

# The past and present role of the Sabin–Feldman dye test in the serodiagnosis of toxoplasmosis

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The dye test for the detection of *Toxoplasma*-specific antibodies was first described by Sabin and Feldman 50 years ago. The test is highly specific and sensitive and considerable information is available on the development and persistence of dye test antibodies after primary *Toxoplasma* infection. However, the test uses live *Toxoplasma gondii* and is now only employed in a few laboratories. It is still the reference method for the serodiagnosis of toxoplasmosis, and a multicentre study comparing dye test results between different laboratories was much needed.

We report in this article the results of a multicentre evaluation of the test involving nineteen laboratories in eight countries. The study revealed overall satisfactory standardization between the laboratories, but there were differences in the test protocols, the use of reference/standard preparations and the interpretation of results. There is still no agreement on the level of dye test values which reflect infection with the parasite, and conversion from titres to international units (IUs) did not improve standardization. However, the results indicated that a value of > 4 IU or a titre of 1:16 met the definition of positivity of most participants.

We recommend that the dye test be retained as a reference method and that interlaboratory standardization be improved by the use of a common protocol and the expression of results in titres.

**Keywords:** antibodies, protozoan; reference standards and values; serological tests; toxoplasmosis diagnosis.

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## Introduction

The serodiagnosis of *Toxoplasma gondii* infection is widely used for screening pregnant women in order to prevent its congenital spread. The Sabin–Feldman dye test was the first test system able to detect specific antibodies to *Toxoplasma gondii* at low levels and to differentiate acute and latent infection (1). The test is based on complement-mediated cytolysis of antibody-coated live *T. gondii* tachyzoites, which is indicated by their inability to take up methylene blue. Selection of the dye is of prime importance in the performance of the test, and it was observed that the immunity phenomenon was inducible only when a so-called accessory factor from human serum was present. Sabin & Feldman suspected that the action of the accessory factor was complement-like (1), but the nature of the activator system was only resolved by Schreiber & Feldman (2) when they described C2 as the active factor in human serum. The finding of a heat-labile, anti-*Toxoplasma* factor led to the recom-

mendation that all specimens should be heat-inactivated. This factor was later referred to by Jettmar (3) as *Toxoplasma*-hostile factor.

Westphal & Knüttgen (4) were the first to question the specificity of the dye test. Its specificity could not be proved as long as the comparatively low-sensitive complement fixation test (egg antigen) was used as the only reference. In 1954, the dye test became reproducible in the USA (blind comparative tests) but was relatively unstandardized in other countries (5). Test performance and interpretation were subsequently standardized (6).

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A modification of the dye test involving the use of microtitration plates was developed in 1966 (7). It required a smaller quantity of parasites and activator serum but did not overcome the difficulties of using the test in routine serology. The development of the immunofluorescence, agglutination, and haemagglutination tests (8, 9), followed by the enzyme-linked immunosorbent assay (10–12), led to commercial test systems for *T. gondii*-specific antibodies.

Fifty years after its introduction, the dye test is still in use and is regarded as the reference test with the highest sensitivity and specificity. By detecting IgM, IgA and IgG antibodies (total immunoglobulins), it permits both very early and late diagnosis of *T. gondii* infection in human and animal sera. A first step towards the standardization of toxoplasmosis serology was taken in 1968 when WHO recommended the expression of dye test titres in international units per millilitre (IU/ml) (13). A first international standard serum for total anti-*Toxoplasma* antibodies was produced, and a second international standard followed in 1980 (14). The estimation of IU in both preparations is based on dye test results or the detection of all Ig classes using membrane antigens on the surface of live *T. gondii*.

At present, fully automated commercial test systems are replacing conventional in-house assays and increasingly fewer laboratories in Europe (15) and the USA (16) perform the dye test routinely.

Interlaboratory modifications in the test performance and possible antigen variations of the original *T. gondii* isolates within the past 50 years have raised concern as to whether the results remain comparable. The aim of the present study was to evaluate the Sabin–Feldman dye test in a multicentre study.

