

## Bulk staining of smears: no demonstrated risk of bacilli transfer from a positive to a negative smear

D. Affolabi,<sup>\*\*</sup> M. Odoun,<sup>\*</sup> C. N. Sanoussi,<sup>\*</sup> B. Tanimomo-Kledjo,<sup>\*</sup> D. Saïzonou,<sup>\*</sup> K. Soumaïla,<sup>\*</sup> L. Kestens,<sup>‡</sup> S. Y. Anagonou,<sup>\*</sup> F. Portaels<sup>†</sup>

<sup>\*</sup> Laboratoire de Référence des Mycobactéries, Cotonou, Bénin; <sup>†</sup> Mycobacteriology Unit, Institute of Tropical Medicine, Antwerp, <sup>‡</sup> Immunology Unit, Institute of Tropical Medicine, Antwerp, Belgium

### SUMMARY

Despite a theoretical risk of transfer of bacilli from a positive to a negative smear, bulk staining is routinely performed in many laboratories. To assess this risk in our laboratory, two smears were made from each sputum specimen and stained with auramine: one smear was stained on a rack and the second using the bulk method. Smears were read blind using a fluorescence microscope.

A total of 811 sputum specimens were analysed. No acid-fast bacilli transfer was observed even when staining solution jars had not been renewed for 3 days. Bulk staining is rapid and cheap, and could be used in laboratories with a high workload in low-resource settings.

**KEY WORDS:** auramine; acid-fast bacilli; bulk staining

ALTHOUGH there may be no published evidence, recommendations for sputum smear microscopy for tuberculosis (TB) state that bulk staining, rinsing, decolourising and counter-staining should be avoided because of the risk of transfer of acid-fast bacilli (AFB) from a positive to a negative smear, leading to a false-positive result.<sup>1</sup> However, despite these recommendations, bulk staining is routinely performed in many laboratories either manually or using a machine.<sup>2</sup> As this method is not recommended, there is no standard technique for bulk staining; for example, the maximum number of days and slides for which bulk staining solutions can be used is not known.

In the present study, we aimed to assess the risk of bacilli transfer from a positive slide to a negative slide. We also analysed the effect of using the stains for several days.

### MATERIALS AND METHODS

#### *Bulk staining with daily replaced stains*

A total of 572 consecutive sputum specimens (for diagnosis or follow-up) were analysed. The smear positivity rate and the proportion of scanty positive and 1+ smears among total positive smears were respectively 17.1% and 47%. Two smears were made from each sputum and care was taken to use the same part of the sputum where possible to make the two smears. The smears were then air-dried, heat-fixed by passing the slides through a flame three or four times with the

smear uppermost, and stained with auramine 0.3% for 10 min, alcohol 74% containing hydrochloric acid 1% for 4 min and potassium permanganate 0.1% for 30 s.<sup>3</sup>

One of the two smears was placed on a slide rack and stained individually by covering the slide with the stain for the required time (rack method, RM). The second smear was stained using the bulk method: 20 slides were simultaneously placed in a rack and left for the required time in containers filled with solutions that were renewed daily (bulk method 1, BM1). The potassium permanganate solution used was always freshly prepared and renewed daily. The slides were rinsed manually with tap water in a container between auramine staining and decolourisation, between decolourisation and counterstaining and after counterstaining. An average of 120 smears were stained per day.

After staining, smears were air-dried and read by fluorescence microscopy at 250× magnification using the internationally recommended scale.<sup>4</sup> Each slide was read blind by two technicians, i.e., the slides were read without knowledge of the staining method used or the result of the other technician. For each slide, results were compared between the two technicians. In case of discrepancy, the readings were repeated by both technicians. Only consensus results were considered. Before declaring a smear negative, one length of the smear (30 low-power fields) was read.

After comparison with RM, all discordant smears (using either with RM or BM1), were re-stained individually with the Ziehl-Neelsen (ZN) technique and

Correspondence to: Dissou Affolabi, Laboratoire de Référence des Mycobactéries, 01 BP 817 Cotonou, Bénin. Tel: (+229) 21 33 15 33. Fax: (+229) 21 33 70 57. e-mail: affolabi\_dissou@yahoo.fr

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carefully read by a biologist at 1000× magnification in brightfield (600 fields).

#### *Bulk staining with fresh solutions simulating high transfer risk*

To simulate a high-risk situation of cross-contamination, 18 smears from sputum samples known to be highly positive (>100 AFB per 250× field) were stained with BM1, together with two smear samples known to be negative. This was repeated for 19 highly positive and one negative sputum samples. All these negative smears were randomly placed between positive smears before staining.

#### *Bulk staining with solutions used for 3 days*

For 199 random sputum specimens, the second smear was stained following the bulk method using auramine and acid alcohol solutions that had already been used for 3 days (bulk method 2, BM2); the stains were covered with a lid every day after use. After comparison with RM results, all discordant smears (using either RM or BM2) were re-stained individually with ZN and carefully read by a biologist at 1000× magnification in brightfield (600 fields).

## RESULTS

#### *Bulk staining with daily replaced solutions*

The comparison between RM and BM1 is summarised in Table 1. Of the 572 sputum specimens, seven were discordant between the two staining methods: three were positive with BM1 but negative with RM, while four were positive with RM but negative with BM1.

The three specimens positive with BM1 but negative with RM were confirmed to be positive by both methods in ZN. Of the four specimens positive with RM but negative with BM1, three were confirmed positive by both methods in ZN. Only one specimen that was scanty positive with RM and negative with BM1 remained negative in ZN by both methods.

