International Journal of Biological, Life and Agricultural Sciences
ISSN: 2415-6612
Vol:5, No:4, 2011

Plaque Formation of *Toxoplasma gondii* in Vero Cells using Carboxymethylcellulose

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Abstract*—Toxoplasma gondii* is an intracellular parasite capable of infecting all nucleated cells in a diverse array of species. Toxoplasma plaque assay have been described using Bacto Agar. Because of its experimental advantages carboxymethyl cellulose overlay, medium viscosity was choosing and the aim of this work was to develop alternative method for formation of *T. gondii* plaques. Tachyzoites were inoculated onto monolayers of Vero cells and cultured at 37° C under 5 % CO_2. The cultures were followed up by microscopy inspection. Small plaques were visible by naphtol blue stain 4 days after infection. Larger plaques could be observed by day 10 of culture. The carboxymethyl cellulose is a cheap reagent and the methodology is easier, faster than assays under agar overlay. This is the first description of the carboxymethyl cellulose overlay use for obtaining the formation of *T. gondii* plaques and may be useful in consequent obtaining tachyzoites for detailed studies.

***Keywords*—**Carboxymethyl cellulose, Cell culture, Plaque assay, *Toxoplasma gondii*.

I. Introduction

TOXOPLASMA gondii (*T. gondii*) is an intracellular eukaryotic parasite capable of infecting all nucleated cells in a diverse array of species. Although most infected individuals show no sign of clinical disease, primary infection during pregnancy can lead to severe congenital disease in humans and other animals [1]. In addition to the importance as a clinical and veterinary pathogen, *T. gondii* provides an attractive experimental system for studying intracellular parasitism, due to its genetic accessibility and ease of culture [2].

Development of molecular genetic tools for *T. gondii* has advanced rapidly in the last decade and transformation of extracellular tachyzoites is a routine procedure now. Both endogenous and heterologous proteins can be expressed in transgenic parasites by transient or stable transfection [3].

Clonal technique is necessary for selection of stable transformants. Different approaches have been described "in vitro" to develop clonal cultures of Toxoplasma parasite as limited dilution method and plaque purification using Bacto Agar [4, 5].

Plaque assays in cell culture monolayers under solid or semisolid overlay media represent a common method for quantification of viruses. In these assays, each virus multiplies under conditions that result in a localized area of infected cells known as plaque. These make use of viscous overlays to cover cells immediately after infection, thus limiting infection spread and restricting growth to foci of cells at the sites of initial infection [6].

One of the methodologies described is to solidify the culture medium with an agar gel. As an alternative to solid gels, for their practical convenient, viscous solutions of soluble hydrophilic polymers, methylcellulose (MC), carboxymethyl cellulose (CMC), can be employed. If strong cytopathogenic effects (CPE) are induced cells in plaques are lysed and plaques can be visualized by staining of the residual intact cells [7, 8].

In order to explorer an alternative simple and handy this report describes, for the first time, a plaque formation of *T. gondii* in Vero cells using CMC overlay, medium viscosity, that may be used for analytical and preparative purposes.

II. Methods

The virulent RH strain of *T. gondii* was maintained by intraperitoneal passages in 6-8-week old, specific pathogen-free male OF1 mice from the National Centre for Laboratory Animals Production. The animals used in the study were cared for in accordance with institutional guidelines.

Tachyzoites were harvested from mice on the third day of

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infection by lavage of the peritoneal cavity with 5 ml of RPMI 1640 medium (Sigma Chemical Co.), containing a mixture of 100 U/ml penicillin and 100 µg/ml streptomycin. Peritoneal exudate was centrifuged at 650 x g during 10 min at 4° C in phosphate buffer saline (PBS) 0.1M pH 7.2, in order to eliminate host cells. The suspension was disrupted by three forced passages through a 27 gauge needle to release the intracellular parasites. Then, it was centrifuged at 160 x g 10 min 4° C and three washes were done with PBS. Viability of the parasites was evaluated by trypan blue exclusion test and parasites counted in Neubauer chamber.