## Materials and methods

**Participants.** Eighteen centres in seven European countries and one laboratory in Israel were identified as routinely performing the Sabin–Feldman test (Table 1). The laboratories received a code number and the study was performed anonymously.

**Samples.** A set of 10 lyophilized serum samples was distributed blindly to each participating laboratory with instructions for reconstitution and use. Samples had to be tested twice on two days. Five individual serum samples (9701, 9702, 9703, 9705, 9706) and five pooled serum preparations (9704, 9707, 9708, 9709, 9710 TOXM) were included. The samples were defined as described below.

- 9701: patient with persisting borderline dye test values during an observation period of 2 years; no clear-cut negative results when tested in a German quality control study; no proof of *Toxoplasma* infection.
- 9702: negative serum.
- 9703: serum taken 27 months after primary acute infection.
- 9704: national standard for anti-*Toxoplasma* antibodies (Germany, ST 85), with an estimated

antibody concentration of 1000 IU/ml, calibrated to the TOXS (Second International Standard preparation, 1980).

- 9705: latent infection with persisting low dye test values (follow-up after 1 year).
- 9706: latent infection with persisting low dye test values (follow-up after 2 years).
- 9707: low positive control serum (Statens Serum Institute, Copenhagen); a mix of a highly positive serum and a negative serum.
- 9708: national standard for anti-*Toxoplasma* antibodies (France, E 6), estimated antibody concentration 900 IU/ml, calibrated to the TOXS (Second International Standard preparation, 1980).
- 9709: low positive serum (France, S 4793), 4 IU/ml by definition.
- 9710: TOXM, Third International Standard for anti-*Toxoplasma* IgG and IgM antibodies.

**Technique.** Dye test performance and test interpretation were determined by the participating laboratories. The following details had to be specified:

- *T. gondii* strain used;
- conditions of parasite *in-vivo* or *in-vitro* culture;
- concentration of tachyzoites in final suspension;
- first serum dilution and dilution steps;
- origin of anti-*Toxoplasma* control serum;
- cut-off value for positivity.

In addition, the samples were analysed by means of any additional anti-*Toxoplasma* Ig/IgG assays available to the participants. The serum panel was also given to manufacturers of *Toxoplasma* antibody assays (Behring Diagnostics, Marburg, Germany; Hoffmann-La Roche, Basel, Switzerland; bioMérieux, Marcy-l’Etoile, France).

**Statistical analysis.** The approximate dye test antibody reactivity of each serum preparation was calculated as the median value of all determinations (single and double) given either in IU/ml or titres. The estimated enzyme immunoassay antibody concentration of each serum preparation was calculated as the arithmetic mean in IU/ml.

## Results

All the laboratories reported dye test results and the majority gave additional test results. Details of dye test performance and interpretation of the test results are shown in Table 1 for each laboratory. Three *Toxoplasma* strains were in use (RH, BK, C-56). Only one laboratory (D 74) used *Toxoplasma* tachyzoites from *in-vitro* culture. The laboratories in Austria and Germany used fourfold dilution steps and gave their results in titres, whereas those in France, Israel, Italy, Norway, and the United Kingdom used twofold dilution steps and reported results in IU/ml. A first serum dilution of 1:4 was predominantly used, but there were disparities between the laboratories in the definition of positivity (2 IU to >8 IU; titre 1:4 to

1:16). The concentration of tachyzoites in the final suspension varied from  $1-3 \times 10^7$  to  $1-6.8 \times 10^4$  /ml.

Fifteen laboratories defined the estimated anti-*Toxoplasma* dye test antibody concentration in titres and IU/ml; four did so only in titres. The resulting median values for each sample preparation, together with the maximum and minimum values, are shown in Table 2. The participants (commercial producers included) reported 12 additional antibody determinations by different enzyme immunoassay systems for each sample. The calculated enzyme immunoassay mean values are shown in Table 3 in comparison to the dye test median values (IU).

