

Impact of Local *Toxoplasma gondii* Strain on Herpes simplex Virus Propagated in Tissue Culture

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Abstract: *Toxoplasma gondii* is an important zoonotic protozoan parasite of warm-blooded animals including humans causing serious health problem in pregnant women and female animals and immuno-compromised people. Cats, including all felines, are its definitive hosts and excrete environmentally-resistant oocysts in their feces. In the present study, isolation of local *T. gondii* strain was accomplished from tissues of sheep intended to slaughter at Cairo abattoir. Two methods for propagation of isolated *T. gondii* tachyzoites in the laboratory were adopted, mouse inoculation technique and tissue culturing in HeLa cells. Meanwhile, Herpes simplex type1 (HSV-1) was propagated in HeLa cells, identified by PCR and the co-infection of *T. gondii* tachyzoites and Herpes simplex virus type1 was also studied. Results showed that HeLa cells were found to be the most suitable for *Toxoplasma gondii* propagation in-vitro system with 76% viability of *T. gondii* tachyzoites. On the other hand, *T. gondii* co-infection decreased the titer of the virus from 10⁷ to 10⁴ compared with control cells contained HSV-1 alone.

Key words: *Toxoplasma gondii* • Propagation • Mice inoculation • Tissue culture • Herpes simplex Virus

INTRODUCTION

Toxoplasmosis is one of the most important worldwide zoonotic diseases, where oocysts survive and caused by an obligatory intracellular protozoan parasite; *Toxoplasma gondii* which can infect humans and almost all warm-blooded animals [1]. The parasite is responsible for major economic losses in human and most classes of livestock through abortions, still birth and neonatal losses. It causes mental retardation and loss of vision in children and abortion in pregnant women and livestock survive [2].

Cats are main reservoir of *T. gondii* because they are the only hosts that can excrete the resistant stage (oocyst) of the parasite in the feces. Humans become infected by eating undercooked meat from infected animals, food and water contaminated with oocysts [3]. Besides vertical infection during pregnancy, humans can get infected post-nataly either by

oral uptake of sporulated *Toxoplasma* oocysts or by ingestion of tissue cysts upon consumption of raw or undercooked meat of infected slaughtered animals [4].

Many studies investigate the *T. gondii* incidence in sheep from different regions in Egypt which were 47% and 50% for ELISA and IFAT respectively [5], 49.5% and 52% in slaughtered sheep in Tanta abattoir using IHAT and IFAT respectively [6], 55.9 and 54.1 % with IHAT and IFAT [7] and 37% by ELISA by mean of patent Ag-coated plates from specific kit [8] and recently, comparative serological examination revealed that, MAT showed the higher prevalence of toxoplasmosis (43.7%) followed by ELISA (41.7%), using antigen prepared from isolated *T. gondii* local strain [9].

In vitro culture systems for *Toxoplasma gondii* are fundamental to toxoplasma research which developed to provide fresh viable tachyzoites as and when required [10, 11].

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Herpes simplex virus type-1(HSV-1) which belongs to the *Herpesviridae* family has large double stranded linear DNA genomes. DNA genome encased within an icosahedral protein cage called the capsid, which is wrapped in a lipid bilayer called the envelope. The envelope is joined to the capsid by means of a tegument [12, 13]. Entry of HSV into the host cell involves interactions of the envelope covering the virus particle, when bound to specific receptors on the cell surface, will fuse with the host cell membrane and create an opening, or pore, through which the virus enters the host cell [14]. HSV-1 considers the least dangerous virus as it usually affect mouth and lips so called fever blister or cold sores [15]. It also may infect animals cause genital herpes [16].

The current study is aiming to propagate locally isolated *Toxoplasma gondii* tachyzoites in the laboratory using mice inoculation and also suitable tissue culture cells as a safe and controlled method for propagating large seed of *Toxoplasma*. Moreover, the dual infection of both *Toxoplasma* and herpes virus type-1 was tested to investigate phenomena of dual infection in vivo.

MATERIALS AND METHODS

The current work was performed in The National research center (NRC) in the period of May to August 2013.

