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A modified Ziehl-Neelsen stain for mycobacteria

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Abstract: For the preliminary detection of mycobacteria in routine pathology specimens heavy reliance is placed on staining methods. Difficulties encountered with a cold-staining method, which had possibly gone un-noticed for several years despite a quality control check programme, are described. An alternative to the 'classical' Ziehl-Neelsen stain is described, which although devised initially as a 'stop-gap' is still in use eight years later.

Key words: Mycobacterium. Quality control. Stains and staining.

Introduction

Despite many advances in medical microbiology in the past decade, early laboratory diagnosis of tuberculosis still relies heavily upon the examination of stained smears. While the laboratory has little control over specimen quality, its choice of staining method can be very important, since for every bacillus seen in a smear 10 000 to 50 000 have to be present in each millilitre of the specimen.

Mycobacteria are classified as acid-fast because of their ability to resist decolourisation by mineral acids after staining with an arylmethane dye. Prior to electron microscopy this acid-fastness was attributed to a hypothetical outer 'waxy layer', but it is now known to depend upon the mycolic acid residues of the peptidoglycolipids of the outer cell wall. During staining, a complex is formed between the acid residues and the dye (*e.g.* carbol fuchsin) which traps the stain intracellularly. Since the mycolic acid-fuchsin complex is resistant to the effect of mineral acids and alcohol, the bacillus remains stained after treatment with these chemicals. The depth of staining depends on the quantity of entrapped fuchsin.

During the 1970s and early 1980s, the cold staining method described by Tam Tham Hok[1] was used extensively in this laboratory. A quality control check was carried out, consisting of preparing two smears from a known positive specimen and comparing the results achieved with old and new batches of stains. However, use of the 'classical' Ziehl-Neelsen method to confirm morphologically atypical acid-fast bacilli in pus from an ischio-rectal abscess, showed a marked increase in the number of stained bacilli, compared to the cold stained smear. A review of the previous year's routine work, and subsequently of the previous ten years, showed a smear-positive/culture-positive rate of 37%. At this time the Communicable Diseases Surveillance Centre was reporting 51% of bacteriologically-confirmed cases of tuberculosis as smear-positive.

A preliminary investigation indicated that the 'classical' Ziehl-Neelsen method gave the most consistently reproducible results when carried out by experienced technical staff. Inexperienced personnel found difficulty in controlling the decolourisation of the slide, and all had problems when using 3% hydrochloric acid in ethanol (acid alcohol). After a series of trials, the 'classical' method was slightly modified to include the counterstain and the decolourising agents. The 'classical' and modified Ziehl-Neelsen methods were then compared with the cold technique.

Materials and methods

The initial experiments were carried out, under Category 3 containment[2], using an homogenised sputum containing approximately 10 acid-fast bacilli *per* high power field. All smear results were verified by

Kw: TB, lab, dx, microscopy, ZN, cold stain, sensitivity

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culturing the samples on Lowenstein-Jensen medium incubated at 37°C for a maximum of ten weeks.

Smears were prepared using a standard loop, and allowed to air-dry before fixing and staining. The following staining methods were used:

'Classical' Ziehl-Neelsen stain. Slides were flooded with carbol fuchsin,[3] which was gently steamed for 5 min. They were then decolourised with 20% sulphuric acid for 10 min and ethanol for 2 min. Slides were washed in running tap water and then counterstained with 0.5 g/l malachite green for 30 s, drained and allowed to air dry.

Modified Ziehl-Neelsen stain. The counterstain was prepared as follows: 5 g of methylene blue powder were dissolved in 160 ml of industrial methylated spirits (IMS). The stain was kept cool while 90 ml of concentrated sulphuric acid was carefully added. It was then diluted to the working concentration by the addition of 250 ml of de-ionised water.

Slides were flooded with carbol fuchsin, gently steamed for 5 min and then washed in running tap water. They were counterstained for 3 min and then washed again in running tap water, drained and allowed to air-dry.

