Microscopy – cold

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COLD STAINING METHODS FOR MYCOBACTERIA

M. ŠLOSÁREK[1], L. MEZENSKÝ[2]

The World Health Organization has been seeking, for use in the developing countries, a simplified method of staining mycobacteria that would not involve specimen heating yet would be sufficiently sensitive to warrant a high rate of detection of acid-fast microorganisms. We accordingly decided to put to the test three cold staining methods for mycobacteria already previously published by different authors and compare their efficiency with that of the Ziehl-Neelsen technique.

The results of our studies are presented in this communication.

MATERIALS AND METHODS

The material used for staining comprised 120 ballotini-homogenized sputa collected from pulmonary tuberculous patients and suspected of positivity for acid-fast bacilli, smears from the nodes of two guinea pigs infected with the human H37Rv strain or a freshly isolated bovine strain, and smears from 10 cultures of different mycobacterium species: *M. tuberculosis*, *M. bovis*, *M. kansasii* (two strains), *M. scrofulaceum*, *M. avium* (two strains), *M. fortuitum*, *M. phlei* and *M. smegmatis*.

Samples of these materials were pipetted in 0.05 ml amounts onto four slides each, spread over a constant diamond-delineated circular area of approx. 4 cm^2 and, prior to the staining proper, fixed, with methanol until its evaporation.

Staining methods employed:

A. Ziehl-Neelsen (in the modification used at the authors' laboratories)

Solution 1		Solution 2
Basic fuchsin	10 g	Sulphuric acid, 25 %
Alcohol, 96 %	100 ml	Solution 3
Phenol, 5 % water solution . .	1000 ml	Malachite green, 1 %

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[1]Institute of Hygiene and Epidemiology, Prague;

[2]Hospital for Tuberculosis and Respiratory Diseases, Žamberk, Czechoslovakia

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The fixed specimen was treated with solution 1, heated three times until appearance of fumes, after 3 min rinsed with water, decolorized with sulphuric acid and, if decolorization appeared, again rinsed with water and counterstained with malachite green for 1 min. It was then rinsed, dried and examined.

B. Lapeyssonnie & Causse (1960)

Solution 1		Solution 2	
Basic fuchsin	5 g	Absolute alcohol	4 volumes
Absolute alcohol	5 g	Concentrated sulphuric acid	1 volume
Phenic acid	10 ml	Methylene blue, 1 %	7 volumes
Distilled water	100 ml		
Tween 80	15 drops		

The fixed specimen was treated with solution 1 for 5 min, rinsed with water and then simultaneously decolorized and counterstained with solution 2 for 3 min. It was then rinsed again, dried and examined.

C. Pottz, Rampey & Benjamin (1964)

Solution 1		Solution 2	
Basic fuchsin	4 g	Malachite green, 2 %	220 ml
Phenol	12 g	Acetic acid, glacial, 99.5 %	30 ml
Glycerol	25 ml	Glycerol	50 ml
Dimethyl sulphoxide	25 ml		
Alcohol, 95 %	25 ml		
Distilled water	160 ml		

The fixed specimen was treated with solution 1 for 3 min, rinsed with water, decolorized and counterstained by immersion in solution 2 for 3 min, rinsed again, dried and examined.

D. Rao, Nagathan & Nair (1966)

Solution 1		Solution 2
Basic fuchsin	1 g	Sulphuric acid, 25 %
Phenol	5 g	**Solution 3**
Absolute alcohol	10 ml	Malachite green, 1 %
Chloroform	0,25 ml	
Distilled water	90 ml	

The staining procedure is as with method A (Ziehl-Neelsen) except that neither stain nor smear were heated. The original method of the Indian authors uses, both for Ziehl-Neelsen staining and for the cold modification, two consecutive decolorization steps (25 % sulphuric acid twice for 2 min and 70 % alcohol or methanol for 2 min) and counterstaining with 0.1 % methyl blue. In the present study, the ususal decolorization and counterstaining techniques employed in our laboratories, as given in Method A, were used.