Isolation of *T. gondii*:

Collection and Preparation of Meat and Tissue Samples: Meat and tissue samples were collected from slaughtered sheep at Cairo abattoir prepared from heart, diaphragm, liver and esophagus as described by Kotula, *et al.* [17], a pooled sample, about 50 -100 grams, from the tissues and organs of each examined sheep was obtained, cut into small cubes and stored at 4°C for few hours until used for bioassay in mice or cat.

Bioassay of Sheep Tissues in Cats: Bioassay of suspected sheep tissues in cats was carried out according to methods described by Davis and Dubey [18]. Fecal samples from each experimentally infected cat were examined parasitologically by concentration floatation technique. The formed sediment was mixed with 2.5 % potassium dichromate solution at room temperature for 5-7 days to allow aeration and after sporulation of the oocysts it was stored at 4°C.

Bioassay of Sheep Tissues in Mice: Bioassay of meat and tissues of slaughtered sheep in mice was carried out according to pepsin-digestion method described by Sharma and Dubey [19]. After 4-6 weeks, mice scarified and impression smear from brain (brain print) prepared, stained with Giemsa and examined to detect the *T. gondii* tissue cysts containing the bradyzoites.

Propagation and Maintenance of Isolated *T. gondii* Strain

Mice Inoculation: Tachyzoites of virulent RH strain of local *T. gondii* strain was propagated by intra peritoneal inoculation in Swiss albino mice maintained according to the procedures of Johnson *et al.* [20]. The peritoneal fluid was re-inoculated into 3 to 5 mice (0.2 ml / each) containing about 2×10^6 tachyzoites and further inoculation every 2 - 4 days depending up on the ambient temperature and the condition of the inoculated mice.

Tissue Culture Cells: HeLa cells (cervix carcinoma cells) obtained from Egyptian Vaccines and Sera Institute (VACSERA, Agoza, Cairo, Egypt). The cell line was grown in 75-cm² flasks (Corning, High Wycombe, UK) in 20 ml of Roswell Park Memorial Institute (RPMI) 1640 media (*Gibco, USA*). The medium contained 10% fetal calf serum (FCS), 100 ig/ml penicillin and 100ig/ml streptomycin. Cells were maintained in a 5% CO₂ humidified incubator at 37°C.

Culturing of *T. gondii* Tachyzoites: HeLa cell monolayers were infected at a 1:1 tachyzoite to cell ratio [10, 21, 22] 5×10^6 HeLa cells was inoculated in 75-cm² flasks with growth medium (RPMI-1640 medium supplemented with 10% FCS) and incubated at 37 °C with 5%CO₂ till forming confluent monolayer. The cells were changed to maintenance medium (RPMI-1640 medium supplemented with 2% FCS) and incubated at 37°C with 5% CO₂ for 16h before infecting with 5×10^6 viable *T. gondii* tachyzoites. The flasks were infected in maintenance medium and after 24 hrs. The medium was replaced with serum-free medium and maintained in this until the tachyzoites were harvested. Cultures were examined by phase contrast microscopy using an inverted microscope to determine the presence of tachyzoites. Cultures were harvested five days later. Tachyzoites were released from the monolayer (biofilm) by vigorous shaking of the tissue culture flasks. The tachyzoites and cells in the decanted supernatant were counted by means of hem-cytometer and viability determined by phase contrast microscopy.

Virological Assay:

Cells: Vero (African Green Monkey) and HeLa cells are supplied from Egyptian vaccines and Sera institute (VACSERA), Agoza, Cairo, Egypt. The cell lines were grown in 75-cm² flasks in 20 ml of culture medium. The media used for Vero cell was Minimum Essential Medium Eagle with Earle's Balanced Salt supplemented with 10% FCS, 100 iug/ml penicillin and 100iug/ml streptomycin while the medium for HeLa cell was propagated as mentioned above.

Virus Propagation: Herpes simplex virus was kindly supplied by VACSERA, Agoza, Cairo, Egypt. The virus was propagated on Vero cells. After several subcultures, the cell was replaced by HeLa cells. The adapted virus was subjected for purification and identification [23].