Two laboratories wrongly classified serum 9702 (considered negative) as borderline (2 IU/ml) by the dye test, but all enzyme immunoassay results were negative. There were also divergent results with sample 9701 (considered nonspecific). Two of the four laboratories that used the BK strain classified it as borderline and one of the four classified it as positive, whereas only two of the fourteen laboratories using the RH strain reported a borderline result. All enzyme immunoassay results on serum 9701 were below the cut-off.

Sample 9709, with a pre-estimated specific *Toxoplasma*-antibody concentration of 4 IU/ml, attained a median dye test value of 2 IU/ml (Table 2) and was considered positive by eight, borderline by six, and negative by four laboratories; the enzyme immunoassay results were also divergent (Table 3). Sample 9707, with a median dye test value of 4 IU/ml (1:16) was assessed as positive by twelve of the eighteen laboratories, borderline by two, and negative by four. Three of twelve enzyme immunoassay results were also borderline. Sample 9705 (median dye test value 8 IU/ml; 1:16) was within the positive range of fourteen laboratories. Distinctly positive enzyme immunoassay results were reported. Sample 9706, with a median dye test value of 16 IU/ml (titre, 1:64) was considered positive by sixteen of the participants. Sample 9703, donated by a chronically infected patient with originally high dye test values, gave 128 IU/ml (titre, 1:256) and was assessed positive by all the laboratories except for one with a very high cut-off.

The French (9708) and German (9704) national anti-*Toxoplasma* reference sera, calibrated against the Second International Standard, TOXS, yielded very similar dye test results, with a median value of 1000 IU/ml (titre, 1:4000). Even the reported enzyme immunoassay mean values were very close and >1000 IU/ml (Table 3). The Third International Standard, TOXM, which was established for the estimation of *Toxoplasma*-specific IgG and IgM antibodies (17), yielded a relative dye test antibody concentration close to the value of the two national control sera but at 824 IU/ml showed weaker enzyme immunoassay reactivity (Table 3).

The *Toxoplasma*-reference/standard preparations used for internal quality control (in-house preparations excluded) are shown in Table 4. On the basis that each dye test was standardized relative to the International Standard (TOXS), one would

Table 1. Dye test performance and interpretation

Participant	<i>Toxoplasma</i> strain	Primary dilution <sup>a</sup>	Dilution step <sup>b</sup>	Result	Cut-off <sup>c</sup>
Austria					
D 12	RH	1:5	2; 10	Titre	1:10
D 29	RH	1:4	4	Titre	1:4
Germany					
D 80	BK	1:4	4	Titre	1:16; 4 IU
D 42	BK	1:4	4	Titre	1:16
D 30	RH	1:16	4	Titre	1:16
D 2	BK	1:4	4	Titre	1:4
D 68	BK	1:4	4	Titre	1:16
D 27	RH	1:4	4	Titre	1:16
France					
D 9	RH	1:10	2	IU	1:8; 2 IU
D 53	RH	1:4	2	IU	>4 IU
D 15	RH	1:2	2	IU	>4 IU
United Kingdom					
D 18	RH	1:16	2	IU	4 IU
D 36	RH	1:8, 1:2	2	IU	8 IU
D 71	RH	1:4	2	Titre	1:4; 2 IU
Denmark					
D 74	RH	1:10	4	Titre	1:10
Norway					
D 48	RH	Undiluted	2	IU	> 6 IU
Italy					
D 19	RH	1:4	2	Titre; IU	> 8 IU
D 66/25	C-56	1:4	2	Titre; IU	>1:32; > 5 IU
Israel					
D 5	RH	1:4	2	IU	1:16; 4 IU

<sup>a</sup> First serum dilution tested.

<sup>b</sup> Serum dilution steps performed in the dye test.

<sup>c</sup> Definition of positivity.

