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Effect of Working Time and Temperature of Sulfuric Ether in the Toluidine Blue O Stain for *Pneumocystis carinii*

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Abstract

Toluidine blue O method sometimes failed to stain *Pneumocystis carinii* cysts especially during high temperatures in the laboratory (over 25°C). As a result of this investigation, it has become clear that the instability of the staining is a direct result of variations in working time and temperature under the sulfation procedure. Various combinations of working time and temperature were set up and tested to get good staining. The results indicated an inverse relationship between working time and temperature in the sulfation process, namely, low temperature needed more time for sulfation to obtain the subsequent satisfactory stain.

Finally, the most recommendable conditions for the sulfation were elucidated to be 4–5 min in time and 5–10°C in temperature. It was also clarified that the activity of sulfuric ether can be maintained for several months when stored in temperatures of 5–10°C.

Key words: *Pneumocystis carinii*, toluidine blue O stain

Introduction

Several staining methods for identification of the cyst of *Pneumocystis carinii* are in common use, namely, sulfation toluidine blue O stain (Chalvardjian and Grawe, 1963, Pifer and Woods, 1978), rapid Gomori's methenamine silver nitrate method (Smith and Hughes, 1972, Churukian and Schenk, 1977, Pintozzi, 1978, Mahan and Sale, 1978, Musto *et al.*, 1982), and cresyl echt violet stain (Bowling *et al.*, 1973). Of these, the toluidine blue O method has proved the most popular, as it is a quick and simple method, applicable to both touch smears and paraffin sections.

This method was developed originally for fungi (Kelly *et al.*, 1962) and adapted for detection of *P. carinii* later by Chalvardjian and Grawe (1963). Also, this method has long been used as a standard metachromatic staining (Mowry, 1958). Our laboratory has used the method for over 15 years.

However, the toluidine blue O method has two weak points; firstly, it requires skill and experience to discriminate *P. carinii* from fungi and, secondly, the color intensity of stained slide samples is not always consistent and the color tone easily fades away with passing time. The first problem is unavoidable as this method is not a specific staining for *P. carinii* but adapted for fungi. This investigation, however, has shown that correct coordination of working time and temperature in the sulfation process can overcome the second weak point. Consequently, the best results obtainable with toluidine blue O staining are seen to be dependent on the optimum conditions for sulfation.

Materials and Methods

Wistar male rats, weighing approximately 200 g, received subcutaneous injections of 25 mg of cortisone acetate twice a week for 8 to 9 weeks. In order to prevent bacterial infec-

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tion, the rats were also given 500 to 1,000 mg/liter of tetracyclin dissolved in their drinking water. Smears of lung tissues of the rats were air-dried, fixed in methanol for 5 to 10 min (not necessary if used immediately), treated with sulfuric ether, and stained by the 0.15% toluidine blue O dye solution.

For the sulfation, the concentrated sulfuric acid (Nakarai Chemicals) and high grade diethyl ether (Nakarai Chemicals) were mixed on the day before use according to the original method of Chalvardjian and Grawe (1963). The sulfation was performed using various combinations of the following working times and temperatures. The working times tried began with 15 sec and 30 sec, then increased to 1 min and by 1 min increments up to 8 min. Likewise, temperatures tried began with 1°C and increased by 5°C increments up to 40°C (Table 1). After sulfation, each of the slides was washed for 10 min in running water (18–22°C in temperatures). All the samples were then stained with toluidine blue O (Merck) for 3 min freshly made at use. After that the slides were dehydrated 3 times, for 30 sec each, in separate trays of isopropyl alcohol. Finally, the slides were immersed 3 times in separate trays of xylene for 5, 5 and 10 min respectively, then mounted.

Results

An inverse relationship between the temperature of sulfuric ether and the period of sulfation can be seen in Table 1. Satisfactory staining of the cysts of *P. carinii* is principally achieved at low temperatures with a long sulfation period, and on the contrary, at high temperatures with a short period. Outside of this zone of satisfactory staining, the cysts became difficult to be recognized, due to insufficient stain, excessive background stain or destruction of cysts by too much sulfation.

Table 1 explains insufficient results we often experienced during the high temperature season (over 25°C). The most usual air-conditioned room temperature during this season may be within 20–30°C. If five minute-sulfation is conducted on samples under these temperatures according to the original method (Chalvardjian and Grawe, 1963), it seems obvious from the data found in Table 1 that most of the cysts will be difficult to identify due to excessive stain of background or to destruction of the cysts.

