

Short reports

Toxoplasma dye test using cell culture derived tachyzoites

D Ashburn, R Evans, J M W Chatterton, A W L Joss, D O Ho-Yen

Abstract

Aims—To assess the diagnostic usefulness of *Toxoplasma gondii* tachyzoites produced by serial passage in HeLa cell culture.

Methods—Tachyzoites derived from serial passage in cell culture were used in the dye test. Human sera were also examined to determine their suitability for use as accessory factor. Using the optimum conditions, the dye test using cell culture derived tachyzoites was compared with the current method of production (animal culture) on 105 randomly selected sera. Start up and maintenance costs of each system were compared.

Results—Tachyzoites in most cell culture harvests (84%) from both early and later passes were useable. Tachyzoite yield and viability were maintained during serial passage in cell culture. Sodium citrate was used to modify accessory factor and improve its suitability. The performance of the accessory factor was improved by the addition of 1% and 3% sodium citrate for the current and cell culture systems, respectively. Under optimum conditions, dye test titres using cell culture and current systems were compared on 105 randomly selected sera. The results from 92 of 105 (87.6%) patients agreed or were within one dilution, but all discrepancies were resolved on re-testing. Start up costs for the current system would be 2.5 times and overall maintenance three times more expensive than cell culture.

Conclusions—Tachyzoites derived from cell culture can be used routinely in the dye test. Production in cell culture is more cost effective than animal culture. It is possible for general hospitals to perform the dye test, thus obtaining faster and more specific results.

(*J Clin Pathol* 2000;53:630–633)

Keywords: toxoplasma dye test; cell culture; citrate

Since it was first developed in 1948, the toxoplasma dye test remains the standard against which other tests are measured.¹ The dye test measures the total amount of specific antibody and has the advantage of being both highly sensitive and specific. However, it has

two major disadvantages: it requires a source of live tachyzoites and a source of human complement as accessory factor. Currently, tachyzoites are produced by serial passage in the peritoneal cavity of mice or cotton rats.² Although convenient and reliable, animal culture is ethically undesirable, and the maintenance of an animal facility is expensive. Previous authors have successfully used cell culture derived tachyzoites for enzyme linked immunosorbent assays (ELISAs)³ and the dye test,⁴ but none have described their use in the dye test for routine diagnosis. We describe the establishment of a dye test method using cell culture derived tachyzoites and its performance on routine diagnostic samples relative to that using animal derived tachyzoites.

Methods

Toxoplasma RH strain tachyzoites were cultured by in vivo and in vitro methods. For animal culture, cotton rats were inoculated intraperitoneally with tachyzoites. After 66 hours the animals were killed and tachyzoites harvested by intraperitoneal washing with sterile normal saline. For cell culture, HeLa cell monolayers in 75 cm² tissue culture flasks (Costar Corning, High Wycombe, UK) were infected with tachyzoites at a multiplicity of infection of 1:1 in maintenance medium (Earle's balanced salt solution with 25 mM Hepes containing 2% fetal bovine serum, 10 mM L-glutamine (Bio Whittaker, Wokingham, UK), 40 000 U gentamicin (Rousell of Ireland, Dublin, Eire), and 1 mg Fungizone (Squibb and Sons, Hounslow, UK) and incubated at 37°C with 5% CO₂ in a Napco incubator (Thomson Scientific, Aberdeen, UK). After 24 hours, the medium was replaced with medium without fetal bovine serum and toxoplasma were harvested three to four days after infection by vigorously shaking the culture and decanting the supernatant. *Toxoplasma* produced by these methods were used to infect further rats or flasks to maintain serial passage. To remove host cell contamination, cell culture harvests were filtered using low pressure vacuum through a polycarbonate filter with a pore size of 5 µm (Whatman International, Maidstone, UK). Harvests containing less than 2 × 10⁶ tachyzoites/ml were concentrated by centrifugation at 1000 ×g for 10 minutes. *Toxoplasma* were then resuspended

Scottish Toxoplasma Reference Laboratory, Microbiology Department, Highland Acute Hospitals NHS Trust, Raigmore Hospital, Inverness IV2 3UJ, UK
D Ashburn
R Evans
J M W Chatterton
A W L Joss
D O Ho-Yen

Correspondence to:
Dr Ashburn
email: microbiology@raigmore.scot.nhs.uk

Accepted for publication
7 March 2000

to approximately 2×10^6 /ml. Animal culture products did not require purification or centrifugation.