Virus Purification: It was prepared according to Wu *et al.* [24] using sucrose gradient ultracentrifugation and stored in 2ml Eppendorf tubes at -70°C till use.

Virus Titration: The HSV-1 was titrated as described by Montanha *et al.* [25] and expressed as 50% tissue culture infective Dose₅₀/ml (TCID₅₀/ ml).

Virus Identification by Nested PCR: The virus DNA was isolated and subjected to nested PCR by QIA amp Viral DNA Mini Kit (Qiagen®, Valencia, USA) and the following primers: first external amplification primers 5' ATC CGA ACG CAG CCC CGC TG 3' and 5' TCC GGS GGC AGC AGG GTG CT 3'; and the second internal primers 5' GCG CCG TCA GCG AGG ATA AC 3' and 5' AGC TGT ATA SGG CGA CGG TG 3', which amplify fragments 413bp of the D-glycoprotein gene, that is present in HSV [26]. The steps were performed according to manufacturer instruction.

Dual Infection by *T. gondii* tachyzoites and HSV-1: HeLa cells were plated in 6-well plates, with growth medium (RPMI-1640 medium supplemented with 10% FCS) and incubated at 37°C with 5%CO₂ until forming confluent monolayer. The confluent cells are washed with PBS and infected with 9×10⁶ viable *T. gondii* tachyzoites and HSV-1 of high titer (10⁷) as described by Cantatore *et al.* [23]. Control negative wells were inoculated with maintenance media and control positive wells where HSV-1 and *T. gondii* tachyzoites each alone were designed. Plates were incubated for 72 hours at 37°C and

5% CO₂ and monitored for cytopathic effect. After 7 days virus titers were determined and Tachyzoites were counted using hem-cytometer.

RESULTS AND DISCUSSION

Isolated *T. gondii* Infective Stages: In the current study, *T. gondii* tachyzoites were detected in the mice peritoneal exudates inside leucocytes (lymphocytes & macrophages) or free in the peritoneal exudates after rupture of leucocytes (Fig: 1 A & B). The tissue cyst stage identified in the impression smears of mice brain (Fig: 1C) and the liberated free bradyzoites obtained after digestion of the infected sheep tissues (Fig: 1D). *T. gondii* oocysts were detected from infected cat feces firstly as fresh unsporulated oocysts (Fig: 1E) and after 3 to 5 days sporulated oocysts were identified (Fig: 1F).

The morphological results of the isolated *T. gondii* infective stages were in accordance with that reported by Dubey and Beattie [27]; Dubey *et al.* [28] and Dubey [29]. Also the isolation and identification results are in agreement with that of Dubey and Frenkel [30], who found *T. gondii* tachyzoites in the impression smears obtained from intestinal lymph nodes on the 6th day post inoculation. However Dubey and Frenkel [31], recognize the *T. gondii* tissue cysts in brain print after (3-4) weeks post infection i.e. earlier than the finding obtained during this study. This could be attributed to the virulence of the strain studied by Dubey and Frenkel [31]. Michael *et al.* [32] and Prickett *et al.* [33] who detected tachyzoites and brain cysts in the impression smears obtained from peritoneal exudates and brain from mice sacrificed at 4 days and (6-8) weeks post-infection respectively. Also Dubey [29], detected *T. gondii* tachyzoites and cysts in the mesenteric lymph nodes, lung and brain after 3 days, 7 days and 2 months post infection respectively. The discrepancy between the results of the present study and those recorded by previous authors might be ascribed to the difference in the strain of the parasite, route of inoculation, age of inoculated mice and the development of its immune system.

