

## ORIGINAL ARTICLE

## Optimisation of acid fast smears for the direct detection of mycobacteria in clinical samples

S J Murray, A Barrett, J G Magee, R Freeman

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See end of article for authors' affiliations

Correspondence to:  
Dr J G Magee, Regional  
Centre for  
Mycobacteriology, Public  
Health Laboratory, Institute  
of Pathology, Newcastle  
General Hospital,  
Westgate Road, Newcastle  
upon Tyne NE4 6BE, UK;  
newjmage@north.phls.nhs.uk

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**Aims:** Despite its long history, the acid fast smear remains unstandardised. Technical variations in both the preparation of clinical material and subsequent staining mean that smear sensitivity relative to culture may vary from 50% to over 80%. This study assessed the sensitivity of acid fast microscopy at each of five stages of sample preparation and by both commonly used staining methods.

**Methods:** Sputum samples thought for varying reasons to be highly likely to be culture positive were used to prepare a series of smears in which the effects of digestion (liquefaction), concentration (centrifugation), and decontamination (sodium hydroxide) could be assessed, together with a comparison of staining by the auramine/phenol and Ziehl-Neelsen techniques.

**Results:** The most effective method for the demonstration of acid fast organisms in sputum was found to be an auramine phenol stain applied to a liquefied, concentrated sample and examined before the decontamination process.

**Conclusions:** The auramine phenol stain applied to a liquefied, concentrated sample and examined before the decontamination process is the most effective method for the demonstration of acid fast organisms in sputum.

The acid fast smear is cheap and rapid and remains a cornerstone of the laboratory diagnosis of tuberculosis. It is particularly useful in the early detection of disease and, when positive, defines the more infectious cases. However, despite its application for over 120 years, this simple technique remains non-standardised, let alone optimised. Variations in both the preparation of clinical material and in the subsequent staining methods result in smear sensitivity relative to culture varying from 50% to over 80%.<sup>1</sup>

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Samples may or may not be digested (liquefied), the digested sample may or may not be concentrated (by sedimentation or centrifugation), and microscopic examination of the resultant material may or may not be undertaken before decontamination with NaOH. Furthermore, although the ease and increased sensitivity of fluorescent staining using auramine/phenol (AP) staining is well established,<sup>2,3</sup> many laboratories adhere to the less sensitive Ziehl-Neelsen (ZN) staining technique. Existing quality control schemes currently test the ability to stain and detect mycobacteria provided as fixed smears, but not the methods by which samples are processed before staining. As a result, laboratories may score well in external quality control assessments and yet suffer a low smear to culture ratio.

## METHODS

Sputum samples from patients with a high clinical probability of tuberculosis but who were not under treatment at the time of sampling, and from patients known to be colonised with non-tuberculous mycobacteria, were identified as they underwent normal processing in the laboratory. Where sample size permitted, part of the sample was set aside and stored at 4-8°C, pending the results of culture. Routine processing in this laboratory comprises the following steps:

- (1) neat sputum
- (2) liquefaction (dithiothreitol)

- (3) concentration (centrifugation)
- (4) decontamination (4% NaOH) followed by neutralisation (K<sub>2</sub> PO<sub>4</sub>)
- (5) centrifugation followed by culture for mycobacteria

The liquefaction agent used is dithiothreitol (Sputasol™, Oxoid Ltd, Basingstoke, UK), which we feel to be superior to N-acetyl-cysteine.<sup>4</sup> Centrifugation is carried out at 3000 *xg* for 20 minutes at both stages 3 and 5. All samples are cultured in the BacT/ALERT 3D automated mycobacterial system and simultaneously on Löwenstein-Jensen slopes, in each case for up to eight weeks. In practice, all positive samples included in our study yielded mycobacterial growth within 28 days. All mycobacterial isolates were identified by standard methods.

Once the results of culture were known, 78 sputum samples were identified from which a mycobacterium had been isolated. The stored material from these specimens was then, in each instance, re-processed as already described, aliquots being removed for staining at each of the five numbered stages, giving a total of 390 aliquots. Two smears were made from each aliquot; one of which was stained by the ZN method,<sup>5</sup> and examined at  $\times 1000$  magnification by transmitted light. The other smear was stained by the AP method<sup>5</sup> and examined at  $\times 400$  magnification using a fluorescence microscope equipped for epifluorescence. In both instances, the numbers of acid fast bacilli seen were converted to a rods/field count. To compensate for the difference in field size of the two methods, 40 fields were examined for AP smears and 200 fields for ZN smears. Each smear was examined by two separate workers and graded<sup>6</sup> by consensus on the following scale: 0, acid fast bacilli not seen; < 1, < 1 rod/field (on average); 1-9, from one to nine rods/field; 10-90, between 10 and 90 rods/field; > 90, uncountable number, greater than 90/field.

Results were analysed and compared with the culture data.

**Abbreviations:** AP, auramine/phenol; MAC, *Mycobacterium avium* complex; MTBC, *Mycobacterium tuberculosis* complex; ZN, Ziehl-Neelsen

**Table 1** Numbers of acid fast bacilli detected in untreated samples

	(n=78)	Auramine (93%)				
		0	<1	1-9	10-90	>90
ZN (73%)	0	13	14			
	<1		12	15	2	
	1-9			1	15	
	10-90				3	3
	>90					

65 positive results by any method from 70 positives in all; detection rates noted in parenthesis.