Table 2. Dye test: *Toxoplasma* antibody concentration<sup>a</sup>

	Titre <sup>b</sup>			IU/ml <sup>b</sup>		
	Reciprocal	Range	<i>n</i>	Min-max	Value	<i>n</i>
ENCT 9703	256	64-64 000	19	25-307	128	15
ENCT 9704	4 000	40-64 000	21	250-16 000	1000	15
ENCT 9705	16	0-32	19	0-32	8	17
ENCT 9706	64	8-128	19	2-65	16	14
ENCT 9707	16	4-128	19	1-15	4	15
ENCT 9708	4 048	1024-65 536	18	800-16 000	1000	13
ENCT 9709	8 0-100	18	0-15	2	13	
TOXM	4 000	1024-262 144	18	400-65 536	1000	13

<sup>a</sup> Positive sera only.

<sup>b</sup> Median value.

expect the same ratio of IU to reciprocal titres to be obtained by the different laboratories. To examine

Table 3. Estimated *Toxoplasma* antibody concentration in IU/ml

	9701	9702	9703	9704	9705	9706	9707	9708	9709	9710 TOXM
Dye test <sup>a</sup>	Neg.	Neg.	128 (25–305) <sup>b</sup>	1 000 (250–16 000)	8 (Neg. –32)	16 (2–65)	4 (1–15)	1 000 (800–16 000)	2 (Neg. –15)	1 000 (400–65 000)
EIA <sup>c</sup>	Neg.	Neg.	154 (70–280)	1291 (579–2110)	11 (5.3–20)	62 (21–131)	8.4 (4–13) <sup>d</sup>	1278 (711–2320)	5.2 (1.8–9.5) <sup>e</sup>	824 (233–1406)

<sup>a</sup> Median value.

<sup>b</sup> Figures in parentheses are minimum-maximum determinations.

<sup>c</sup> Arithmetic mean value of 12 antibody determinations.

<sup>d</sup> Sample classified as borderline by three laboratories.

<sup>e</sup> Sample classified as negative by eight laboratories and borderline by 10 laboratories.

the laboratory-specific coefficients, we selected one low positive serum (9705) and the national control sera (9704 and 9708). Only results from laboratories that had reported IU and titres were considered. The resulting coefficients varied from 1 to 0.25. The coefficients also differed in laboratories using the same reference serum (Table 4).

## Discussion

Fifty years after its introduction the dye test is performed in only a few laboratories but it is still

considered an important reference test with high sensitivity and specificity. The results of the European multicentre study reported here revealed satisfactory overall agreement between the participants but also demonstrated variations in test performance. The study with ten selected sera confirmed that the interpretation of low titres was still difficult, as reported in 1952, when titres ranging from 1:16 to 1:64 were regarded as possibly significant (6). In the past it was suspected (18, 19) or denied (20, 21) that cross-reactivity with other protozoa could cause low positive titres in human serum. A definite discrimination between nonspecificity and low positivity was possible only after experimental infections were established in animals (22), when titres of 1:4, 1:6, 1:8 and 1:12 were found to be specific.

Disagreement continues on the level of dye test values which reflects infection with the parasite. However, an exact definition of the term "serum dilution" is necessary since the titre may represent the final dilution of serum after the addition of activator (accessory factor) and the undiluted *Toxoplasma*-suspension (6), or, as in the original description of the test (1), may represent the primary dilution of a serum. Recently, false-negative dye test results in comparison to IMx values (enzyme immunoassay, Abbott, Illinois, USA) were resolved by using lower (< 1:16) serum dilutions (23). In a French study on the evaluation of commercial anti-*Toxoplasma*-IgG assays, a range of 5.9 IU to 10 IU was considered to be borderline positive and a value of >10 IU was taken to be positive (24). The dye test should be used in a confirmatory capacity for resolving true or false-positive or negative results. The findings of the present study indicated that a value of >4 IU or a titre of 1:16 met the definition of positivity of most of the participants (serum 9705). This serum dilution was previously indicated as an acceptable cut-off value (23).