***Toxoplasma gondii* Propagation on HeLa Cells:** HeLa cells were used for propagation of *T. gondii* tachyzoites as it was found to be the most suitable cell culture for *Toxoplasma* propagation in-vitro system [21, 22]. The infection rate was 1:1 and in a concentration of 5×10⁶ cell because it gave the best results (78.7%) during

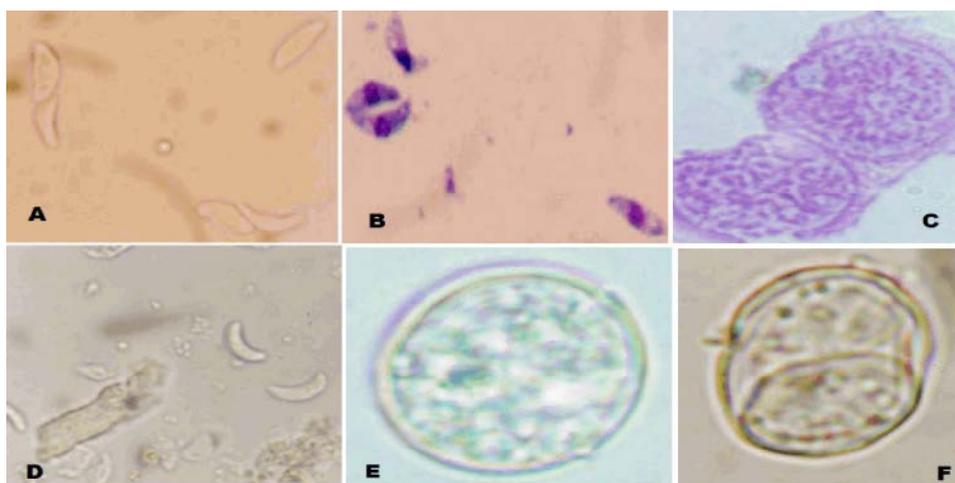


Fig. 1: *T. gondii* infective stages (1000X); unstained tachyzoites (A), Giemsa stained tachyzoites (B), Tissue cysts(C) free *T. gondii* bradyzoites (D), un-sporulated *T. gondii* oocyst (E) and sporulated oocyst (F).

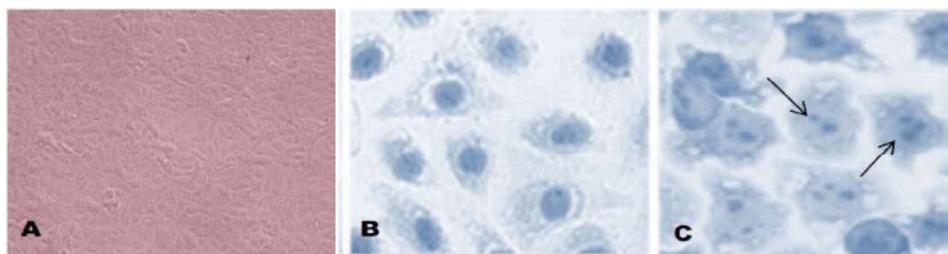


Fig. 2: Confluent HeLa cells appears on the left (A), the same cells stained with Trepan blue (B) and stained HeLa cells contain the tachyzoites of *Toxoplasma gondii* 3 days post infection (C).

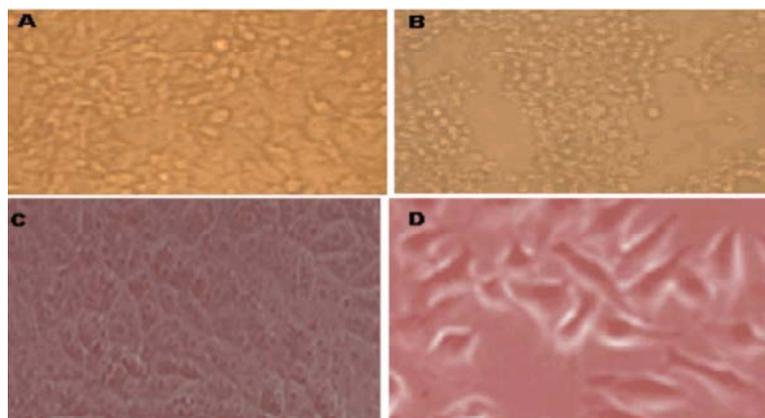


Fig. 3: Normal Vero cells (A), the same cell infected with HSV-1 72hrs PI (B), normal HeLa cells (C) and infected HeLa cells with herpes simplex virus 72hrs PI, where the cells undergone necrosis and pyknosis (D).