Cold method (Tan Tham Hok). Slides were flooded with carbol fuchsin for 3 min, washed in running tap water and then counterstained with Gabbett's stain[4] for 4 min, washed again in running tap water, drained and air-dried.

Comparability study of the three staining methods

Smears were prepared from each dilution of a known positive specimen, using a standard loop. Slides were chosen randomly from each dilution for staining by one of the methods. For the purpose of grading the smears the following arbitrary system was used.

++ = 10 or more acid-fast bacilli *per* 10 high power fields
+ = 1–9 acid-fast bacilli *per* 10 high power fields
± = < 1 acid-fast bacilli *per* 10 high power fields
− = acid-fast bacilli NOT seen.

Comparison of the 'classical' and modified Ziehl-Neelsen stains

To determine the sensitivity of the stain for routine work, a series of trials was carried out, each of one calendar month's duration:

Month 1. All specimens received were stained by the 'classical' Ziehl-Neelsen method, and only positives examined by the modified method.
Month 2. All specimens were stained in parallel by both hot methods.
Month 3. All specimens were stained by the modified method and only positive smears were confirmed by the 'classical' Ziehl-Neelsen method.

Results

In all methods the acid-fast bacilli stained red, whereas the background varied according to the counterstain. The use of Gabbett's counterstain overcame the problem of red/green colour blindness. With the cold stain, even experienced staff found difficulty decolourising the smears.

Table 1 shows the results of the comparability study. The slight discrepancy between the 'classical' Ziehl-Neelsen method and the modified method is consistent with sampling error. However, a marked difference was seen between the two hot methods and the cold method.

Table 2 shows the results of the trial where all specimens were stained in parallel by both hot methods. Of the 122 specimens examined, 104 were smear-negative/culture-negative, and 16 were smear-positive/culture-positive; the remaining two were smear-positive by the modified method, but negative by both the 'classical' Ziehl-Neelsen method and on culture. All smear-positive specimens were examined by the cold method and only one of the 19 was positive (5.3%).

In addition, 209 specimens were examined solely by the 'classical' Ziehl-Neelsen stain, of which 207 were smear-negative/culture-negative, and two were smear-negative/culture-positive. Similar results were obtained in the 195 specimens examined by the modified method alone, with 193 smear-negative/culture-negative and two smear-negative/culture-positive.

Discussion

Many different staining methods have been described since Koch's original method,[5] the majority having disappeared into oblivion. One of the few exceptions is Neelsen's[6] modification of the original stain of Ziehl.[7]

Table 1. Comparison of the number of organisms seen in dilutions of a known positive specimen against the type of staining technique used

		Dilution of positive smear						
Staining method	Smear	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
'Classical' Ziehl-Neelsen	1	++	++	+	+	+	±	±
	2	++	++	+	+	±	±	−
	3	++	++	++	+	+	−	−
	4	++	++	+	+	±	±	−
	5	++	++	++	±	±	±	−
Modified Ziehl-Neelsen	6	++	++	++	+	±	±	−
	7	++	++	++	±	±	±	±
	8	++	++	++	+	+	±	−
	9	++	++	+	+	±	−	−
	10	++	++	++	+	±	−	−
Cold method	11	+	±	−	−	−	−	−
	12	+	±	−	−	−	−	−
	13	+	±	−	−	−	−	−
	14	±	±	−	−	−	−	−
	15	±	−	−	−	−	−	−

++ = more than 10 bacilli *per* 10 hpf: + = 1–9 bacilli *per* 10 hpf: ± = < 1 bacillus *per* 10 hpf: − = bacilli NOT seen

Table 2. Results of staining by both 'classical' and modified Ziehl-Neelsen methods in 122 specimens

	Mycobacterium tuberculosis culture	
Stain	Positive	Negative
'Classical' and modified *positive*	16	0
'Classical' *positive*, modified *negative*	0	0
'Classical' and modified *negative*	0	104
'Classical' *negative*, modified *positive*	0	2

This staining method is still used in the majority of laboratories as the primary microscopy method to detect mycobacteria, or as confirmation of another staining technique.