The results indicate that national protocols lead to variations in test performance and interpretation. In Germany a first attempt at dye test standardization was published in 1966 (25), and this was followed by an official recommendation for dye test performance using microtitration plates in 1976 (26). A standar-

Table 4. Dye test standardization data

Participant	Reference sera	Coefficient <sup>a</sup>
Germany		
D 80	ST85 <sup>b</sup>	0.25
D 42	ST85	0.25
D 2	ST85	0.25
D 68	ST85	0.25
France		
D 9	E6	0.25
D 53	E6 <sup>c</sup>	0.5
D 15	E6	0.25
United Kingdom		
D 18	UK national	0.5
D 36	International Standard	1
D 71	UK national	0.5
Denmark		
D 74	International Standard	0.25
Norway		
D 48	International Standard	0.5
Israel		
D 5	International Standard	0.25

<sup>a</sup> Antibody concentration in IU per reciprocal titre values for sera 9704, 9705, and 9708.

<sup>b</sup> German national standard.

<sup>c</sup> French national standard.

dized methodology for the United Kingdom was published in 1980 (27).

Variations in test results were explained by test inhibition due to the presence of a soluble component (soluble antigen) in the peritoneal exudate from mice, which depended on the number of parasites in the exudate and the *Toxoplasma* strain used (5). In the early 1950s at least four different lines (RH Sabin, RH Frenkel, RH CDC, EK) of the original RH Sabin isolate (6) were in use in the USA, two of which produced inhibition (5). Another *Toxoplasma* strain was isolated in the Netherlands at the University of Leiden from the cerebrospinal fluid of a 6-week-old child (28). This isolate, named BK, showed a close relationship to Sabin's RH strain when tested in biological and serological assays (28). Today, both strains, passaged in mice for more than 50 years, are in use in different European laboratories. Our study shows that participants using the BK strain in the dye test obtained relatively more nonspecific reactions with serum 9701 than laboratories using the RH strain. This finding emphasizes that results may vary between centres using different strains and may indicate antigenic diversity among different *Toxoplasma* strains, as reported elsewhere (29).

Minor variations in the IgG concentration (IU) are not necessarily reflected by changing DT titres (samples 9705, 9707). It is remarkable that medium dye test values (IU) were always below the enzyme immunoassay mean values (IU) in serum samples with low IgG concentrations. An overestimation of IU by enzyme immunoassay systems compared with dye test values has been reported previously (15).

The two national reference sera (9704, 9708), which had been calibrated independently against the Second International Standard, TOXS, proved to have similar estimated antibody concentrations, with little difference between the dye test and enzyme immunoassay values. The Third International Standard TOXM (9710) contains IgG antibodies of low avidity. As shown in this study its dye test activity was close to TOXS (1000 IU/ml), as previously defined in a collaborative study (29), but its IgG enzyme immunoassay concentration was lower than defined (30).

*Toxoplasma* antibody values are not absolute but are a function of the type of antigens presented and

the antibody classes detected. We therefore believe, in contrast to a previous report (31), that the IU system is not suitable for all *Toxoplasma* antibody assays. The use of the IU gives an impression of high precision but this is never obtained in practice. As shown in this study the conversion from titres to IU (32) did not improve dye test standardization or facilitate the interpretation of results. Today, *Toxoplasma* serodiagnosis in Europe is dominated by commercial kits, especially enzyme immunoassays, with separate detection of IgG and IgM antibodies. A valid extrapolation from observed test results of reference samples to results in unknown serum samples is easier for enzyme immunoassay systems but expression in IU will not necessarily improve interassay comparability. For better standardization there is a great need for international standards permitting the quantification of IgG antibodies with low and high avidity as well as differing membrane and cytoplasm antigen targets.

Fifty years after its first description (1) the dye test has become a rare test system. However, it is important to retain it as a reference test and an additional confirmatory test for the validation of commercial kits. A better standardization of the dye test in European laboratories should be possible by the use of a common protocol and the same *Toxoplasma* isolate and reference/standard serum, together with the expression of results in titres. ■

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## Résumé

### Rôle passé et présent du test de lyse des toxoplasmes dans le sérodiagnostic de la toxoplasmose

Il y a 50 ans Sabin et Feldman décrivaient pour la première fois un test de lyse des toxoplasmes pour la recherche des anticorps antitoxoplasmiques spécifiques. C'est un test extrêmement sensible et spécifique, qui reste le test de référence reconnu pour la mise en évidence des anticorps spécifiques anti-*Toxoplasma gondii*.

