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Double-staining procedure for the fluorescent treponemal antibody absorption (FTA-ABS) test

ELIZABETH F. HUNTER, ROGER M. McKINNEY, SHIRLEY E. MADDISON, AND DONNA D. CRUCE

From the Center for Disease Control, Atlanta, Georgia, USA

SUMMARY The fluorescent treponemal antibody absorption (FTA–ABS) double-staining procedure was reproducible, comparable to the conventional test, and easy to read. We recommend the use of the FTA–ABS double-staining procedure for microscopes with incident illumination, the 100×/1·30 oil achromatic objective and the 6·3× ocular to obtain optimal fluorescence, and the KP560 as a barrier filter to exclude rhodamine emission when fluorescein fluorescence is read. With this system, errors related to poor focusing or failure to visualise treponemes on all smears should be eliminated.

Introduction

A double-staining technique has been developed for use in the fluorescent treponemal antibody absorption (FTA–ABS) test (National Communicable Disease Center, 1969). This procedure allows the new improved microscopy equipment to be used, specifically the incident illumination microscope. In the incident illumination system described by Ploem (1967) dichroic mirrors placed in the microscope tube reflect the exciting light through the objective; the objective then serves as the condenser. This system eliminates the necessity for proper alignment and focusing of the conventional darkfield condenser, and the microscope is much easier to use. The equipment offers a number of advantages and is especially useful for quantitative fluorescence measurements. For the present FTA–ABS test, however, unless the microscope is also equipped for transmitted darkfield illumination (thereby increasing cost), the reader is unable to verify the presence of treponemes on non-reactive slides. It is to solve the latter problem that our attention was directed to the incident illumination microscopes, which have become increasingly popular.

Materials and methods

Treponema pallidum antigen and sorbent were the reference reagents used for the conventional FTA–ABS test with transmitted illumination and were obtained through the Biological Products Division, Center for Disease Control (CDC), Atlanta, Georgia. Sera for preparation of controls and evaluation of the test were characterised samples from syphilitic and non-syphilitic subjects obtained through the Venereal Disease Serum Bank, CDC, or were fresh sera from a public health service outpatient clinic.

The primary antibody was goat anti-Fc fragment of pooled normal human IgG. The secondary antibody was IgG syphilitic serum obtained from sera of patients with clinically documented untreated, latent, or untreated secondary syphilis. The IgG of both sera was obtained by DEAE Sephadex chromatography with 0·1 mol/l NaCl containing 0·05 mol/l boric acid buffer adjusted to pH 8·0 with NaOH. The primary and secondary globulins were labelled with either fluorescein isothiocyanate or tetramethyl rhodamine isothiocyanate according to procedures described by McKinney *et al.* (1976). Additionally, a class-specific fluorescein labelled anti-human IgG reagent was obtained from Burroughs Wellcome Inc. Specificity for IgG was established as described earlier (Reimer *et al.*, 1970; Hunter *et al.*, 1972; Hunter *et al.*, 1976).

DOUBLE-STAINING PROCEDURE

Two methods of double-staining were examined.

Method A

The procedure is essentially the same as the conventional FTA–ABS test (National Communicable Disease Center, 1969). Absorbed human serum was incubated with *T. pallidum* antigen in the first step. The preparation was washed, and a fluorescein-labelled anti-human IgG globulin was added to

Address for reprints: Dr E. F. Hunter, Center for Disease Control Atlanta, Georgia 30333, USA

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demonstrate syphilitic antibody attachment. Slides were rinsed in phosphate-buffered saline. Then, in an additional step, the smears were stained with rhodamine-labelled human anti-*T. pallidum* globulin. In this step all treponemes are stained; therefore, with incident illumination, reactive and non-reactive treponemes could be visualised without a darkfield condenser.

Method B
The procedure is the same as that for method A, except that the label of the two globulins was reversed. The primary reagent was rhodamine-labelled anti-human IgG, and fluorescein-labelled anti-*T. pallidum* globulin was used in the final step.

MICROSCOPE
The Leitz Ortholux II microscope we used was equipped with the Ploemopak multi-wavelength fluorescence illuminator and a high pressure mercury arc lamp (HBO-200). The basic filter system for fluorescein consisted of the BG-38 red-suppressing filter, the K480 edge filter, two KP490 exciter filters, the TK510 dichroic beam splitter, and the K515 barrier filter. Additionally, a KP560 filter was inserted on the slider as a barrier filter to exclude rhodamine emission (Haaijman, 1977). The filters for rhodamine excitation included the red-suppressing BG-38, the BG-36 band-absorption glass filter to absorb the strong mercury line at 578 nm, the KP560 as the exciter filter, the K530 edge filter, the TK580 dichroic beam splitter, and the K590 barrier filter. The optical equipment included 10× and 6·3× oculars, a fluorite (Fl) 40×/1·30 oil, and a 100×/1·30 oil achromatic objective.

Results and discussion

When two fluorescent dyes are present in the same preparation the fluorescence emission must be filtered to allow the observer to visualise both dyes selectively. With the fluorescein and rhodamine dyes excitation of fluorescein will result in some excitation of rhodamine. Without a selective emission filter, staining results may be interpreted incorrectly (Haaijman, 1977). In the figure we show the emission spectra for fluorescein- and rhodamine-labelled conjugates. By adding a KP560 as a barrier filter we are able to observe fluorescein staining, and rhodamine staining is completely excluded.