**Table 1** Comparison between results of smears stained with BM1 and RM

BM1	RM					Total
	Negative	Scanty*	1+	2+	3+	
Negative	470	4	0	0	0	474
Scanty	1	5	7	3	1	17
1+	1	3	10	13	2	29
2+	1	2	9	13	4	29
3+	0	0	1	2	20	23
Total	473	14	27	31	27	572

\* Scanty = 1–29 AFB/30 LPF; 1+ = 1–9 AFB/LPF; 2+ = 10–100 AFB/LPF; 3+ = >100 AFB/LPF.

BM1 = bulk method with daily replaced stains; RM = rack method; AFB = acid-fast bacilli; LPF = low-power field.

**Table 2** Comparison between results of smears stained with BM2 and RM

BM2	RM					Total
	Negative	Scanty*	1+	2+	3+	
Negative	161	1	0	0	0	162
Scanty	1	2	2	0	0	5
1+	1	1	3	8	0	13
2+	0	0	5	3	4	12
3+	0	0	0	0	7	7
Total	163	4	10	11	11	199

\* Scanty = 1–29 AFB/30 LPF; 1+ = 1–9 AFB/LPF; 2+ = 10–100 AFB/LPF; 3+ = >100 AFB/LPF.

BM2 = bulk method with daily replaced stains; RM = rack method.

#### *Bulk staining with highly positive smears*

All three negative smears remained negative even when the whole smear was read.

#### *Bulk staining with solutions used for 3 days*

The results of the comparison between RM and BM2 are summarised in Table 2. Of the 199 specimens, two were positive with BM2 but negative with RM, while one specimen was positive with RM and negative with BM2.

Of the two specimens positive with BM2 but negative with RM, one was confirmed positive with both methods after ZN re-staining. The second specimen remained positive with the BM2 smear and negative with the RM smear. However, another smear from the same specimen individually stained with ZN was positive. The specimen positive with RM but negative with BM2 was confirmed positive by both staining methods in ZN.

Considering together all smears stained with RM, BM1 and BM2 (Tables 1 and 2), the same number of positive specimens (134) was detected using bulk versus individual staining. Quantifications were also similar.

## DISCUSSION

Bulk staining has always been prohibited because of the theoretical risk of false-positive results, without any scientific basis. To the best of our knowledge, no publications have documented its occurrence. On the contrary, all studies published to date suggest that the risk may be very low.<sup>2,5</sup> The results obtained in our study suggest that this risk is negligible. If the smear is well fixed, the risk of AFB dislodged from a positive smear becoming fixed on another smear, remaining there throughout the staining process, and being detected in the limited number of fields read, may be very low.

The maximum number of days during which the solutions can be used not known. We found that auramine and acid alcohol could be used with consistently good results for 3 days, after which the level of the solutions became too low; topping up or renewing was therefore necessary. The potassium permanganate

solution was replaced every day, as oxidation causes a background, rendering reading difficult. We therefore recommend daily changing of the solution.

Bulk staining is cheap, as 250 ml of each solution (auramine and acid alcohol) could be used for 120 slides for an average of 3 days (360 slides), which is about seven times less the solution needed for RM. However, the main advantage of the bulk staining is that the method is rapid and less laborious, and it could be useful for busy laboratories where staining quality is often poor. As shown by Fodor in 1984, this method could also be used for ZN.<sup>5</sup>

## CONCLUSION

No transfer of bacilli was observed in our study. The bulk method could be used safely and efficiently in laboratories with a high workload. Our findings could therefore contribute to the re-evaluation of international recommendations on bulk staining.

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## RÉSUMÉ

En dépit d'un risque théorique de transfert de mycobactéries d'un frottis positif à un frottis négatif, plusieurs laboratoires utilisent en routine la coloration en cuve pour la recherche des bacilles acido-alcool-résistants au microscope. Dans le but d'évaluer ce risque dans notre laboratoire, nous avons réalisé deux frottis par échantillon de crachat, tous deux colorés à l'auramine : un frottis coloré en utilisant un pont de coloration et l'autre

coloré en cuve avec d'autres frottis. Les frottis ont été ensuite lus à l'aveugle au microscope à fluorescence. Au total, 811 échantillons de crachats ont été testés. Aucun transfert de mycobactéries n'a été mis en évidence même lorsque les flacons de colorants n'ont pas été renouvelés depuis 3 jours. La coloration en cuve est rapide et peu coûteuse. Elle convient donc aux laboratoires ayant une charge élevée de travail dans les pays à faibles revenus.

## RESUMEN

Pese al riesgo teórico de traspaso de bacilos de un frotis de esputo positivo a uno negativo, en muchos laboratorios se utiliza en forma sistemática la coloración simultánea de múltiples muestras para baciloscopia. Con el objeto de evaluar este riesgo, se realizó un estudio de 811 muestras de esputo analizando dos frotis por cada muestra, mediante tinción con auramina. El primer frotis se tiñó solo en una rejilla y el segundo en una rejilla con

múltiples muestras. Se llevó a cabo la lectura con anonimato de las laminillas en un microscopio de fluorescencia. No se observó ninguna transferencia de bacilos acidorresistentes, aun cuando no se renovaran las cubetas con soluciones de tinción durante 3 días. La tinción múltiple es rápida y de bajo costo y se puede emplear en laboratorios con un alto ritmo de trabajo en medios con recursos limitados.