During the 1970s and early 1980s this laboratory used the cold staining method described by Tan Tham Hok.[1] The advantage was that smears could be left unattended during the staining period. Although the author claimed that Kinyoun's carbol fuchsin could be used cold for three minutes, Engbaek[8] considered it necessary to use the stain for a minimum of three hours to ensure adequate staining of the bacilli. Despite these reservations, Collins[9] suggested using a cold staining method with a one minute counterstain (Gabbett's). In our experience, insufficient decolourisation always occurred at under three minutes.

The longer method for the 'classical' Ziehl-Neelsen technique was used because of difficulties in obtaining consistent results when 3% hydrochloric acid in ethanol (acid-alcohol) was used as the decolourising agent. Comparison with a fluorescent stain was not made because of unfamiliarity with the technique. The comparative study indicated that if heat is applied during the initial staining there is little variation in the results. However, if the primary stain is used cold the number of stained bacilli is noticeably reduced.

During the three month trial, 527 smears were examined by at least one of the hot staining methods: of these, 16 (3%) were smear-positive/culture-positive and 504 (95%) were smear-negative/culture-negative. Of the remaining seven, four were smear-negative but culture-positive. Three were sputa, two from patients already on treatment and the third was the second of a series of three sputa, the previous and subsequent samples being smear- and culture-positive. This result was due to the quality of the specimen. The remaining smear-negative/culture-positive specimen was a lymph node biopsy, culture-positive at ten weeks, the patient already receiving anti-tuberculosis chemotherapy. Of the remaining three specimens from the original group of seven, two

were smear-positive by the modified method but negative by the 'classical' Ziehl-Neelsen stain and culture-positive. These were sputa from a patient already on anti-tuberculosis treatment because of an earlier slide positive result and the clinical picture. The remaining specimen, which was smear-positive by both hot staining methods and culture-negative, was a post-mortem lung. This result was probably a failure to isolate antibiotic-damaged mycobacteria.

The results emphasise the unreliability of using cold staining methods for acid-fast bacilli. The modified Ziehl-Neelsen stain is still used as the primary staining method in this laboratory. Many unqualified staff have worked in the area and have been responsible, under supervision, for staining the smears. All appeared to have little difficulty mastering the technique.

With the increasing emphasis on time-saving in the laboratory, and the employment of unqualified staff, there is a necessity to assess critically any staining method or technique before it is adopted and also a need to scrutinise carefully all current methods. This report highlights the requirement for a continuous review of any current quality control checks used.

References

1 Tan Tham Hok. A simple and rapid cold-staining method for acid-fast bacilli. *Am Rev Respir Dis* 1962; 85:703–4.

2 Advisory Committee on Dangerous Pathogens. *Categorisation of Pathogens According to Hazard and Categories of Containment*. 2nd edn. London: HMSO, 1990.

3 Cruickshank R, Duguid JP, Marmion BP, Swain RHA. *The Practice of Medical Microbiology*. Vol. 2. 12th edn. Edinburgh: Livingstone, 1975; 38.

4 Gabbett HS. Letter. *Lancet* 1887; 2:1090.

5 Koch R. Die aetiologie der Tuberculose. *Berliner Klinische Wochenschrift* 1882; 19:221–30.

6 Neelsen F. Ein casuistischer Beitrag zur Lehre von der Tuberkulose. *Centralblatt fur die medizinischen Wissenschaften* 1883; No. 28: 497.

7 Ziehl F. Zur Färbung der Tuberkelbacillus. *Deutsche Medizinische Wochenschrift* 1882; 8:451–4.

8 Engbaek HC, Bennedsen J, Christensen S. Comparison of various staining methods for the demonstration of tubercle bacilli in sputum by direct microscopy. *Bull Int Union Tuberc* 1967; 42:94–110.

9 Collins CH, Grange JM, Yates MT. *Organization and Practice in Tuberculosis Bacteriology*. London: Butterworth, 1985; 41.