The dye test was performed as previously described using a micromodification of the Sabin-Feldman dye test.^{5,6} Briefly, a suspension of 2×10^6 tachyzoites in 50% accessory factor was added to doubling dilutions of patients' and control sera in sterile normal saline in flat bottomed microtitre plates (Costar Corning). Accessory factor was toxoplasma specific, antibody negative serum supplied by the North of Scotland Blood Transfusion Service, collected by anonymous donation, and previously checked for suitability in the test. After incubation at 37°C for one hour, titres were read as the end point dilution for 50% tachyzoite killing assessed by phase contrast microscopy and confirmed by the addition of methylene blue. We tested 105 randomly selected sera. Sera with discrepant results were also tested using in house IgG and IgM ELISAs.⁷ The start up and maintenance costs for producing toxoplasma tachyzoites from the cell culture and current (animal culture) systems were compared. Statistical analysis (χ^2 test, *t* test with 95% confidence interval (CI), and calculation of bias⁸) was performed where appropriate. Analysis of dye test titres was performed after \log_{10} transformation of data.

Results

Toxoplasma gondii tachyzoites were successfully maintained for up to 80 serial passages in cell culture. One hundred and fifty two of 181 (84%) harvests were successful in the dye test. The remaining harvests failed in the dye test owing to non-specific killing of tachyzoites. To be useful as an accessory factor, sera must be *T. gondii* specific antibody negative and must not cause non-specific killing in the dye test. Using animal derived tachyzoites, 10 of 14 seronegative sera were suitable as accessory factor, but only three of 21 were suitable using cell culture tachyzoites ($p < 0.001$). It was found that the addition of sodium citrate (as a 50% solution of trisodium citrate in sterile normal saline) to the accessory factor reduced or prevented non-specific killing. The optimum amount to obtain correct dye test titres was 1% sodium citrate for animal derived tachyzoites (table 1), but this had to be increased to 3% for tissue culture derived tachyzoites (table 2). Using these modifications, 15 previously unsuitable sera were re-evaluated and converted to useable accessory factor by addition of the appropriate concentration of sodium citrate. Modified

Table 1 Effect of sodium citrate on dye test titre and toxoplasma viability using tachyzoites derived from animal culture

Sodium citrate (%)	Dye test titre (IU/ml) in		Viability (%) in negative control
	30 IU/ml control	<4 IU/ml (negative) control	
0	65	Unreadable	36
0.5	30	<4	94
1	30	<4	100
5	8	<4	100

Viability estimates are mean values using two different accessory factors.

accessory factors were almost equally suitable for use with tachyzoites from cell culture (85%) or from animals (86%).

Cell culture harvests were considered to be of dye test quality when the yield was $> 1 \times 10^6$ /ml and viability was $> 90\%$. Fulfilment of these criteria was to some extent dependent on the pass number of the harvest; between passes 1 and 20 (low pass), 74 of 204 (36.4%) harvests failed to meet these criteria, and between passes 21 and 40 (high pass), 55 of 184 (29.9%) failed. This difference was not significant ($p > 0.05$). However, the mean (2 SD) yield was greater for high pass harvests (4.29 (4.54) *v* 2.37 (2.50) $\times 10^6$ /ml; $p < 0.0001$; 95% CI, 1.47 to 2.37×10^6). Each harvest yielded 20 ml so that from an inoculum of 3×10^6 tachyzoites the mean increase was 15.8-fold and 28.6-fold for low and high passes, respectively. The mean (2 SD) viability of low pass harvests was 94.9% (5.7%) and was similar to that for high pass harvests (95.5% (2.50%); $p = 0.09$; 95% CI, -0.126% to 1.326%).

Costs to start up and maintain an animal house are greatly influenced by the number of users of this facility. In our case (the worst case) we were the sole users. The start up costs for cell culture equipment (incubators, pipettes, etc) were similar to those for animals (cages, racks, and sinks), and safety cabinets were common to both systems. However, the additional costs for licences and administration added an extra 25% to the animal system. Although the laboratory space required for both systems was similar, the animal system required additional security, which added a further 25% to the start up costs. In addition, the laboratory space required for a cell culture system can easily be incorporated into a virology laboratory, whereas the animal system requires dedicated space and costs twice as much. Thus, the start up cost for an animal system is at least 2.5 times greater. Consumable costs are 2.5 times greater for cell culture; staff costs are similar; licences, vets, and administration make the overall costs of the cell culture system 80% of that for animals. However, because dedicated laboratory space is necessary for the animal system, the overall maintenance of the animal system is three times that for cell culture.

