

# LONG TERM MAINTENANCE OF *TOXOPLASMA GONDII* (RH STRAIN) IN VERO CELL LINE AND USE OF HARVESTED ANTIGENS FOR IMMUNODIAGNOSIS

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**Abstract.** Thirty *in vitro* serial passages of *Toxoplasma gondii* cultures in Vero cell line performed once in every five days had a mean increase in parasite count of  $74.4 \pm 14.8$  times from that of initial counts. Long term cultures in Vero cell line did not alter the virulence of the parasite. The good correlation ( $r = 0.99$ ) between the IFA titer and ELISA OD values using the parasite antigens from *in vitro* sources indicates that long term maintenance of *T. gondii* in culture does not affect significantly the ability to recognize antibodies to surface and soluble antigens. The results also show that soluble antigens containing host cells can be directly used for immunodiagnostic purposes without purification. The *in vitro* maintenance of *T. gondii* is safer and cheaper when compared to the *in vivo* method.

The parasite antigens harvested from such an *in vitro* source are a good substitute for those of the *in vivo* source for immunodiagnostic purposes.

## INTRODUCTION

*Toxoplasma gondii* (Nicolle and Manceaux) is one of the most widespread protozoan parasites, infecting humans and domestic as well as wild animals. As many as 500 million people worldwide show serological evidence of *Toxoplasma* infection (Kean, 1972).

The yearly cost for care of children and adults with the sequelae of congenital toxoplasmosis is in the hundreds of millions of dollars (Wilson and Remington, 1980). Thus the economic implications of *T. gondii* infection as well as its increased importance due to the increasing numbers of people with acquired immune deficiency disease, makes it pertinent to have a better understanding of the immunology and biology of this parasite.

A major problem encountered by most workers is the production of sufficient quantities of pure *T. gondii* tachyzoites without host cell contamination for serological and other studies. The objectives of the present study were to maintain *T. gondii* tachyzoites *in vitro* using Vero cell line and to explore the use of the antigens derived from such a culture system for

immunodiagnostic purposes.

## MATERIALS AND METHODS

*Toxoplasma* tachyzoites were harvested under sterile conditions from Balb/c mice infected intraperitoneally with *T. gondii* (RH strain) 4 days earlier. They were washed thrice with RPMI 1640 containing 100 µg/ml penicillin (P) and 100 µg/ml of streptomycin (S), then adjusted to a concentration of  $10^6$  tachyzoites per ml of medium. One ml of the suspension was inoculated into each of three 25 mm<sup>3</sup> flasks containing Vero cells grown in RPMI 1640+P+S+10% fetal calf serum (FCS). The flasks were maintained at 37° C in a CO<sub>2</sub> chamber.

At every fifth day, the culture fluid from each culture flask containing tachyzoites released from infected Vero cells was collected and centrifuged at 3000 rpm for 10 minutes. About  $10^6$  viable tachyzoites (viability determined by trypan blue dye test to be greater than 99%) in 1 ml of culture supernatant were further inoculated into each of 3 culture flasks containing Vero cells. Similar passages were made every fifth day over a period of five months.

At every fifth passage, 1 ml of culture medium containing  $10^6$  tachyzoites was also inoculated into each of 5 mice to assess the viability and virulence of the parasite. The remaining tachyzoites were separated from the culture fluid, pooled and stored at  $-20^\circ\text{C}$  until further use. The culture fluid was likewise stored at  $-20^\circ\text{C}$ .

The pooled tachyzoites separated from the culture fluids were washed several times with 0.85% normal saline, with centrifugation being carried out at 3000 rpm for 10 minutes. The final suspension of tachyzoites in 0.5 ml of normal saline was sonicated and stored at  $-20^\circ\text{C}$ .

Uninfected Vero cells grown in control flasks were also harvested every fifth passage, pooled, spun, sonicated and stored at  $-20^\circ\text{C}$ .

The protein concentrations of soluble *T. gondii* antigens from *in vivo* and *in vitro* sources, soluble uninfected Vero cell antigens (UV) and uninfected mouse peritoneal exudate cells (UP) were determined using the Bio-Rad assay kit and found to be 0.370, 0.190, 0.075 and 0.050 mg/ml respectively.

At every fifth passage, fresh tachyzoites from the culture were also used for preparing antigen slides for the indirect fluorescent antibody (IFA) test. The enzyme-linked immunosorbent assay (ELISA) and the IFA test for 30 normal patient sera, and 12 and 16 patient serum samples with known IFA titers of 1:16 and 1:64, respectively (Table 2), were performed according to the method of Voller *et al* (1976) and the modified method of Chessum (1970) respectively, using antigens obtained from the *in vivo* and *in vitro* infections described above.