**Table 2** Numbers of acid fast bacilli detected in liquefied samples

	(n=78)	Auramine (86%)				
		0	<1	1-9	10-90	>90
ZN (60%)	0	18	17	1		
	<1		14	10	2	
	1-9			5	9	1
	10-90				1	
	>90					

60 positive results by any method from 70 positives in all; detection rates noted in parenthesis.

**Table 3** Numbers of acid fast bacilli detected after liquefaction and concentration of samples

	(n=78)	Auramine (100%)				
		0	<1	1-9	10-90	>90
ZN (80%)	0	8	14			
	<1		8	18	1	
	1-9			1	14	1
	10-90			2	3	7
	>90					1

70 positive results by any method from 70 positives in all; detection rates noted in parenthesis.

## RESULTS

Of the mycobacteria isolated from the 78 culture positive sputum samples, 52 were members of the *Mycobacterium tuberculosis* complex (MTBC), 12 were of the *M avium* complex (MAC), nine were *M malmoense*, four were *M kansasii*, and one was *M xenopi*. The possibility of cell wall deterioration leading to reduced smear sensitivity was considered even though samples were stored at low temperature before inclusion in our study. However, 70 of the 78 samples were smear positive by at least one method, so that storage did not appear to impact on the results. Mycobacteria isolated from the eight smear negative, culture positive samples comprised three MTBCs, one MAC, three *M kansasii*, and one *M malmoense*. Tables 1-5 detail the results of the smear comparisons.

In all instances, smears stained by the AP method showed a higher level of detection of acid fast bacilli than those stained by the ZN method, and higher counts of acid fast rods within positive smears were noted using the AP method. The highest detection rate by both methods was in the examination of liquefied and concentrated samples (table 3), and the lowest detection rate by both methods was in the examination of post-NaOH treated samples (tables 4, 5). Material examined after NaOH decontamination, whether concentrated or not, gave particularly poor results in comparison with other methods, and samples were more difficult to fix to slides.

**Table 4** Numbers of acid fast bacilli detected in NaOH treated samples

	(n=78)	Auramine (40%)				
		0	<1	1-9	10-90	>90
ZN (7%)	0	50	23			
	<1		1	1		
	1-9			2	1	
	10-90					
	>90					

28 positive results by any method from 70 positives in all; detection rates noted in parenthesis.

**Table 5** Numbers of acid fast bacilli detected after NaOH treatment and concentration of samples

	(n=78)	Auramine (83%)				
		0	<1	1-9	10-90	>90
ZN (44%)	0	20	22	4	1	
	<1		6	10	1	
	1-9			1	10	
	10-90				2	1
	>90					

58 positive results by any method from 70 positives in all; detection rates noted in parenthesis.

Comparatively poor results were also obtained in the examination of neat (that is, untreated sputum).

## Statistical analysis

The real results were compared with the expected results for the null hypothesis of no difference between the methods (and between the treatment regimens) using the  $\chi^2$  test. The AP method was significantly better than the ZN method with all pretreatment regimens and the differences between pretreatment regimens were also significant. The liquefied and concentrated samples performed best for both staining methods. All differences were significant at  $p > 0.01$ —for example, AP versus ZN on liquefied and concentrated samples,  $\chi^2 = 15.3$  with 4 degrees of freedom,  $p = 0.0041$ .

## DISCUSSION

Our results strongly suggest that the optimum detection of acid fast organisms is achieved by the application of auramine/phenol fluorescent staining to digested (liquefied) and concentrated samples. The consistent superiority of the AP stain over the ZN stain in all the settings tested shows that the common practice of trying to “confirm” a positive AP smear by ZN is unscientific.

“A suboptimal smear performance may significantly compromise or delay isolation and contact tracing measures”

Two other important points must be borne in mind. First, it has been suggested that smear positivity should be used as the criterion for selecting sputum specimens for examination by continuous automated mycobacterial liquid culture,<sup>7</sup> a much more rapid method for mycobacterial isolation, the inference being that smear negative samples will, by default, receive conventional solid culture. Suboptimal smear performance will mean that some patients' samples that should receive immediate attention do not do so, and diagnosis will be

### Take home messages

- The auramine phenol stain applied to a liquefied, concentrated sample and examined before the decontamination process was the most effective method for the demonstration of acid fast organisms in sputum
- All laboratories should attempt to standardise and optimise their the acid fast technique

delayed. Second, the smear is often used as an index of infectivity. It is evident that smear positive patients will be more infectious than smear negative patients. However, in studies in which the smear positivity versus the culture rate is over 85%,<sup>8</sup> a clear risk of transmission from smear negative culture positive patients has been demonstrated. A suboptimal smear performance may significantly compromise or delay isolation and contact tracing measures.

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### Authors' affiliations

S J Murray, A Barrett, J G Magee, R Freeman, Regional Centre for Mycobacteriology, Public Health Laboratory, Institute of Pathology, Newcastle General Hospital, Westgate Road, Newcastle upon Tyne NE4 6BE, UK

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