Nous avons évalué les résultats du test pratiqué par dix-neuf laboratoires situés dans huit pays. Le degré

de standardisation atteint par les laboratoires était dans l'ensemble satisfaisant. La plupart utilisent comme titre initial une dilution 1:4 du sérum et définissent la séropositivité par l'observation d'une réaction positive aux dilutions 1:4 à 1:16, ce qui équivaut à 2-8 UI/ml. D'après les résultats, un taux supérieur à 4 UI ou un titre de 1:16 sont conformes à la définition de la positivité.

Quinze laboratoires ont exprimé le taux d'anticorps estimé avec le test de lyse des toxoplasmes à la fois

par le titre et par le nombre d'UI/ml; quatre laboratoires n'ont indiqué que le titre. La conversion du titre en unités internationales n'a pas amélioré la standardisation.

Trois souches de *T. gondii* étaient utilisées (RH, BK, C-56) et seul un laboratoire employait des tachyzoïtes de toxoplasme obtenus sur culture *in vitro*.

Les sérums de référence nationaux, français et allemands, dirigés contre les toxoplasmes et étalonnés par rapport au deuxième étalon international de sérum antitoxoplasme humain de l'OMS (TOXS) ont donné des résultats très comparables dans le test de lyse, avec une médiane de 1000 UI/ml ou un titre de 1:4000. Le

troisième étalon international de sérum antitoxoplasme humain (TOXM) pour le dosage des IgG et des IgM antitoxoplasmes a donné une concentration relative en anticorps proche de la valeur fournie par les deux sérums témoins nationaux, mais une réactivité plus faible s'établissant à 824 UI/ml avec les méthodes immuno-enzymatiques.

Nous proposons de garder le test de lyse des toxoplasmes comme méthode de référence et d'améliorer la standardisation interlaboratoires en appliquant un protocole commun et en exprimant les résultats par le titre en anticorps.

## Resumen

### Pasado y presente de la prueba de coloración de Sabin-Feldman en el serodiagnóstico de la toxoplasmosis

La prueba de serocoloración fue descrita por primera vez hace 50 años por Sabin y Feldman como método de detección de anticuerpos antitoxoplasma específicos. Es muy específica y sensible y sigue siendo el método de referencia aceptado para la detección de los anticuerpos específicos contra *Toxoplasma gondii*.

Evaluamos el funcionamiento de la prueba en 19 laboratorios y ocho países. La normalización de la técnica entre los laboratorios fue satisfactoria en general. La mayoría de ellos empleaban una dilución de suero de 1:4 como título inicial, y la seropositividad se definía como una reacción positiva a diluciones de 1:4 a 1:16, equivalentes a 2-8 UI/ml. Los resultados indican que un valor > 4 UI o un título de 1:16 satisface la definición de positividad.

Quince laboratorios expresaban en títulos y en UI/ml la concentración de anticuerpos antitoxoplasma detectada mediante la prueba; cuatro laboratorios proporcionaban sólo los títulos. La conversión de títulos a unidades internacionales no mejoraba la normalización.

Se empleaban tres cepas de *T. gondii* (RH, BK, C-56), y sólo un laboratorio empleaba tachyzoïtes procedentes de cultivos *in vitro*.

Los sueros francés y alemán empleados como referencia nacional para toxoplasma, calibrados frente al segundo patrón internacional de suero antitoxoplasma humano de la OMS (TOXS), dieron resultados de serocoloración muy parecidos, con una mediana de 1000 UI/ml o un título de 1:4000. El tercer patrón internacional (TOXM) establecido para la estimación de IgG e IgM antitoxoplasma, mostró en la serocoloración una concentración relativa de anticuerpos próxima al valor de los dos sueros controles nacionales, pero presentó una reactividad más débil, 824 UI/ml, en los inmunoensayos enzimáticos.

Proponemos que se mantenga como método de referencia la prueba de serocoloración y que se mejore la normalización interlaboratorios empleando un protocolo común y expresando los resultados en títulos.

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