The findings in sputa and guinea pig node specimens were evaulated in terms of the acid fast bacillus (AFB) count in fifty fields, as follows: up to 50 AFB = +, 51-250 AFB = ++, above 250 AFB = +++. Up to the number of 50 the bacilli were counted individually.

All the sputa used for microscopy were simultaneously cultivated. After homogenization, each sputum was taken on three swabs and, following decontamination by standard technique using 1 N hydrochloric acid, was inoculated into six culture media (four liquid Šula media and two Löwenstein-Jensen egg media). Cultures were read after incubation for 3, 6 and 9 weeks at 37 °C.

RESULTS

The numbers of sputa positive by the individual staining methods and the results of verification by culture are presented in Table 1. It is evident from this table that the best results were obtained by the Ziehl-Neelsen method (A). The cold staining methods proved less sensitive, in the order (descending) of D, B and C.

The results obtained in specimens prepared from the nodes of guinea pigs infected with human or bovine mycobacteria are presented in Table 2. In this trial, method A also proved to be the most sensitive, the order of the three cold staining methods according to the count of bacilli detected being practically the same as in the sputum smears (D, B and C). The order of the quality of the staining (intensity of stain, contour of bacilli) by the cold methods, according to the results obtained in both sputum and node specimens, was: B, D and C. The difference between the Ziehl-Neelsen method (A) and the best of the cold staining methods from the above sequence (B) as found in smears from guinea pig nodes is well documented by Figs. 1—4.

The quality of staining cultures of the different mycobacterium species by the methods employed showed considerable variation. With all the 10 strains tested, the hot carbolfuchsin method (A) again yielded the best results. Only the *M. smegmatis* culture contained a small proportion (approx. 10 %) of non-acidfast cells; the other nine cultures exhibited practically only rich red, well delimited bacilli of varying shape. The cold staining methods yielded relatively good results in pure cultures of *M. tuberculosis* and *M. bovis* (with the exception of

Table 1. Comparison of Three Cold Staining Methods for Sputa with the Ziehl-Neelsen Method

Sputa, total	Total positive microscopy	Method	Positive by microscopy	Cultivat. confirmed	Positive by microscopy only by method	Of these, posit. by cultivat.	Positive* by microscopy only
120	40	A	36	32	8	7	4
		B	20	20	0	0	0
		C	15	14	1	0	1
		D	27	23	3	2	4

Explanations: A = Ziel-Neelsen; B, C, D = cold staining methods

*) There were six sputa positive by microscopy only; three of them were positive simultaneously by methods A and D and one each by methods A, C and D.

Table 2. Findings of Acid-fast Bacilli in Smears from Nodes of Guinea Pigs Infected with Human or Bovine Mycobacteria Stained after Ziehl-Neelsen and by the Three Cold Methods

Guinea pig node contained		Method used			
		A	B	C	D
Human mycobacteria	smear 1	+++	++	+ (40)	++
	smear 2	+++	++	+ (25)	++
Bovine mycobacteria	smear 1	+++	+ (18)	+ (1)	+ (45)
	smear 2	+++	+ (6)	+ (8)	+ (40)

Explanations: A = Ziehl-Neelsen; B, D, C = cold staining methods
+++ = above 250 bacilli in 50 fields
++ = 51—250 bacilli in 50 fields
+ = 1—50 bacilli in 50 fields
(figures in parentheses = bacillus counts)

method C, which especially in bovine mycobacteria produced only partial and very weak staining). Method B gave very weak staining results in *M. phlei, M. fortuitum, M. smegmatis* and *M. kansasii* (only some bacilli were weakly red, most were not stained at all); other cultures stained fairly well by this method. The method of the Indian authors (D) yielded, in practically all the cultures tested, along well-stained AFB, a greater or smaller proportion of non-acid-fast cells (from 10 % in *M. smegmatis* to 90 % in *M. kansasii*). The greatest variation of results was observed with method C, which frequently displayed only single AFB of nuclear contour *(M. kansasii, M. bovis, M. phlei)* even in massive culture smears, whilst in other cases some bacilli stained well but a major part of the population was made up of non-acid-fast cells *(M. fortuitum, M. smegmatis)*. An example of the different stainability of *M. kansasii* culture by the methods under trial is presented in Figs. 5—8.