Vero cells (DTCC-CCL 41) were grown in Minimum Essential Medium (MEM) nonessential amino acids supplemented with 10 % foetal bovine serum (FBS) (Biochrom AG), 100 U/ml penicillin, 100 µg/ml streptomycin and 1 % of L-glutamine 200 mM and were cultivated at 37° C in a 5 % CO_2 atmosphere in 24-well plates at approximately 1. 5 x 10^5 cells/ml until the monolayer was confluent.

The cells were infected with *T. gondii* parasites using the medium described above with the concentration of FBS reduced to 1 % (infection medium). At this serum concentration, confluent monolayer cultures maintained their normal morphology. The assays were made in duplicate.

Serially dilutions of tachyzoites (3 x 10^7 to 3 x 10 parasites/ml) were done. Parasite inoculum in 100 µL infection medium was added to each well. The infection was allowed for 16 h at 37° C as described previously [4]. The medium of infected culture was removed and the cells were washed twice with infection medium.

The CMC, medium viscosity powder (Sigma-Aldrich) was hydrated in water at 3 % (w/v) and stirred at room temperature until homogenous. CMC solution was sterilized by autoclaving and stoked at 4° C until use. CMC overlay (1,5 % final concentration) were prepared by mixing 1:1 CMC solution with an equal volume of infection medium, 1 ml of the mixture was added to each well. It was referred as experimental medium. Plaque assays were kept undisturbed at 37° C and 5 % CO2 for 1 to 10 days.

The cultures were examinated by inspection under an inverted microscope to determine the cytopatic effects on the monolayer. Overlay was removed daily and the cells were washed and stained 30 min with 1 % naftol blue solution. The experimental medium without parasite addition was used as experimental control. Plaques diameters were measured with aid of ruler using background white.

III. Results and Discussion

Different overlay techniques, including CMC, have been reported for plaque assays for virus [7, 8]. Previously were demonstrated [5, 9] that *T. gondii* parasite both grows and makes plaque in tissue culture using agar overlay. In the present work the CMC, medium viscosity, use was choose because it is easy to prepare and to store at 4° C until use; likewise, it is easier to manipulate than Bacto Agar medium. The latter requires being freshly prepared, melting at 56° C and cooling to 41° C before addition.

The results showed their suitability on *T. gondii*. As Fig. 1 shown, four days after infection with 3 x 10^4 to 3 x 10^2 parasite/ml, small rounded plaques (least than 1mm) were macroscopically visible by naphtol blue stain. The highest parasite concentrations destroyed the monolayer completely.

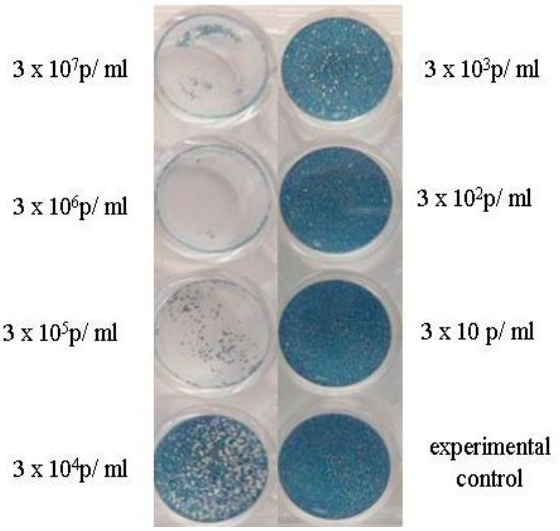

Fig. 1 Plaque assay for *T. gondii* using carboxymethyl cellulose. Tachyzoites was serially diluted on Vero cells (3x 10^7 until 3x 10 parasites/ml) and 16 h post infection CMC overlay was added. After 4 days cell were stained with 1 % naftol blue solution. The parasite concentration used for infection is shown.

Some reports describe the *T. gondii* growth efficiently in Vero cells support [3]. In this work, four days after infection Vero monolayer starting showed clearly cytopathogenic effects. Cells became detached from the surface, rounded and showed morphological signs of cell death (Fig. 2).

