% confluent VERO cells clearly resulted in lowered percentage of viable tachyzoites and increased percentage of dead host cells compared to using other host cell confluences.

A similar pattern was observed when DMEM was used as the culture medium

Table 1. Viability, yield and degree of host cell contamination of *in vitro grown T. gondii* using flasks with various confluences of VERO cells in RPMI-1640 medium

No.	VERO cell confluence	Tachyzoite			VERO cell contamination	Multiplication of tachyzoites from the initial inoculum
		seed count	harvesting count	viability		
1	50%	1×10^6	5.0×10^7	100 %	3 %	50 ×
2	50%	1×10^7	2.7×10^8	100 %	2 %	27 ×
3	50%	1×10^8	2.2×10^8	100 %	1 %	2.2 ×
4	85%	1×10^6	4.7×10^7	95 %	5 %	47 ×
5	85%	1×10^7	6.2×10^8	99 %	3 %	62 ×
6	85%	1×10^8	6.4×10^8	98 %	4 %	6.4 ×
7	100%	1×10^6	1.0×10^7	92 %	5 %	10 ×
8	100%	1×10^7	3.7×10^8	89 %	10 %	37 ×
9	100%	1×10^8	4.2×10^8	90 %	7 %	4.2 ×

Table 2. Viability, yield and degree of host cell contamination of *in vitro grown T. gondii* with flasks with various confluences of VERO cells in DMEM medium

No.	VERO cell confluence	Tachyzoite			VERO cell contamination	Multiplication of tachyzoites from the initial inoculum
		seed count	harvesting count	viability		
1	50%	1×10^6	5.4×10^7	100 %	6%	54 ×
2	50%	1×10^7	2.0×10^8	100 %	4%	20 ×
3	50%	1×10^8	1.8×10^8	100 %	1%	1.8 ×
4	85%	1×10^6	6.0×10^7	97 %	2%	60 ×
5	85%	1×10^7	7.8×10^8	99 %	3%	78 ×
6	85%	1×10^8	8.0×10^8	99 %	5%	8 ×
7	100%	1×10^6	2.3×10^7	75%	7%	23 ×
8	100%	1×10^7	4.0×10^8	70 %	7%	40 ×
9	100%	1×10^8	5.4×10^8	80 %	7%	5.4 ×

whereby 1×10^7 tachyzoites seeded onto 85% confluent host cell monolayer produced the highest parasite multiplication (78×), at harvesting count of 7.8×10^8 per flask, 99% viability and 3% host cell contamination ($p < 0.05$). When the inoculum on 85% confluent cells was increased to 1×10^8 tachyzoites, the yield based on the harvesting count was the highest (8.0×10^8 per flask), however this only constituted an 8× multiplication of the tachyzoites, and the host cell contamination was undesirably increased to 5% (Table 2). Similar to the results with RPMI-1640, an initial 50% and 100% confluent VERO cells produced the highest and the lowest tachyzoite viabilities respectively; however both could not achieve the multiplication rates produced with 85% confluent VERO cells.

Comparison of the optimised conditions using RPMI-1640 and DMEM medium showed that the latter is more suitable since

both produced 99% viable tachyzoites and 3% host cell contamination but the latter yielded 1.3× higher yield of viable tachyzoites ($p < 0.05$).

The *in vitro* production of tachyzoites is essential for many kinds of toxoplasma research, such as production of excretory-secretory antigen which is useful to improve the diagnosis of toxoplasmosis, either for *in vitro* diagnosis or when an accurate indication of cellular immunity is required (Hughes *et al.*, 1986). Tachyzoites derived from cell culture can also be used routinely in the dye test (Evans *et al.*, 1999; Ashburn *et al.*, 2000; Mavin *et al.*, 2004) as well as in the development of in-house enzyme immunoassays (Joss *et al.*, 1989; Ashburn *et al.*, 1992).

It was estimated that the total cost of the *in vitro* culture system for *T. gondii* is one third to one half of the cost of the *in vivo* method (Ashburn *et al.*, 2000). In addition to avoiding ethical issues, the use of *in vitro* culture system avoids the risk of human infection during animal inoculation. Furthermore, the *in vitro* culture system for *T. gondii* tachyzoites production is flexible, vigorous and provides a means whereby tachyzoites of standard quality can be produced regularly or stored for use in various experiments and/or for application in diagnostic tests.

