# In Vitro Cultivation of *Toxoplasma Gondii* in Various Cell Cultures

## *Toxoplasma Gondii*'nin Çeşitli Hücre Kültürlerindeki İn Vitro Üretimi

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**ABSTRACT Objective:** The purpose of this study was to evaluate various cell cultures such as African green monkey kidney cells (Vero), human cervix adenocarsinoma cells (HeLa) and primary fetal bovine kidney cells (FBK), which we cultured and subcultured in our laboratory for Toxoplasma growth rate and yield. **Material and Methods:** *Toxoplasma gondii* tachyzoites were obtained by washing peritoneal cavities of albino mice which were inoculated intraperitoneally three days prior to cell culture infection. The tachyzoites in the peritoneal exudates were purified, pooled and used to infect three types of cells: Vero, HeLa and primary fetal bovine kidney cells. Tachyzoite multiplication rate and yield of these three cells were evaluated microscopically both in the cell culture flasks and from the harvests. **Results:** All of the cell types gave reproducible results, with over $1x10^6$ tachyzoites in the harvests, more than 90% of which were viable. Tachyzoite yield from Vero cells was significantly higher than HeLa cells. Vero and FBK cells were infected with tachyzoites faster than HeLa cell culture and the harvest times were more stable. **Conclusion:** *Toxoplasma gondii* tachyzoite growth was higher in Vero cells than in HeLa cells and it is a continuous cell line, differing from primary FBK cells. Therefore, Vero cell line was preferable among three cell types as a tachyzoite source for consecutive diagnostic and investive use.

**Key Words:** Toxoplasma; cell culture techniques

**ÖZET Amaç:** Bu çalışmada, laboratuvarımızda primer kültür ve pasajlarını yaptığımız Afrika yeşil maymun böbrek hücreleri (Vero), insan serviks adenokarsinom hücreleri (HeLa) ve primer fetal dana böbrek hücreleri (FBK) gibi çeşitli hücre kültürlerini, *Toxoplasma gondii*'nin üreme hızı ve verimlilikleri açısından değerlendirmeyi amaçladık. **Gereç ve Yöntemler:** Toxoplasma gondii takizoitleri, hücre kültürlerine ekilmesinden üç gün önce intraperitoneal olarak inoküle edilen albino farelerden, periton boşluğunun yıkanması ile elde edilmiştir. Periton eksüdalarındaki takizoitler ayrıştırılmış, birleştirilmiş ve Vero, HeLa ve FBK hücre kültürlerine ekim için kullanılmıştır. Hücre kültürlerindeki takizoit çoğalma hızı ve miktarı, hem hücre kültür tabakalarının hem de toplanan besiyerlerinin mikroskopik olarak değerlendirilmesiyle belirlenmiştir. **Bulgular:** Tüm hücre tipleri, $1x10^6$'nın üzerinde ve %90'ından fazlası canlı olan takizoit üreme sonuçları vermiştir. Vero hücrelerinde HeLa hücrelerinden anlamlı oranda daha fazla takizoit çoğaltılmıştır. Vero ve FBK hücreleri takizoitlerle HeLa hücrelerinden daha hızlı enfekte olurken, çoğalma süreleri daha istikrarlı olmuştur. **Sonuç:** *T. gondii* takizoitleri, Vero hücrelerinde HeLa hücrelerinden daha fazla üremiştir ve Vero hücreleri, primer FBK hücrelerinden farklı olarak, devamlı bir hücre dizisidir. Bu nedenlerle, Vero hücre kültürü üç hücre kültürü arasında daha sonraki tanı ve araştırma çalışmaları için daha tercih edilir bulunmuştur.

**Anahtar Kelimeler:** Toksoplazma; hücre kültürü teknikleri

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*Toxoplasma gondii* is an intracellular protozoan parasite which is globally distributed.[1] Felidae is the certain host for *T. gondii* whereas rodents and birds are important intermediate hosts for the life cycle.

Toxoplasmosis is an infection of warm-blooded vertebrates including humans. Sheep and bovine infections cause detrimental effects for economy as well as being important for the human contamination by ingestion of raw or undercooked meat of these animals.[2] Although the infection is mostly asymptomatic in healthy adults, it can be of vital importance for non-immune pregnant women by inducing embryopathy and for immunocompromised persons by causing encephalitis and leading to death. Early diagnosis is significant for these cases in order to prevent serious consequences. [3]

The production of tachyzoites is essential for isolation of the parasite, all experimental models for genetic, biochemical pathway and drug studies as well as the development of serological tests.[4,5] Besides, viable tachyzoites are needed to perform Sabin-Feldman test which is still considered to be the "gold standard" among serological tests for the detection of toxoplasmosis.[6] Therefore, both in vitro and in vivo culture systems for *T. gondii* are fundamental to Toxoplasma diagnosis and treatment research.