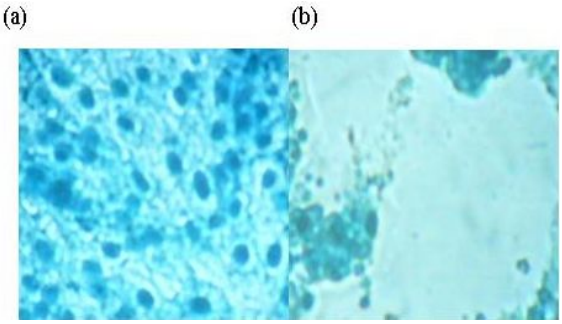

Fig. 2 Cytopathogenic effect of *T. gondii* on Vero cells. (a): experimental control. (b): Vero cells 4 days after infection with 3 x 10^3 parasites/ ml in MEM medium with CMC overlay. Photographs were taken at 40 fold magnification and ocular 10 x.

In order to test whether parasite plaque diameters could be related with incubation times, ten plaque dilution assays were done using CMC overlay. On days 1 and 2, no plaques were visible (Fig. 3a). From day 5 onward, around 1mm plaques

were visible in 3 x 10^3 and 3 x 10^2 parasite/ml (Fig. 3c). The larger plaques (2 mm approximately) were observed on 10 day at a concentration of 3 x 10^2 parasites/ ml (Fig. 3e).

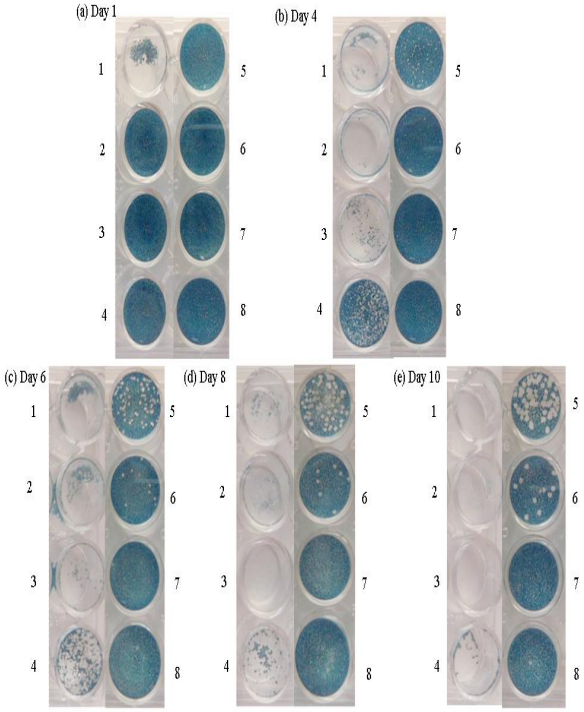

Fig. 3 Plaque assays with different incubation times. Plaque assays were performed with CMC overlay and incubated for 1 to 10 days. The parasite concentration used for infection is shown. The parasite concentration used for infection is shown as 1: 3 x 10^7p/ ml, 2: 3 x 10^6p/ ml, 3: 3 x 10^5p/ ml, 4: 3 x 10^4p/ ml, 5: 3 x 10^3p/ ml, 6: 3 x 10^2p/ ml, 7: 3 x 10 p/ ml and 8: experimental control.

To our knowledge this is the first report about the use of CMC overlay method to detect the formation of *T. gondii* plaque in Vero cells. This alternative is simple and handy and may be useful, for example, to generate cloned population of this protozoan.

Clone cultures would be the ideal tool to address general questions about pathogenicity of the parasites, and performance of genetic analysis. Different approaches are reported to develop clonal cultures of parasites ([10]- [13]).

In the case of *T. gondii*, cloned populations can be obtained using different methods. "In vivo" they include the single oocyst isolation technique [14], the isolation of a single tissue cyst from brain tissue [15] and "in vitro", the limited dilution method and plaque purification using Bacto Agar, mentioned previously [4, 5].

The use of CMC described in this report may be an advantaged alternative for the plaque formation of *T gondii*. It is a practical "in vitro" tool that could be useful to obtaining tachyzoites for others detailed studies.

IV. Conclusion

This is the first description of the carboxymethyl cellulose overlay use for obtaining the formation of *T. gondii* plaques, an alternative simple and this may be useful for analytical and preparative purposes.

Acknowledgment

We are grateful to Dianeya Mendoza for her technical support with the Vero cell and to Mr. Armando Martinez for his assistance in the preparation of the manuscript.

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