Table 1: The amount of obtained *T. gondii* tachyzoites in HeLa cells

Amount of HeLa cells in 75cm ² flask (×10 ⁶ /ml) ^a	<i>T. gondii</i> tachyzoites in 75cm ² flask (×10 ⁶ /ml) ^a	Ratio between HeLa cells & <i>T. gondii</i> tachyzoites	Amount of viable <i>T. gondii</i> tachyzoites 5 days PI (×10 ⁶ /ml) ^a	Dead tachyzoites 5 days PI (×10 ⁶ /ml) ^a	Viability rate	HeLa cells contamination (×10 ⁶ /ml) ^a
5	5	1:1	7.5	2.5	76%	2

a: 20 ml maintenance medium was used in 75-cm² flasks. The amount of HeLa cells and tachyzoites are average values obtained from triplicate flasks.

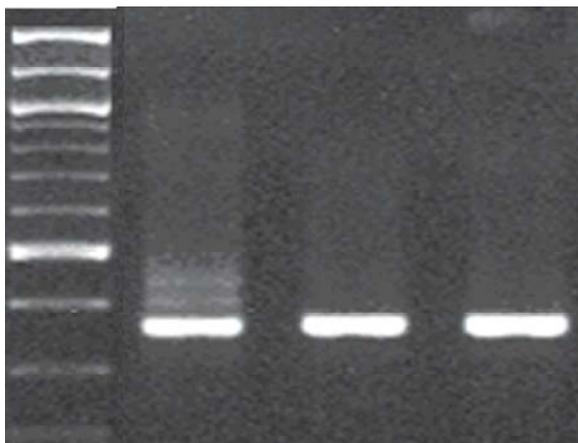


Fig. 4: Amplified HSV-1 after agarose gel electrophoresis, ethidium bromide staining and UV trans illumination. Lane 1, contain 100 bp marker while lane 2, 3 and 4 contain HSV-1 where an illuminating band at 280 bp.

optimization of HeLa cell culture for *In vitro* *T. gondii* production [22]. The result shows 76% viability of *T. gondii* tachyzoites (Table 1), this result comes in agreement with [22]. Microscopic examinations on the following days post infection with *Toxoplasma* tachyzoites revealed development of parasites in the giant cells which proceeded until complete cell degeneration (Fig: 2 A, B&C).

Propagation of HSV-1 in Tissue Culture: The virus showed multinucleated cell formation and cell necrosis in Vero cells. Meanwhile the virus showed vacuolation, necrosis and pyknosis in HeLa cells ((Fig. 3; A, B, C & D)

Diagnosis of Herpes Simplex Virus by PCR: Specific band at 280 pb amplify fragments of the D-glycoprotein gene was monitored, which matches the results of Del Prete *et al.* [26] (Fig. 4).

Dual Infection of HeLa Cells with *T. Gondii* and HSV-1: To investigate whether *T. gondii* and HSV-1 affect each other's replication in co-infected tissues ex-vivo, matched cells (HeLa cells) were inoculated with a combination of these two microbes and with each of them individually. *T. gondii* replication was mildly but significantly suppressed HSV-1 infection. *T. gondii* co-infected HeLa cells decreased the titer of the virus to 10^4 compared with control cells contained HSV-1 alone. This may be attributed to its genome which encodes a cyclophilin that may binds to cell receptor or it may be able to suppress

HSV-1 replication in HeLa cells. This result matches the result obtained by *T. gondii* which suppressed HIV as reported by Golding *et al.* [34].

CONCLUSION

This study proves the isolation of the local *T. gondii* strain infective stages from Egyptian sheep tissues and successful maintenance of the strain through mice inoculation. The study also investigated the propagation of *T. gondii* tachyzoites in HeLa cells which is the first record in Egypt and gave very promising results for controlled and safe way for production of tachyzoites. Moreover, it investigated the co-infection of *T. gondii* tachyzoites and HSV-1 which is also the first of its kind the suppression of HSV by *T. gondii* tachyzoites need further monitor to study this phenomena.

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