Table 1. The relationship between the temperature of sulfuric ether and the period of sulfation in the toluidine blue O stain for *P. carinii*: □, Hard to recognize the cysts and trophozoites due to excessive stain of background or to destruction of the organism by too much sulfation; △, Hard to recognize the cysts and trophozoites due to insufficient stain; ●, Both the cysts and trophozoites are satisfactorily stained; ○, Only the cysts are satisfactorily stained; –, Not examined

Minutes for sulfation	Temperatures(°C) of sulfuric acid ether reagent at use								
	1	5	10	15	20	25	30	35	40
8	○	●	–	□	□	□	□	–	–
6	○	●	●	□	□	□	□	–	–
5	△	○	●	●	●	□	□	–	–
4	△	○	○	●	●	□	□	□	–
3	–	–	△	●	●	●	□	□	□
2	△	△	–	○	●	●	□	□	□
1	–	–	–	△	○	●	●	●	●
1/2	–	–	–	–	○	○	○	●	●
1/4	–	–	–	–	△	△	△	○	○

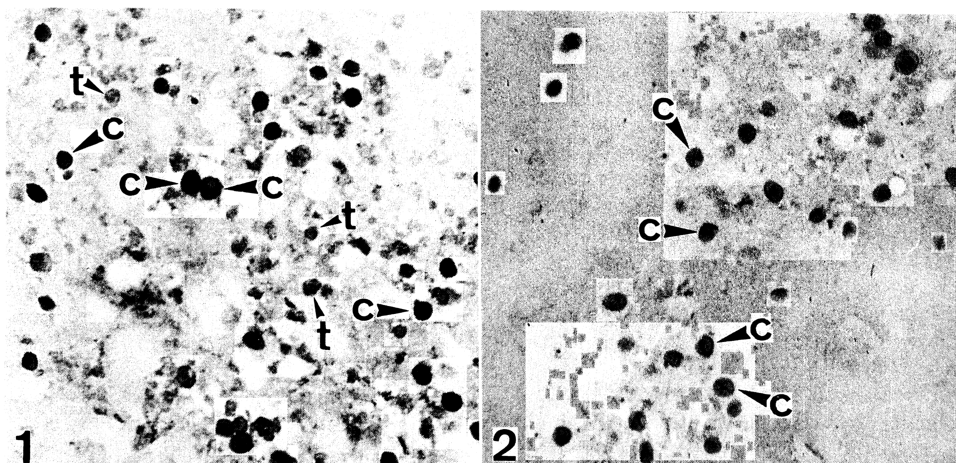

Fig. 1 Both cysts and trophozoites of *P. carinii* stained for 5 min in temperatures of between 10 and 20°C. (x600) c: cyst, t: trophozoite

Fig. 2 Cysts of *P. carinii* stained for 5 min in temperatures of between 5 and 10°C. (x600) c: cyst

It can be seen from the table that the zone indicating satisfactory staining is widest for sulfation periods of 30 sec to 1 min with ether temperatures between 20 and 40°C. However, these parameters are unsuitable for practical use, because such hurried handling may lead to inaccuracies. The most convenient time for sulfation, therefore, is 4–5 min in ether temperatures of between 5–20°C.

Moreover, it was found that this staining could reveal not only cysts but also trophozoites and immature cysts of *P. carinii.* The conditions of sulfation for obtaining better staining results are 5 min at 10–20°C for both cysts and trophozoites (Fig. 1), while 5 min at 5–10°C for cysts alone (Fig. 2). All procedures require only 10–15 min.

Discussion

We found that the toluidine blue O staining method for identifying the cyst of *P. carinii* can be highly satisfactory, provided that the temperature of the sulfuric ether and the time of sulfation are taken into account. This procedure is also good for both urgent and routine diagnosis of cysts of *P. carinii.*

Moreover, it is interesting to note that the trophozoites and immature cysts of *P. carinii* also showed a consistent staining pattern. The stability of color intensity of *P. carinii* by this method seems good as far as observed for several years.

The inhibition for this stain has also been reported as a cause of inconsistencies (Settnes and Larsen, 1979). The inhibition is caused by the addition of stabilizers in the diethyl ether which slows down evaporation. However, the diethyl ether which we used did not contain the stabilizers, and we did not find such evidence in our own experiments but felt that the relation of the temperature of sulfuric ether and the period of sulfation was of prime importance.

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