DISCUSSION

Apart from the usual Ziehl-Neelsen technique, various simplified procedures for the microscopic demonstration of mycobacteria that do not require specimen heating and in some instances combine decolorization and counterstaining into a single step have been described; this would especially offer advantage to laboratories with minimal equipment. The elimination of specimen heating from the staining procedure would also contribute to greater standardness of the method and the possibility of its employment as one of the parameters in the international comparison of tuberculosis epidemiology.

The cold staining methods that have been designated B and C in the present work had been compared with the Ziehl-Neelsen technique already by Moučka and Kaňkovská (1967), and the experiences of these authors were similar to ours. Both of these methods are based on the assumption that by increasing the concentration of the principal dye (carbolfuchsin) and adding Tween 80 (B) or

dimethyl sulphoxide (C), satisfactory staining of mycobacteria will be obtained without the necessity of heating the specimen. As decolorization and counter-staining have moreover been combined into a single step, the authors recommend these techniques for routine use. However, comparison of these methods with the classical staining after Ziehl-Neelsen provides unequivocal evidence that simplification of procedure is in them accompanied by a substantially lower rate of mycobacterium detection.

The method of the Indian authors (D) involves practically the same procedure as the Ziehl-Neelsen method (A), except that specimen heating is omitted and replaced by addition of 0.25 % chloroform to the carbolfuchsin. Although better results were obtained by this method than by either of the above cold methods, the quality of bacillus staining and mycobacterium demonstration in sputa whose positivity was confirmed by cultivation was much inferior to hot carbolfuchsin staining (A).

Thus, the simplification of the staining procedure and omission of specimen heating, with the time of stain exposure retained more or less as it was, lead to inferior quality of the stain, the cells frequently showing only an unclear contour and hence being in danger of being overlooked or considered an artifact. Since only certain developmental stages of mycobacteria stain in the cold as a rule, it is certainly — especially in paucibacillary material — much more difficult to demonstrate the very presence of AFB by the cold staining methods. All the eight sputa which in microscopy displayed AFB solely by the Ziehl-Neelsen method (A) were positive at the + level, i.e. exhibited only 1—50 bacilli in fifty fields. The four sputa microscopically positive by one or another of the cold methods only (one by method C and three by method D) also contained but a small number (1—3) of weakly stained bacilli each. It cannot be precluded that in the case of the AFB finding solely by method C, the least successful of the methods, in a sputum negative by cultivation, an artifact might have been involved.

Apart from the three cold staining methods already previously described, we tested as additives a number of organic agents in different concentrations whose presence in carbolfuchsin could possibly facilitate stain penetration into mycobacterial cells even in the cold; these agents were ethylene glycol, glycol benzoylacetate, lysol, lauryl sulphate, and some others. Moreover, we tested other stains than the commonly used carbolfuchsin, such as sudan, night blue, etc., in tentative cold trials. However, results that in the quantitative or qualitative respect would equal those yielded by the classical method of Ziehl-Neelsen were not obtained in a single case.

We furthermore explored the staining effect of carbolfuchsin without any additive (after Ziehl-Neelsen), but in the cold and at different exposure intervals (5, 15, 30, 60, 120 min, 4 and 24 h). The specimens comprised positive sputa and some mycobacterium strains (*M. avium, M. kansasii* and *M. smegmatis*). As compared with the hot method, AFB were less intensively stained at the 5 and 15 min intervals, but from 30 min on there was practically no difference from sputum specimens stained by hot fuchsin (5 min). *Mycobacterium smegmatis*

specimens displayed more AFB units after 60 min in the cold than after 5 min of hot staining. In *M. avium,* correspondence between the classical Ziehl-Neelsen and cold staining was apparent from 120 min of cold exposure upward. However, these results still require verification on a larger set of specimens.