We use mice for the maintenance of *T. gondii* tachyzoites by serial subcultures twice weekly in order to carry out Sabin-Feldman test in our laboratory. As cell culture method is more ethical than use of mouse inoculation, in a preliminary study, we aimed to investigate various cells such as African green monkey kidney cells (Vero), human cervix adenocarsinoma cells (HeLa) and primary fetal bovine kidney cells (FBK) in the view of their suitability to permit efficient growth of *T. gondii*.

## MATERIAL AND METHODS

Vero and HeLa cells were obtained from the Department of Microbiology. Cells were thawed: 25cm$^2$ flasks (Greiner-cellstar) were seeded with 1x10$^6$ Vero and HeLa cells and incubated in DMEM/Hepes supplemented with 10% fetal calf serum (Sigma) at 37°C with 5% $CO_2$ for 24 hours. Cells were routinely subcultured every 2-3 days by trypsinisation of confluent monolayers washed with the medium.

FBK cells were primarily cultured in our laboratory as follows: Five months old bovine fetus kidneys were taken out aseptically. The outside of the kidney was freed of all adhering tissue. The renal capsule was cut and peeled off. The kidneys were sliced in two halves to remove pelvis and calices as only the cortex was used to prepare the cell culture. The cortex was cut with a scalpel into pieces of 3-4 mm. These pieces were rinsed several times to remove as much blood as possible. Then prewarmed 0.25% trypsin solution was added. Trypsinisation was carried out with a magnetic stirrer at room temperature. Every 20 minutes, the trypsin solution was decanted and replaced by an equal amount of trypsin solution. The decanted fluid which contained kidney cells was collected, kept at 4°C and was centrifuged at 1300xg for five minutes to eliminate trypsin. After the cells were counted, they were seeded to a culture flask and incubated in the growth medium at 37°C with 5% $CO_2$.[7]

Swiss albino mice were inoculated intraperitoneally with *T. gondii* tachyzoites three days prior to the infection of cell cultures. Mice were sacrificed and peritoneal exudates were aspirated by washing the peritoneal cavity with 5 ml of Dulbecco's Modified Eagle's Medium Hepes Modification (DMEM/Hepes, Sigma) containing 100 IU/ml penicillin (Merck) and 100µg/ml streptomycin (Biological Industries). The exudate was centrifuged at 50xg for five minutes to eliminate host cells. Then supernatant was washed three times by centrifuging at 1300xg for five minutes with DMEM containing antibiotics. The amount and viability of tachyzoites in the pellet were determined by using a hemocytometer under phase contrast microscopy (x400 magnification) and the pellet was diluted to contain 1x 10$^6$ tachyzoites/ml.[8]

After the confluent monolayer for each cell culture type had formed, the medium was aspirated, confluent cells were washed with fresh medium and the cells were infected with 1x 10$^6$ tachyzoites in 1 ml of maintenance medium (DMEM/Hepes supplemented with 2% fetal calf serum) within two hours from the time tachyzoites were obtained from mice. Flasks were incubated for 30 min at 37°C with 5% $CO_2$. Then 4 ml of maintenance medium was added and the flasks were incubated at 37°C with 5% $CO_2$ .

Cultures were examined by phase contrast microscopy using an inverted microscope to observe the presence of *T. gondii* tachyzoites, their entrance to the cells and multiplication. The yield was determined after 48 hours. The flasks were tapped from the sides to release the tachyzoites. The number and the viability of tachyzoites in cell culture supernatants harvested from the flasks were determined with a haemocytometer under phase-contrast microscopy. The tachyzoites obtained from cell cultures were used to infect five consecutive subculture of each cell type following the above protocol.

The difference of three cells due to Toxoplasma yield and plague numbers were evaluated by Kruskal-Wallis test. When the difference was found statistically significant, Dunn multiple comparison test was used to know which cell differs from the other. SPSS for Windows 11.0 pocket program was used for statistical analysis.

## RESULTS

The viability rate of the tachyzoites obtained from mice was over 95% at the time of the infection of cell cultures. The release of tachyzoites in cell culture, especially in HeLa, was unpredictable and the optimum time of harvest could vary from 48 to 120 hours after infection of the monolayer.