The *T. gondii* has been reported to be able to grow with varying success in many continuous cell lines, which included Hep2, MDBK, VERO, BHK, RK13, RDA, CER, MRC-5, AGMPK, HeLa, LLC, but, there was no consensus on the best cell line to use (Hughes *et al.*, 1986; Evans *et al.*, 1999; Chatterton *et al.*, 2002; Diab & El-Bahy, 2008). In a preliminary experiment, we observed that the growth in VERO cell line produced tachyzoites multiplication rate similar or greater than what has been reported, thus this cell line was used in this study (data not shown).

The choice of cell line to use is also dependent on the intended use of the tachyzoites, and also whether the tachyzoites needed to be kept for a long time. In a study in which the investigators aimed at prolonging the survival of the parasites for several weeks at 4°C, and use them for animal infection, the best cell line was found to be Hep2 (Diab & El-Bahy, 2008). In another study where regular predictable and consistent supplies of fresh tachyzoites of greater 1×10^6 per ml and >90% viable were needed for the Sabin-Feldman dye test, HeLa cells was found to fulfil this requirement (Evans *et al.*, 1999; Chatterton *et al.*, 2002). In the present study, the tachyzoites were intended for subsequent use to produce excretory-secretory antigen for proteomic studies, thus emphasis was placed on obtaining maximum yield of tachyzoites, with $\geq$99% viability and minimum host cell contamination.

Other than the type of cell line, there are other factors that have been studied as to its effect on the quantity and quality of the parasites, such as lowering temperature at maximal infection and serum supplementation (Chatterton *et al.*, 2002; Diab & El-Bahy, 2008). To the best of our knowledge, previous reported studies did not compare the effects of different levels of initial host cell confluence, or RPMI versus DMEM on the *in vitro* tachyzoite production.

In this study, *T. gondii* tachyzoites were introduced into VERO cells with several degrees of confluence, maintained with either RPMI-1640 or DMEM, and then inoculated/seeded with different number of tachyzoites. Using both medium, monolayer cell confluence of 85% produced greater number of parasite multiplication and less host cell contamination as compared to 100% cell confluence since it probably allowed room for the host cells to multiply concurrently with the propagation of the tachyzoites, thus minimizing the accumulation of 'older' host cells. As mentioned earlier, it took about 6 hours for a flask with 85% host cell confluence to reach 100% confluence, thus the results were most probably not due to a difference in 'age' of the cells. Host cell confluence of 50% in DMEM inoculated with 1×10^6 cells produced more than 50× tachyzoites multiplication but generated high host cell contamination.

This study showed the importance of optimizing the size of seed count (inoculum) since highest number of tachyzoites (1×10^8) produced lowest parasite multiplication when seeded into flasks with all three host cell confluences. On the other hand low seed counts, did not necessarily lead to minimum host cell contamination, which would be an important condition to achieve in the production of *Toxoplasma* excretory-secretory antigen. The yield and viability of *T. gondii* tachyzoite collected was found to reach an optimum level when 85% confluent of VERO cell monolayer maintained in either RPMI-1640 or DMEM was inoculated with 1×10^7 tachyzoites.

The results of this study also demonstrated that better yield of viable tachyzoite was harvested when the host cells were maintained in DMEM, compared to RPMI medium. It has been demonstrated that the components present in the growth medium are likely to influence the cell proliferation and cell viability of many cell lines (Wu *et al*., 2009). RPMI-1640, at first designed for use with lymphocytes, is a medium which is less nutrient-rich and simple in ingredients whereas DMEM which is modified from Eagle's basal medium is rich in nutrition. Moreover, DMEM contains a higher concentration of glucose (4.5 g/l) than RPMI-1640 (2.0 g/l). Since glucose is a central source of energy for cells, perhaps this composition in the DMEM medium is a factor that allowed the propagation of tachyzoites in the host cells more efficiently and hence produce higher yield of tachyzoites.

In conclusion, this study has successfully optimized the method of propagation of *T. gondii* in VERO cells which produced high yield and viability of tachyzoites, with minimal host cell contamination.

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