The wells of micro ELISA plates (Immulon II, Dynatech(R)) were coated with 200  $\mu\text{l}$  of soluble antigens (UP + parasite) and UP diluted in coating buffer in alternating column of the plates. 200  $\mu\text{l}$  of inactivated FCS diluted in PBS Tween 20 (1:400) were added to all wells to block non-specific binding. The optimal dilution of serum and IgG goat anti-human peroxidase as determined by checker-board titration was 1:400 and 1:10,000, respectively.

The test was repeated using soluble antigens (UV + parasite) and UV from the *in vitro* culture system.

## RESULTS

Table 1 shows the partial growth profile of *T. gondii* in Vero cell line through 30 serial passages performed once every five days. Except for an initial 43.6-fold increase in parasite count at the first passage, all other increases were between 53.8 and 107.8 fold, the mean increase being  $74.4 \pm 14.8$  fold. (Only viable parasites were counted).

The mean time of death for batches of five mice inoculated with  $10^6$  *T. gondii* tachyzoites harvested from the *in vivo* and *in vitro* cultures was  $4.5 \pm 0.5$  days. Batches of five mice died either on Day 4 or Day 5 post-inoculation.

The geometric mean IFA titers of human serum samples corresponded closely with the ELISA Optical Density (OD) values (Table 2), for the parasite antigens harvested from both *in vivo* and *in vitro* systems. An increase in the IFA titer was reflected by an increase in OD value;  $r = 0.99$  using parasite antigens harvested from *in vivo* and  $r = 0.71$  using antigens from the *in vitro* system.

The effect of host cell contaminants on the ELISA for detecting anti-*Toxoplasma* antibodies in human sera is presented in Table 3. The correlation between the ELISA OD values using soluble *Toxoplasma* + host cell antigens and soluble *Toxoplasma* antigens alone from *in vivo* and *in vitro* sources were 0.97 and 0.99, respectively. For the *in vitro* source, the ELISA OD values were 40 to 63% lower when only *Toxoplasma* antigens were present, compared to when host cell antigens were also present. Likewise, for the *in vivo* source, the OD values were 27–46% lower when only *Toxoplasma* antigens were present. Similar differences were also observed between the *in vitro* and *in vivo* sources of antigens, due to the protein concentration in the *in vivo* sources being much higher.

## DISCUSSION

An *in vitro* culture system of *T. gondii* can be used for studies to provide valuable information on parasite growth, replication and fine structure (Azab and Beverley, 1974; Scholtyseck and Mehlhorn, 1970).

Table 1

Partial growth profile of *Toxoplasma gondii* in Vero cell line through 30 serial passages performed once every 5 days.

Passage No.	Mean tachyzoite inoculum/ml ( $\times 10^6$ )	Mean tachyzoite yield/ml ( $\times 10^6$ )	Increase (multiple of initial count)
1	1.1 $\pm$ 0.1	48.0 $\pm$ 16.6	43.6
5	0.9 $\pm$ 0.1	100.0 $\pm$ 35.6	107.5
10	1.2 $\pm$ 0.2	120.0 $\pm$ 24.5	100.0
15	1.1 $\pm$ 0.3	90.0 $\pm$ 16.3	81.8
20	1.1 $\pm$ 0.3	83.3 $\pm$ 17.0	73.7
25	2.2 $\pm$ 0.2	143.3 $\pm$ 24.9	66.1
30	1.7 $\pm$ 0.3	106.7 $\pm$ 12.5	61.7

\* Only viable parasites were counted.

Table 2

Use of *Toxoplasma gondii* antigens from parasites maintained *in vivo* and *in vitro* to detect antibodies in human sera by the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA).

No. of human serum samples (IFA titer)	* GM IFA titer		ELISA OD value	
	Antigen source		Antigen source	
	<i>in vivo</i>	<i>in vitro</i>	<i>in vivo</i>	<i>in vitro</i>
30 (NEG)	neg	neg	0.39+0.18 (0.15-0.75)	0.19+0.06 (0.11-0.36)
12 (1:16)	15.99	14.21+1.73 (8.17-24.73)	0.89+0.24 (0.44-1.20)	0.38+0.15 (0.24-0.72)
16 (1:64)	63.97	41.48+1.61 (25.62-66.69)	1.64+0.67 (0.83-2.40)	0.63+0.17 (0.42-0.96)

\* GM = Geometric Mean

In the present study *T. gondii* was successfully cultured in Vero cell line through 30 serial passages over a period of five months. The Vero cell line was chosen because in previous studies it was shown to be a very susceptible cell line giving a high parasite yield with low amounts of host cell contamination.