If a general estimate is to be given of the three cold staining methods on the basis of our comparison with the classical Ziehl-Neelsen technique, the conclusion that must be drawn is unequivocal: staining of mycobacteria by these methods is much inferior to their staining with hot carbolfuchsin, and hence these methods cannot be recommended for the routine microbiological diagnosis of mycobacteria.

SUMMARY

Three available cold staining methods for mycobacteria were compared with the Ziehl-Neelsen method. The material for staining comprised smears from 120 sputa suspected of mycobacterial positivity, two nodes from guinea pigs infected with human and bovine mycobacteria and ten different mycobacterium cultures. The comparative experiments proper were supplemented by tentative trials involving cold staining of mycobacteria with carbolfuchsin plus admixture of different organic agents that could facilitate the penetration of the stain into the mycobacterial cell. However, in no case did a cold method yield results that could match qualitatively or quantitatively with mycobacterium staining with hot carbolfuchsin after Ziehl-Neelsen.

RÉSUMÉ

L'étude comparative de trois méthodes, publiées auparavantes, de la coloration froide des mycobactéries d'après Ziehl-Neelsen. A titre pour la coloration on se servait des frottis de 120 crachats suspectés d'être positifs aux bacilles tuberculeux, de 2 ganglions de cobayes infectés de bacilles tuberculeux humaines et bovins comme aussi de 10 cultures différentes des bacilles tuberculeux. Les expériences comparatives propres étaient complétées par des épreuves d'orientation en colorant des bacilles tuberculelux à froid par la fuchsine de Ziehl à l'acide de différentes substances organiques ajoutées facilitant pénetration de matière colorante dans les cellules des bacilles tuberculeux. En aucun cas on n'a obtenu à froid, tels résultats qui seraient qualitativement ou quantitativement comparables à ceux obtenus en colorant les bacilles tuberculeux à l'acide de la fuchsine chaude d'après Ziehl-Neelsen.

ZUSAMMENFASSUNG

Es wurden drei schon früher veröffentlichten Methoden der Kaltfärbung von Mykobakterien nach Ziehl-Neelsen verglichen. Für die Färbung wurden Ausstriche des Sputums von 120 Personen die wegen Mykobakterien verdächtig waren, zwei Lymphknoten der Meeresschweinchen die mit humanen und bovinen Stämmen der Mykobakterien infiziert wurden und zehn verschiedene Kulturen der Mykobakterien. Die eigenen Vergleichungsversuche wurden weiter durch Orientierungsprüfungen mit Kaltfärbung der Mykobakterien durch Karbolfuchsin mit einem Gemisch verschiedener organischer Stoffe die das Durchdringen des Farbstoffes in die Zellen der Mykobakterien ermöglichen könnte, ergänzt. Es ist nicht gelungen bei der Kaltfärbung solche Resultate zu erzielen, die kvalitativ und kvantitativ der Färbung der Mykobakterien mit heissem Karbolfuchsin nach Ziehl-Neelsen gleich waren.

RESUMEN

Realizamos la comparación de tres, anteriormente publicados métodos fríos de la tinción de los mycobacteria, con el método de Ziehl-Neelsen. Como el material para la coloración empleamos las muestras de 120 esputos sospechosos de la presencia de los mycobacteria, 2 ganglios de cobayos infectados con los mucobacteria humanos y bovinos y 10 varios cultivos de los mycobacteria. Los experimentos comparativos completamos con las pruebas de orientación con la tinción de frío de los mycobacteria por la carbolfuscina en la mezcla con varias substancias orgánicas que podrían facilitar la penetración del colorante en las células de los mycobacteria. En ningún caso obtenemos con el método frío los resultados comparables cualitativamente o cuantitativamente con la tinción de los mycobacteria por la carbolfuscina caliente según Ziehl-Neelsen.

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M. Šlosárek, Institute of Hygiene and Epidemiology,
Šrobárova 48, Praha 10, Czechoslovakia