

# IS SABIN-FELDMAN DYE TEST USING *T. GONDII* TACHYZOITES FROM ANIMAL INOCULATION STILL THE BEST METHOD FOR DETECTING *TOXOPLASMA GONDII* ANTIBODIES?

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**Abstract.** Although the Sabin-Feldman dye test is the gold standard for detecting *Toxoplasma* antibodies in human, it is performed only in reference laboratories because live virulent *T. gondii* are used for the test. We collected 210 human serum samples and tested them by the dye test using *in vivo* tachyzoites (conventional method) then compared these results with three other methods: a dye test using cell culture-derived *T. gondii* tachyzoites and indirect immunofluorescent antibody tests (IFAT) using *in vivo* and *in vitro* tachyzoites. We found the conventional dye test detected the highest percent of cases (4.3%), followed by the IFAT using parasites from mice (3.8%), then the dye test and the IFAT using cell culture tachyzoites (both 2.8%). Agreement with the dye test when using mouse and cell culture derived tachyzoites was 96.7%. Using *in vivo* tachyzoites for the dye test and the IFAT gave 94.3% agreement, while using *in vitro* tachyzoites gave 94.8% agreement. When compared with the conventional dye test, the IFAT had 75% sensitivity and 100% specificity. The *T. gondii* tachyzoites obtained from cell culture had a lower virulence, as indicated by a three times longer survival period in the inoculated mice. We favor the conventional dye test as the gold standard for *Toxoplasma* antibody detection. *In vitro* tachyzoites can be used routinely in the dye test but false negative results may occur in some cases. The IFAT, using either *in vivo* or *in vitro* tachyzoites, are alternatives for laboratories where provision of live tachyzoites is limited.

**Keywords:** *Toxoplasma gondii*, mouse and cell culture derived tachyzoites, dye test, IFAT

## INTRODUCTION

*Toxoplasma gondii* is a protozoan parasite which infects almost one-third of the

world's population usually causing mild, non-specific clinical features (Dubey and Beattie, 1988; Tenter *et al*, 2000). It is an important opportunistic pathogen in immunocompromised persons and pregnant women, causing severe clinical manifestations and congenital defects. Undercooked meat containing cysts of the parasite or oocysts contaminating water or food are the main sources of transmission.

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The detection of *Toxoplasma* antibodies is the mainstay diagnostic method for toxoplasmosis, especially in acquired infection and in pregnant women. Many serological tests have been used as screening tests, such as the indirect immunofluorescent antibody test (IFAT), latex agglutination (LA), indirect hemagglutination (IHA) and ELISA with high sensitivity and specificity. However, confirmation with the gold standard, Sabin-Feldman dye test, in questionable cases is recommended (Sukthana, 2006).

The dye test is still the gold standard for detecting *Toxoplasma* antibodies (Reiter-Owona *et al*, 1999). This method was first described in 1948 (Sabin and Feldman, 1948). It gives the most reliable results with a high sensitivity and specificity but is performed only in some reference laboratories, since it needs live, virulent *Toxoplasma* tachyzoites derived from inoculated mice.

The IFAT is another widely used method, but similar to the dye test, requires intact tachyzoites to carry it out. Therefore, it is more sensitive and specific than IHA, LA and ELISA because during infection the first significant increase in immunoglobulin is against cuticular antigens (Piergili, 2004). Unlike the dye test, the IFAT does not require the live tachyzoites for the test.

*T. gondii* maintained in mouse peritoneal fluid is reliable for use in the dye test (Obwaller *et al*, 1995). In our laboratory, we have continuously inoculated *T. gondii* RH strain into mice for more than 15 years and have used it as a source of tachyzoites for the dye test. However, it is laborious and time-consuming. It requires an experienced technician and from an ethical viewpoint it may be undesirable. Culture of *Toxoplasma* tachyzoites *in vitro* has been successfully performed and is recom-

mended for routine use in the dye test (Ashburn *et al*, 2000; Chatterton *et al*, 2002). We studied the agreement between the results of the dye test, and different sources of *in vivo* and *in vitro* tachyzoites. The IFAT using mouse and cell culture derived tachyzoites was also compared with the conventional dye test to see if it could be used as an alternative method, where the dye test is not available or is impracticable.

## MATERIALS AND METHODS

### Sample collection

At the Bangkok Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Thailand we carried out the Sabin-Feldman dye test using live tachyzoites derive from mice as a routine service for detecting *T. gondii* antibodies. Between January 2007 and February 2008, 210 blood samples were sent to our laboratory for *T. gondii* antibody testing. The male: female ratio was 1:10.4, the average age was 31.5 years (19 - 57 years) and all the samples were from immunocompetent hosts. After informed consent was obtained, all the samples were kept at -20°C until the dye test and IFAT using *T. gondii* tachyzoites from an inoculated mouse and from a cell culture were performed.