The size of the inoculum used was  $10^6$  tachyzoites per ml of medium as this was the optimal

infective dose established by other workers (Srinivasa *et al*, 1982; Hughes *et al*, 1984). The study by Srinivasa *et al* (1982) showed that the mean parasite increase after 54 serial passages over six months in Vero cell line was 117 fold compared to this study with 74.4 fold after 30 serial passages (Table 1). In their study the maintenance medium was changed within 24 hours of inoculation of cultures whereas in the present study parasite harvest was made once

Table 3  
Effect of host cell contaminants in enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies in human sera.

No. of human serum samples IFA titer	ELISA OD value $\pm$ SD			
	Parasite <i>in vivo</i>		Parasite <i>in vitro</i>	
	X	Y	X	Y
30 (NEG)	0.30 $\pm$ 0.18	0.17 $\pm$ 0.10	0.19 $\pm$ 0.16	0.08 $\pm$ 0.05
12 (1:16)	0.89 $\pm$ 0.23	0.48 $\pm$ 0.19	0.38 $\pm$ 0.15	0.14 $\pm$ 0.10
16 (1:64)	1.64 $\pm$ 0.64	1.19 $\pm$ 0.52	0.63 $\pm$ 0.17	0.38 $\pm$ 0.08

X = Soluble *Toxoplasma* antigens + host-cell antigens

Y = Soluble *Toxoplasma* antigens only

every five days without any media change. This could have resulted in a lower mean parasite increase. However, if the cultures were extended as in their study, similar results may be obtained.

The present study indicates that long term maintenance of *T. gondii* in Vero cell cultures does not cause significant change in the virulence of the parasite. Hughes *et al* (1986) showed that there were no significant changes in the mean survival time (3–4 days) when Balb/c mice were inoculated intraperitoneally with  $10^6$  parasites at various time intervals throughout the period of continuous culture in the African green monkey primary kidney (AGMPK) cells compared with parasites that had been serially passaged solely in mice. The present findings are also similar to those of Srinivasa *et al* (1982).

The present results (Table 2) suggest that parasite antigens from *in vitro* cultures can also be effectively used for diagnostic purposes. In the ELISA procedure, the protein concentration of the antigen harvested from the *in vitro* system was almost half that harvested from the *in vivo* system. This resulted in the ELISA OD value in *in vitro* antigens to be almost half that of the *in vivo* antigens.

The good correlation ( $r = 0.99$ ) between the IFA titer and ELISA OD value using the parasite antigens from *in vitro* sources indicates that long term maintenance of *T. gondii* in culture does not affect significantly its ability to recognize antibodies to surface and soluble antigens.

A major problem encountered by most workers is the production of pure tachyzoites without host cell contamination for serological and biochemical studies. Dempster (1983) compared eight established methods of purifications and showed that such purification processes could alter and affect the antigenicity of the parasite.

In the present study, the ELISA OD values with soluble *Toxoplasma* antigens alone were 27–63% lower than those with *Toxoplasma* antigens + host cell antigens. Further purification of parasites from host-cell contaminants could possibly decrease the reading further. This could be due to the partial loss of reactive antigens from parasites itself or from those of parasite origin attached to host cells. A further reason may be that a large portion of the reaction is non-specific and directed to host-cells.

Although the present results show that soluble antigens containing host cells can be directly

used for diagnostic screening purposes without purification, further studies should be carried out to determine the interpretation of these results. The *in vitro* cultured parasites and their soluble products have also been used by other workers for the preparation of soluble antigens for use in ELISA (van Loon, 1983) and lymphocyte transformation tests (Hughes *et al*, 1984).

The *in vitro* culture of *Toxoplasma* offers advantages over the *in vivo* system for the production of *T. gondii* parasites. The risk of human infection during inoculation is comparatively minimized when the *in vitro* system is used. Secreted parasite antigens can be used to improve the diagnosis of toxoplasmosis when an accurate indication of cellular immunity is required (Hughes *et al*, 1984). Parasite antigens harvested from *in vitro* culture would also be free of mouse proteins but still contain other cell contaminants which would interfere with the serological tests. The cost of maintaining tachyzoites in the *in vitro* system is however very much cheaper compared to maintaining tachyzoites in mice. Last but not least, the *in vitro* system takes into account the ethical considerations of maintaining parasites in animals in a situation where an *in vitro* system is available.

The study has shown that *T. gondii* can be maintained in Vero cell line for long durations without losing its virulence. *T. gondii* antigens harvested from such an *in vitro* culture system can be good substitutes for those from *in vivo* sources for diagnostic purposes.

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