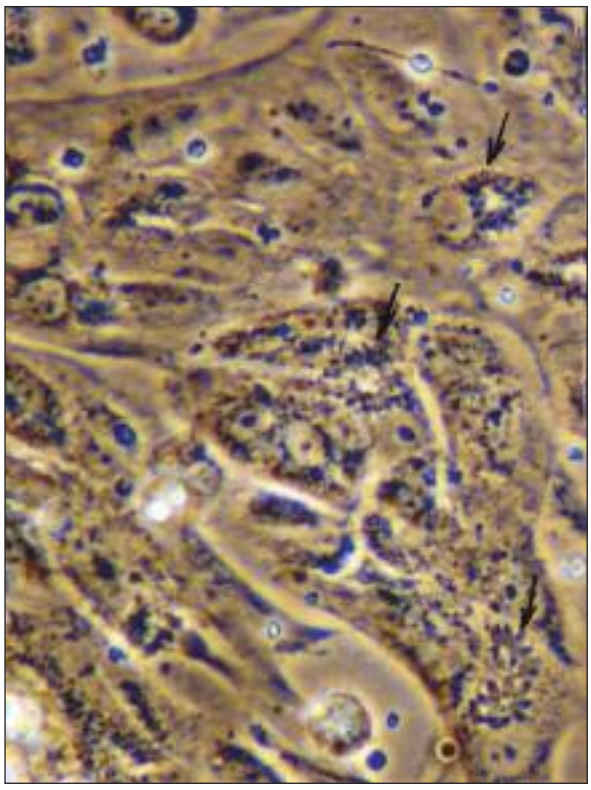

**FIGURE 1:** HeLa cells infected with T. gondii.

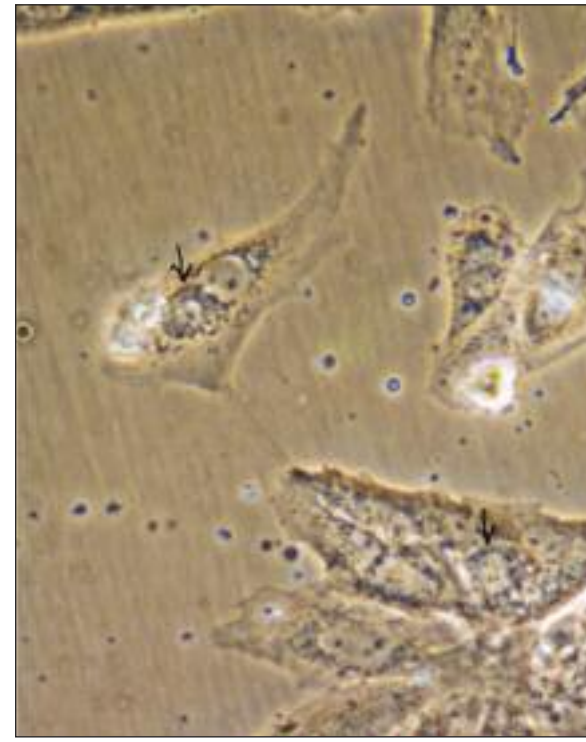

**FIGURE 2:** Vero cells infected with T. gondii.

Infected cells with one or two and rarely four tachyzoites were observed in Vero cell culture at 6 hours post-infection while mostly one or two tachyzoites were present in FBK and HeLa cells. Multiplication rate of tachyzoites in Vero and FBK cells seemed to be faster than HeLa cells, although the difference was not statistically significant (p=0.148). Both Vero and FBK cells were heavily infected with tachyzoites between 18-24 hours after *Toxoplasma* inoculation to the flasks and then tachyzoites started to be released (Figure 1, 2, 3). However, both the number of HeLa cells infected and the multiplication rate of tachyzoites within HeLa cells were slightly less than Vero and FBK cells at 24 hours post-infection. Monolayers of Vero and FBK cells began to be disrupted when an average of 5-6 tachyzoite plagues per field of 400x magnification were just observed in HeLa cell culture.