### Preparation of tachyzoites

**Mouse inoculation.** ICR mice aged 3-4 weeks were used in this study under a protocol accepted by the Faculty of Tropical Medicine Animal Care and Use Committee (FTM-ACUC 011/2007). A 0.2 ml sample of *T. gondii* tachyzoites, at a concentration of 30,000-50,000 cells/mm<sup>3</sup>, was intraperitoneally inoculated into each mouse. Three days post inoculation (pi) the number of multiplying tachyzoites in the inoculated mouse peritoneal fluid was high enough to use for the conventional

dye test and for further infections as well. The inoculated mice were euthanized instead of allowing them to die of severe acute toxoplasmosis. The peritoneal fluid containing parasites was harvested, washed with sterile normal saline and the concentration was adjusted in a hemacytometer before use.

#### Cell line cultivation

African green monkey cells (Vero) were grown in 75 cm<sup>3</sup> tissue culture flask with modified eagle medium (1x). This was supplemented with 4,500 mg/l glucose, 110 mg/l sodium pyruvate and fetal bovine serum (FCS) at a concentration of 10% and incubated at 37°C in 5% CO<sub>2</sub> until there was a completely confluent monolayer of cells. The cells were then transferred into a 2% FCS maintenance medium and incubated at 37°C in 5% CO<sub>2</sub> for 24 hours. *T. gondii* tachyzoites at a concentration of 1x10<sup>6</sup> were added to the cell culture, which was then incubated at 37°C in 5% CO<sub>2</sub> for 48 hours. The medium without FCS was then replaced and left until the tachyzoites were harvested at approximately 48 hours.

#### Sabin-Feldman dye test

The Sabin-Feldman dye test and its modification were described previously (Sabin and Feldman, 1948; Feldman and Lamb, 1996; Reiter-Owona *et al*, 1999; Udonsan *et al*, 2008). The test is based on complement mediated cytolysis of antibody coated live *T. gondii* tachyzoites. The accessory factors, and suspension of live *T. gondii* used as antigen, were added to a two-fold serial dilution of serum and incubated at 37°C for 1 hour. Methylene blue was used to stain the live tachyzoites. If ≥55% were stained, the result was negative, but if ≥55% were unstained the result was positive.

#### Indirect immunofluorescent antibody test (IFAT)

Killed *T. gondii* tachyzoites were fixed on Teflon printed slides. The diluted sera were incubated on antigen coated slides for 1 hour at 37°C, then the slides were washed with rinse buffer. Goat anti-human IgG/FITC conjugate was added and incubated for 1 hour and then washed again; the slides were mounted and interpreted with an UV light microscope.

#### Virulence testing

*Toxoplasma* virulence was classified by mouse susceptibility. RH strain *Toxoplasma* maintained in our laboratory is highly virulent and can kill an inoculated mouse within a week. The *Toxoplasma* tachyzoites from inoculated mouse were harvested Day 3 pi, adjusted to a concentration of 30,000-50,000 cells/mm<sup>3</sup> and used for the dye test and IFAT as mentioned above. Zero point two milliliter of the remaining parasite containing solution was inoculated into an uninfected mouse each week, for 3 weeks. A similar procedure was performed using cell culture tachyzoites. All 6 infected mice were reared in a standard animal house until death.

## RESULTS

The results obtained by two serological methods, the dye test and IFAT, using *T. gondii* tachyzoites from cell culture and mouse inoculation are shown in Table 1. The mouse derived tachyzoites gave the highest seropositivity at 4.3% with the dye test, and 3.8% with the IFAT. When we used cell-culture-tachyzoites for both methods the results gave a lower seroprevalence of 2.8%. The seropositivity rate detected with the *in vitro* tachyzoite dye test was lower than the conventional method; the agreement between them was

96.7% (203 out of 210 samples). When the IFAT was performed with mouse and cell culture tachyzoites, the agreement between them was 99% (204 out of 210 samples). The agreement between the conventional dye test and the IFAT, using *in vivo* and *in vitro* tachyzoites were 94.5% and 94.8%, respectively (198 and 199 out of 210 samples). The IFAT gave a 75% sensitivity and 100% specificity when compared with the gold standard conventional dye test.

Fig 1 shows survival time with virulence testing. Mice infected with *in vitro* tachyzoites survived three times longer than those infected with tachyzoites harvested from mice (3-4 days pi vs 9-10 days pi), indicating the virulence of the tachyzoites decreased when passing through the cell culture.