To demonstrate that diagnostic usefulness was maintained during serial passage, tachyzoites derived from both low and high pass cell

Table 2 Effect of sodium citrate on dye test titre and toxoplasma viability using tachyzoites derived from tissue culture

Sodium citrate (%)	Dye test titre (IU/ml) in		Viability (%) in negative control
	30 IU/ml control	<4 IU/ml (negative) control	
0	>1000	>1000	0
0.5	>1000	>1000	52
1	65 (trail)	8	87
2	30	<4	91
3	30	<4	96
4	15	<4	98
5	15	<4	98

Viability assessments are mean values using five different accessory factors.

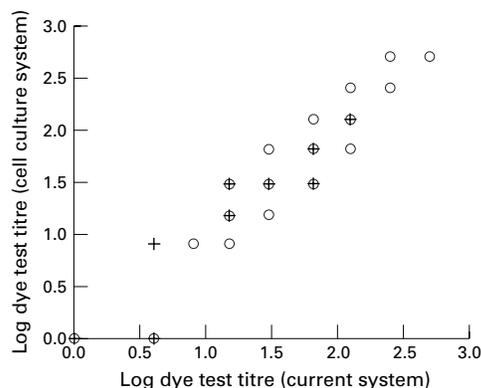


Figure 1 Relation between dye test titres produced using the current (animal culture) and cell culture systems. Open circles, low pass harvests ($n = 61$); crosses, high pass harvests ($n = 44$).

culture harvests and from the current system were used to test 105 randomly selected sera. The dye test titres ranged from < 4 to 500 IU/ml ($39 < 4$ IU/ml, $66 > 4$ IU/ml). When tested in parallel, 63 of 105 (60.0%) results agreed and a further 29 of 105 (27.6%) were within one dilution. Repeat testing of the remainder led to full agreement in 10 of 13 and in three of 13 to within one dilution; six of 13 producing the same result as before with animal derived tachyzoites and three of 13 with cell culture derived tachyzoites ($p = 0.135$; 95% CI, 1.02 to 1.04; fig 1). Dye test titres of patients' sera produced using each system were correlated. For both low and high pass cell culture harvests, the correlation coefficient $r = 0.983$. Comparability of results was also assessed by calculating bias. For low pass harvests, the cell culture system produced results 0.93 IU/ml higher than the current system (limits of agreement 0.39 to 2.23 IU/ml and 0.92 IU/ml for titres ≥ 65 IU/ml). Similarly, high pass harvests produced results 0.95 IU/ml higher (limits of agreement 0.43 to 1.93 IU/ml). The range of bias in high pass harvests was 0.84 to 1.07 IU/ml. Two sera had very low titres (≤ 4 IU/ml) with animal derived tachyzoites and were negative (< 4 IU/ml) using cell culture derived tachyzoites. Results of other toxoplasma specific antibody tests on these two sera were negative.

Discussion

The performance of the dye test with tachyzoites produced in cell culture has been described previously, but such tachyzoites have never been used for routine diagnosis in the dye test.⁴ Our method of tachyzoite production in HeLa cells has been adapted to serial passage to provide a continuous supply of viable parasites in sufficient numbers to perform the dye test routinely. Furthermore, the yield significantly increased with serial passage ($p < 0.0001$), which reduced the need to centrifuge harvests before use in the dye test. Tachyzoite viability was acceptable but not significantly different in low and high pass harvests ($p = 0.09$). Thus, the test can be performed using parasites both from early and later pass numbers, confirming the diagnostic usefulness of the tachyzoites produced. In

our experience, significantly less of the accessory factors used as a source of complement can be used for performing the dye test with tachyzoites grown in cell culture (14%) compared with those from animals (71%). It was not clear whether this is a function of the tachyzoites, the accessory factor, or both.

Because the accessory factor is the source of complement required for antibody mediated killing in the test,⁹ its contribution is crucial. Although a smaller proportion of sera than plasma can be used as accessory factors, many sera can be converted to useable accessory factors by the addition of sodium citrate.⁹ Our results confirm that the addition of sodium citrate increases the proportion of sera that can be used as accessory factors, but that a higher concentration (3%) is required for cell culture derived tachyzoites than for animal derived tachyzoites (1%). Excess sodium citrate reduces the sensitivity of the test, which is not surprising because its ability to chelate calcium and magnesium ions will inhibit activation of the classical complement pathway, which is essential to dye test function. It is possible that at optimum concentration, citrate removes sufficient magnesium to inhibit the alternative complement pathway, which might be responsible for non-antibody mediated killing, but leaves sufficient calcium for classical pathway activation. The reason that different citrate concentrations are required for the two types of tachyzoite probably arises from the fact that cell culture tachyzoites are collected in EMEM/Hepes, which contains magnesium and calcium, whereas animal derived tachyzoites are collected in saline.