Traditionally, the FTA-ABS test has been read with a 10× ocular and a 40× dry objective. We attempted to read the double-stained slides with a 10× ocular and a Fl 40×/1·30 oil objective, but

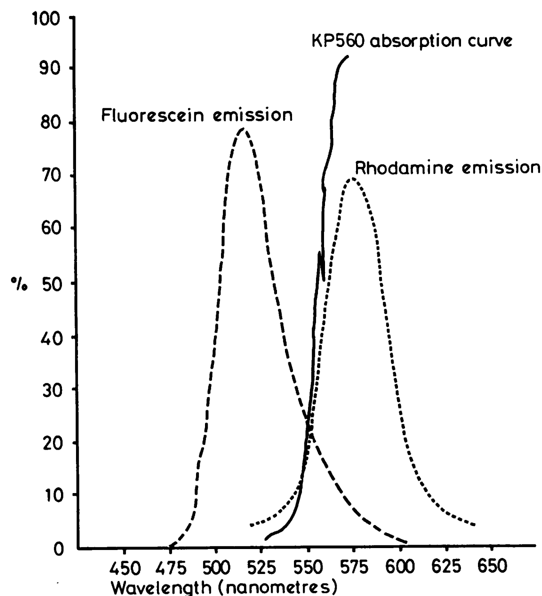

Figure *Emission spectra for fluorescein and rhodamine conjugates and KP560 absorption curve*

image quality was poor and there was excessive background when non-reactive slides were read with the fluorescein filters (Table 1). The fluorescein filters without the KP560 barrier should pass sufficient rhodamine emission for the 4+ rhodamine counterstaining on non-reactive treponemes to be visualised as brick-red in colour. This would offer

Table 1 *Quality of FTA-ABS test results read by incident illumination on a Leitz Ortholux II microscope equipped with a 10× ocular and a Fl 40×/1·30 oil objective* (500×)

	Microscopical evaluation		
Filter system	*Fluorescence*	*Image quality*	*Background*
Fluorescein BG38, K480, 2KP490, TK510, K515	S	U	U
Above filters +KP560	S	S	S
Rhodamine BG38, BG36, KP560, K530, TK580, K590	S	S	S

S Satisfactory
U Unsatisfactory

another possibility for observing treponemes on non-reactive FTA-ABS test smears without the added cost of the multiple Ploem filter system. These optics were considered unsatisfactory for this purpose perhaps because of spherical abberration of the objective and eyepiece magnification (Haaijman, 1977). All other readings were satisfactory.

For the FTA-ABS double-staining procedure a 100×/1·30 oil achromatic objective with a 6·3× ocular is recommended. We obtained excellent fluorescence, excellent image quality, and an excellent background (Table 2). With this combination non-reactive treponemes could be seen with the single fluorescein filter system because image quality and background were much improved. The brick-red colour was not as easy to read as the maximum red fluorescence obtained with the rhodamine filter system, but the system was satisfactory.

The addition of rhodamine-labelled anti-*T. pallidum* globulin in the final step of method A stained all treponemes red. We recommend, therefore, that treponemes be located and focused with the rhodamine filter system. The 40× oil or 40× dry objective may be easier to use in finding the field. After the field is focused, the 100× oil objective is moved in place. Then, with the selector knob available on the Ploemopak the fluorescein filters are switched in position, the KP560 is inserted, and fluorescein fluorescence is correctly read. After fluorescence intensity has been recorded, slides can be interchanged with very little focus adjustment. The slides are much easier to interchange and focus with this equipment than with the darkfield condenser.

We prepared reading controls for the double-staining procedure and evaluated the test with FTA-ABS test results obtained by reading with transmitted light. We obtained essentially the same results as were obtained in the conventional test on sera from 92 syphilitic subjects (Table 3). The differences were between a borderline and a non-reactive and a borderline and a reactive result. Results of tests on sera from 108 non-syphilitic subjects were the same (Table 4).

In the early part of our study we chose method A for several reasons: (1) individuals were trained for reading fluorescein fluorescence; (2) individuals may already have an incident illumination microscope with only the fluorescein filter system; and (3) the Ploemopak was expensive. Now that less expensive microscopes accept up to three filter modules we have to reconsider our choice. We examined method B with a class-specific rhodamine-labelled

Table 2 *Quality of FTA-ABS test results read by incident illumination on a Leitz Orthulux II microscope equipped with a 6·3× ocular and a 100×/1·3 oil achromatic objective* (800×)

	Microscopical evaluation		
Filter system	*Fluorescence*	*Image quality*	*Background*
Fluorescein BG38, K480, 2KP490, TK510, K515	E	E	E
Above filters +KP560	E	E	E
Rhodamine BG38, BG36, KP560, K530, TK580, K590	E	E	E

E Excellent

Table 3 *Comparison of results obtained with the FTA-ABS double-staining procedure and with the conventional test of sera from 92 syphilitic subjects*

	FTA-ABS test					
Category of syphilis	*Double-staining test*			*Conventional test*		
	R	*B*	*N*	*R*	*B*	*N*
Primary	30		4	30	1	3
Secondary	26			26		
Latent	31	1		32		
Total	87	1	4	88	1	3

R Reactive
B Borderline
N Non-reactive

Table 4 *Comparison of results of the FTA-ABS double-staining procedure and the conventional test with sera from 108 non-syphilitic subjects*

	FTA-ABS test					
Category	*Double-staining test*			*Conventional test*		
	R	*B*	*N*	*R*	*B*	*N*
Presumed normals			70			70
Diseases other than syphilis			18			18
Biological false-positive			20			20
Total			108			108

R Reactive
B Borderline
N Non-reactive

anti-IgG conjugate and a fluorescein-labelled anti-*T. pallidum* globulin. This appears to be a workable system, but further evaluation is needed.

Conclusion

We recommend use of the double-staining procedure in laboratories where microscopes with incident illumination are available. The FTA-ABS double-staining procedure described here was highly satisfactory and easy to perform. We do not recommend the use of these microscopes with the single-staining FTA-ABS procedure (National Communicable Disease Center, 1969).

Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the US Department of Health, Education, and Welfare.

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