DISCUSSION

Although the dye test has been used since 1948 (Sabin and Feldman, 1948), it is

still the reference method for serological diagnosis of toxoplasmosis (Reiter-Owona *et al*, 1999). This method is used in few laboratories because of the need for *in vivo* virulent tachyzoite production for test procedure. We are one laboratory routinely

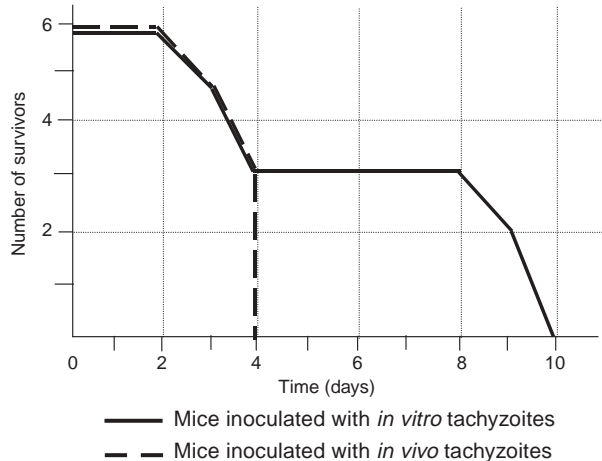


Fig 1-Survival time of the tested mice inoculated with *in vitro* and *in vivo* tachyzoites showing virulence.

Table 1

The results of *T. gondii* antibodies in 210 human serum samples tested using the dye test and IFAT with *in vitro* and *in vivo* *T. gondii* tachyzoites.

Results	Dye test		IFAT	
	Source of tachyzoites		Source of tachyzoites	
	Mouse	Cell culture	Mouse	Cell culture
Number positive (%)	9 (4.3)	6 (2.8)	8 (3.8)	6 (2.8)
Number negative (%)	201 (95.7)	204 (97.1)	202 (96.2)	204 (97.1)
Number of agreement (%)	203 out of 210 (96.7)		208 out of 210 (99.0)	
Results	Tachyzoites derived from mouse		Tachyzoites derived from cell culture	
	Dye test	IFAT	Dye test	IFAT
	Number positive (%)	9 (4.3)	8 (3.8)	6 (2.8)
Number negative (%)	201 (95.7)	202 (96.2)	204 (97.1)	204 (97.1)
Number of agreement (%)	198 out of 210 (94.3)		199 out of 210 (94.8)	

performing the dye test using animal culture tachyzoites. It is time consuming, laborious and we are faced with ethical difficulties. The results of the present study are promising showing a high agreement between the dye test with different sources of tachyzoites (96.7%) and between the dye test and the IFAT with either *in vivo* or *in vitro* tachyzoites (94.3% and 94.7%). These findings are consistent with those of Chatterton *et al* (2002) who suggested using tachyzoites derived from cell culture in the dye test, because it was cheaper than animal culture and gave 87.6% agreement. They also suggested improving the quality of accessory factors by increasing the sodium citrate from 1% to 3% when using tachyzoites derived from cell culture.

Evans *et al* (1999) suggested HeLa was the cell line of choice, giving higher tachyzoites when compared with Vero cells ( $p < 0.05$ ). They also recommended continuing inoculation into 25 cm<sup>3</sup> flasks can provide a regular supply of viable *T. gondii* tachyzoites at a concentration of 1x10<sup>6</sup> tachyzoites/ml, by 96-120 hours of infection. In our study, we used Vero cells in 75 cm<sup>3</sup> flasks and yielded similar numbers of tachyzoites but with a shorter harvest time (48 hours after infection). Loss of virulence of *T. gondii* tachyzoites, was also seen by Nischik *et al* (2001) who showed a 1,000-fold higher dose of *in vitro* tachyzoites was required to cause 100% lethality in mice. Diab *et al* (2008) also found the infectivity of tachyzoites maintained in cell culture was lower and killed only 40% of inoculated mice, corresponding with another study that showed the size of the tachyzoites decreased when passing through cell culture and the virulence in mice also decreased (Döskaya *et al*, 2006). Our study found the virulence of cell culture tachyzoites was three times lower than mouse derived tachyzoites

(Fig 1). This may be a reason for the lower positivity prevalence detected with cell culture derived tachyzoites than that detected with the conventional dye test (2.8% vs 4.3%). Therefore, a false negative result is more likely to occur when using the former method. To overcome this problem we suggest re-inoculating cell culture tachyzoites into mice for 3-5 passages, every 2-3 months. By doing so, *T. gondii* virulence will be maintained. Continuous use of cell culture derived tachyzoites in the dye test is feasible.

Unlike the dye test, the IFAT does not need live tachyzoites. The present study found 99% agreement with the IFAT using cell culture and mouse derived tachyzoites. Therefore, either *in vivo* or *in vitro* tachyzoites may be used as a source for tachyzoites for the IFAT. Our IFAT gave 75% sensitivity and 100% specificity compared to the conventional dye test. This figure is fair for screening purposes and good for excluding disease. It should be used with caution in some groups of patient and in low prevalence areas (Sukthana *et al*, 2001). *Toxoplasma* seropositive immunocompromised hosts need close attention to prevent toxoplasmic reactivation. Using the IFAT for screening this group will give some false negative results and severe disease may occur without primary prophylaxis. In pregnant women, the seronegative group is more concerned with preventing infection during pregnancy. The IFAT rarely misclassifies pregnant women who have no *T. gondii* antibodies.

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