For the cell culture derived tachyzoites to replace animal derived ones in the dye test, results must be comparable. In our comparative study using both low and high pass cell culture harvests, 87.5% of dye test results agreed or were within one dilution, and all of the discrepancies could be resolved on re-testing ($r = 0.983$). This is not unexpected because it is accepted that each test is only readable to within one dilution, and the results confirm that diagnostic usefulness was maintained during serial passage. A small bias was observed using cell culture derived tachyzoites (< 1 IU/ml higher), which was well within the limits acceptable for a doubling dilution test. However, on re-testing it was clear that both systems had contributed to the initial discrepancies. Bias was similar at low and high titres using both low and high pass cell culture harvests. Two sera differed in that very low titres were recorded using the current system but results were negative using tissue culture derived tachyzoites. For testing immunity and when testing immunocompromised patients it is important to detect any specific antibody.⁷ Because the discrepant low positive results recorded by the current methods were equivocal and of doubtful importance, there are no concerns that the test using cell culture derived tachyzoites might fail to detect toxoplasma antibody.

Although a move from antigen production by the current system to cell culture has clear ethical advantages, there should also be clear financial benefits. We found that the start up cost for an animal system was 2.5 times greater than cell culture. Furthermore, the maintenance costs for animals were three times greater. These results are similar to those of other workers, who estimated that toxoplasma production by cell culture is only one third to one half the cost of that of animal culture.³ This would improve the cost effectiveness of the service provided.

We have established that in the dye test tachyzoites from cell culture systems can be comparable to those from animal culture. Thus, it is now possible for general hospital laboratories with cell culture facilities to perform the dye test routinely, without referral. Clinicians will therefore be able to obtain faster and more specific results, especially important in the management of pregnant and immunocompromised patients.

We are grateful for the help and expertise of Mrs June Steen; to the North of Scotland Blood Transfusion Service, especially Sheila Ross; and to our secretaries (Ms Morag Forbes and Ms Teresa Cruickshank). We are indebted to the Endowment Fund's Committee of Raigmore Hospital, Inverness without whose support this work could not have been undertaken.

- 1 Sabin AB, Feldman HA. Dyes as microchemical indicators of a new immunity phenomenon affecting a protozoan parasite (*Toxoplasma*). *Science* 1948;108:660-3.
- 2 Obwallner A, Hassl A, Picher O, *et al*. An enzyme linked immunosorbent assay with whole trophozoites of *Toxoplasma gondii* from serum-free tissue culture for detection of specific antibodies. *Parasitol Res* 1995;81:361-4.
- 3 Hughs HPA, Hudson L, Fleck DG. In vitro culture of *Toxoplasma gondii* in primary and established cell lines. *Int J Parasitol* 1986;16:317-22.
- 4 Feldman HA, Lamb GA. A micromodification of the *Toxoplasma* dye test. *J Parasitol* 1966;52:415.
- 5 Fleck DG, Kwantes W. *The laboratory diagnosis of toxoplasmosis*. London: HMSO.0 11 887104 8, 1980.
- 6 Joss AWL. Diagnosis. In: Ho-Yen DO, Joss AWL, eds. *Human toxoplasmosis*. Oxford: Oxford University Press, 1992;79-118.
- 7 Ashburn D, Davidson MM, Joss AWL, *et al*. Improved diagnosis of reactivated toxoplasmosis. *J Clin Pathol: Mol Pathol* 1998;51:105-9.
- 8 Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986;1:307-10.
- 9 Payne RA. The effect of citrate radical on the dye test for toxoplasmosis. *Monthly Bulletin of the Ministry of Health and the Public Health Laboratory Service* 1966;25:161-4.

WHAT'S YOUR VIEW?

***Pathology Interactive*: useful or useless?**

To make *Pathology Interactive* a more useful tool to help you in your CPD

... we need your views

Please take time to complete a self completion questionnaire and fax it back to us

Questionnaire available for printing out on:

Pathology Interactive CD Vol 1 issue3 (Dec 1999)

Pathology Interactive CD Vol 2 2000

Journal of Clinical Pathology website - www.jclinpath.com

Fax completed forms to Natalie Davies, Editorial Assistant, *Journal of Clinical Pathology* (+44 (0)20 7383 6668).

Your time and help are appreciated. We especially thank all those ACP members who returned questionnaires circulated in the September 1999 journal issue for their valuable feedback.

***Pathology Interactive* 2000 offers up to three CPD credits per article; later issues feature case study and picture quiz formats**