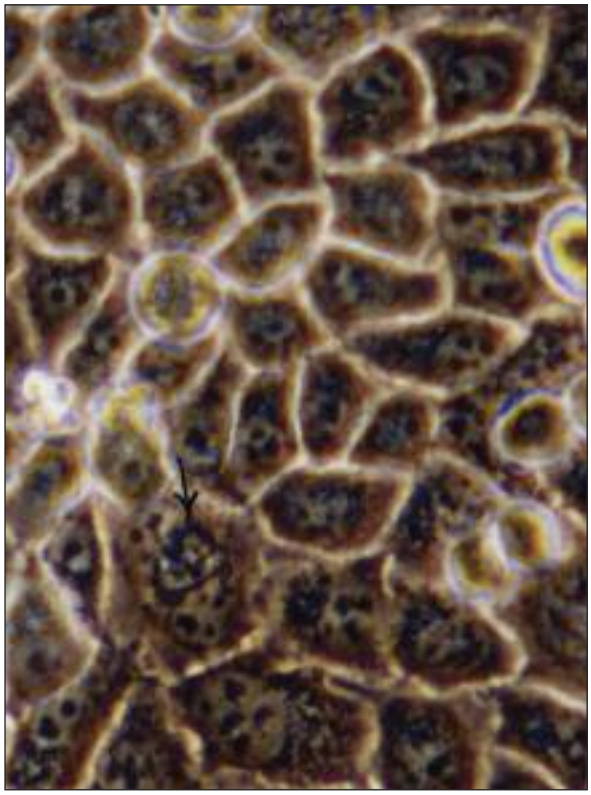

**FIGURE 3:** FBK cells infected with T. gondii.

The tachyzoite yield and viability in the harvests from each cell type were evaluated by phase contrast microscopy. While tachyzoites from Vero and FBK cells were harvested at 48-72 hours post-infection, HeLa cells were suitable for harvest later than these two cells except one subculture when the tachyzoites were harvested after 72 hours. All cell types gave tachyzoite yields more than $1x10^6$ tachyzoites of which more than 90% were viable and sufficient to be used as a source for Sabin-Feldman test. The average viable tachyzoite count at the time of the harvest from Vero, FBK and HeLa cells were $2.8x10^6$, $2.62x10^6$ and $2.35\ x10^6$, respectively. The difference between Vero and HeLa cells was found to be statistically significant ($p<0.05$). Host cell contamination of the harvests was more with Vero and FBK cells than with HeLa cells. In these cell lines, late harvest of tachyzoites affected the viability.

## DISCUSSION

Many investigators have been using various cell cultures to isolate and maintain *T. gondii* for both diagnostic and investigational purposes.[9,10] Although in vitro cultivation is reported to be more complicated and labor intensive than mouse inoculation, it is considered as more cost-effective and last but not the least animal protective. Our aim was to determine the most suitable and satisfactory cell line among Vero, HeLa and primary FBK cells in respect to tachyzoite yield, in order to use them for diagnostic purposes in Sabin-Feldman test in the future. In the light of our findings in this study, we plan to optimize the cell culture method for long time continuous maintenance using the most appropriate cell type as a tachyzoite source.

Evans et al. compared HeLa, LLC and Vero cells to investigate a suitable tachyzoite source for diagnostic use.[11] They found the optimal multiplicity of infection was reached at 1:1 tachyzoite to cell ratio. We also used this ratio during our study. They concluded that HeLa cells gave higher tachyzoite yield than the other two cells. However they also expressed that the cells had unpredictable growth cycles. Controversially, Doskaya et al. found that there was no statistically significant difference between the tachyzoite yields of Vero and HeLa cells. [10] We observed that the average tachyzoite yield of Vero cells was significantly higher than HeLa cells. We also found that HeLa cells permit a slightly slower multiplication rate of tachyzoites than Vero and primary FBK cells in some experiments although the difference between the average rates was not statistically significant. The number of subcultures or the condition of the cells used in these two different studies may have interfered with the results. On the other hand, our consecutive experiments with different HeLa cell lines gave similar results so we do not expect the discordance of the results in respect to HeLa cells would be a result of the above mentioned factors. The reason for HeLa cells to permit a slower growth of *T. gondii* tachyzoites in this study can be due to RH strain lineage of our stock. Mavin et al. reported that the genetic variability in different stocks of *T. gondii* RH strain can have an impact on the incorporation into the continuous culture system and Sabin-Feldman test.[12]

Hughes et al. reported successful continuous cell culture of *T. gondii* in primary African Green

Monkey Kidney cells.[13] In our study, primary FBK cells were also shown to be good hosts for *T. gondii*. However, as it is difficult to propagate this primary host cell line, it would not be suitable for continuous supply of tachyzoites especially for Sabin-Feldman test.

## CONCLUSION

The *T. gondii* tachyzoite growth in Vero and FBK cell cultures were comparable to and slightly faster than HeLa cell culture. The harvest time was longer for HeLa cells. In our experiments, Vero cell line was the best due to harvest yield of *T. gondii* tachyzoites among the cells tested in order to isolate and maintain *T. gondii* tachyzoites, as it is a continuous cell line and tachyzoite yield is satisfactory. However cell contamination in the harvests would make it difficult to interpret Sabin-Feldman test results. Success in long term continuous passages and Sabin-Feldman test performances of these cells should be the subject of the next study in the attempt to use cell culture as a tachyzoite source for Sabin-Feldman test.

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