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Bulk acid-fast staining of sputum smears: time to end a taboo

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SUMMARY

SETTING: A high-throughput laboratory routinely performing fluorescence microscopy for acid-fast bacilli (AFB) smear with automated bulk staining.

OBJECTIVES: To determine the risk of false-positive AFB sputum smears from bulk staining showing as smear-positive, culture-negative specimens, or a decrease in smear- and culture-positives.

DESIGN: Direct AFB smear and Löwenstein-Jensen culture were performed for a total of 39 350 routine sputum specimens. Of these, 6633 were randomly selected for individual AFB staining, while the remaining 32 717 were processed by bulk machine staining. Positives for smear and culture were compared.

RESULTS: Overall, 111 specimens yielded a positive individually stained smear; of these, 100 (90.1%, 95%CI 83.0–95.0) were also culture-positive compared to 504/543 smear-positives after bulk staining (92.8%, 95%CI 90.6–95.0). The proportions of smear-positive, culture-negative and smear- and culture-positive specimens were respectively 1.8% vs. 2.2% and 90.1% vs. 92.8%, for individual and bulk staining (non-significant).

CONCLUSIONS: The risk of transferring AFB from positive to negative smears during bulk AFB staining is negligible, if it occurs at all. Bulk staining should not be discouraged, as even in low-income countries this method will save significant resources, particularly manpower, and improve staining results in laboratories with a high workload.

KEY WORDS: microscopy; fluorescence; staining; tuberculosis; cross-contamination

FLUORESCENCE MICROSCOPY (FM) using light-emitting diode (LED) lamps holds considerable promise for optimising the detection of acid-fast bacilli (AFB). These robust and user-friendly systems may allow widespread, permanent use of the more efficient, and often also more effective, FM technique.[1,2] However, guidelines for the use of FM in the field, and for its quality assurance under such conditions, are not yet well developed, and several questions and controversies remain. As it is primarily the largest laboratories of the network that are concerned, where sheer workload and not lack of expertise constitutes the main obstacle to obtaining high quality results, optimisation of each of these details is clearly necessary.

An important issue concerns bulk (manual or automated) staining. The World Health Organization (WHO) and International Union Against Tuberculosis and Lung Disease guidelines recommend that smears be processed individually, with sufficient distance on staining racks, etc., to prevent transfer of AFB from true-positive to negative smears.[3,4] This recommendation seems to apply only to AFB microscopy networks in low-income countries, as bulk staining is widespread in laboratories in high-income countries, including some renowned mycobacterium reference laboratories. The obvious advantages are considerable reductions in workload and salary costs, as well as the better quality, at least for automated machine staining, reported by some reputed groups.[5] Furthermore, to the best of our knowledge no publications have documented AFB transfer between smears resulting in false-positives. This issue may be of particular interest for FM, not only because it often concerns high-volume laboratories, but also because the cold auramine staining technique is more readily adapted to bulk or machine staining.

In this study, we have tried to estimate the rate of AFB transfer from positive to negative smears by comparing the frequency of smear-positive, culture-negative specimens in a prospective study using individual vs. bulk machine staining. This would be the result of cross-contamination alone affecting smears but not the sputum samples used for culture; it is also known to occur in a small proportion of smear-positives due to non-viable AFB, over-harsh decontamination or culture failure, and it is thus often designated as false-negative culture. It can be frequent when patients take certain specific drugs, particularly rifampicin, before sample collection.[6] Although these common causes would not readily be distinguishable

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from false-positive smears due to AFB transfer during bulk staining, the rate of smear-positive, culture-negative results (from any cause) would be significantly higher if the risk of such a transfer existed.

Our study was performed as part of routine practice in the Hong Kong Supranational Tuberculosis Reference Laboratory, which is equipped with high quality FM as well as culture, has an extremely high workload (about 8000 specimens per month), and bulk machine staining has been a routine technique for about 20 years.

MATERIALS AND METHODS

Samples

A total of 6633 sputum specimens were randomly selected from routine sputum specimens (n = 39 350) for individual staining between November 2007 and May 2008. The other 32 717 specimens were stained by the bulk machine staining method. Briefly, direct smears were prepared from sputum using a 10 μl plastic disposable loop. All smears were air-dried and then heat-fixed by passing the slides through a flame several times, smear side up.

Bulk staining

Bulk staining was performed using a staining machine (Leica ST 5020, Solms, Germany). A total of 30 slides were loaded onto 500 ml staining buckets containing different staining solutions and distilled water in the following order: 0.3% auramine-O in 3% phenol for 10 min, distilled water (DW) for 1 min, 1% acid alcohol for 1 min, DW for 1 min, 1% acid alcohol for 4 min, DW for 1 min, 0.1% potassium permanganate for 1 min, DW for 1 min. All solutions and DW were renewed daily, and an average of 200 smears were stained in the same solutions.

Individual staining

Stains and solutions used for individual staining were obtained from the same source as for bulk machine staining. Smears were first stained with auramine-O for 15 min, rinsed with water, destained with acid alcohol for 5 min, rinsed with water again and finally counterstained with potassium permanganate for 1 min.

AFB smear examination

After staining, smears prepared from bulk or individual staining were air-dried inside a 65°C hot air oven before microscopic examination. During the study period, well trained laboratory staff performed AFB smear microscopy. Slides prepared from bulk or individual staining were distributed to the technicians for blind examination using mercury vapour lamp fluorescence microscopes (Zeiss, Gottingen, Germany). Briefly, all smears were first screened using a 20× objective lens. Whenever suspected AFB were spotted, 63× objective magnification was used for confirmation. A positive smear was defined as at least three AFB in 300 optical fields. All positive smears were re-examined by another medical technologist with more than 10 years' experience in AFB smear microscopy. The same technicians were responsible for staining, microscopy and culture processing for both individual and bulk staining.

AFB culture and identification

Standard *N*-acetyl-L-cysteine-sodium hydroxide methods for AFB culture and *Mycobacterium tuberculosis* identification were followed for all specimens after AFB smear had been performed.[7,8] Any number of AFB smear-positive colonies were interpreted as a positive culture.

Statistical analysis

Parameters, such as proportions of smear- and culture-positive, *M. tuberculosis*-positive and smear-positive, culture-negative (culture false-negative) were compared for smears prepared by bulk and individual staining. Pearson's χ^2 test was used to determine the significance of the differences between the proportions. The study hypothesised that culture false-negatives would occur significantly more often in the machine staining arm as a consequence of AFB transfer. Assuming a maximum of 2% culture false-negatives using individual staining, an increase to 5% (possibly due to false-positive smears) was set as the maximum acceptable loss of smear specificity, given the considerable reduction in workload, and possibly also increased smear sensitivity.

RESULTS

All specimens submitted for diagnosis by presumably untreated patients were included in the analysis.

Table 1 shows the number of patients enrolled and results of AFB smear and culture by staining method (individual or bulk machine-stained). A total of 6633

Table 1 Numbers of specimens and results of microscopy and culture by staining process

Staining process	Total enrolled *n*	Smear-positive *n* (%)	Culture-positive *n* (%)	Culture-negative *n* (%)	Culture-contaminated *n* (%)
Individual*	6633	111 (1.7)	494 (7.5)	5853 (88.2)	286 (4.3)
Bulk†	32 717	543 (1.7)	2523 (7.7)	28 875 (88.3)	1319 (4.0)

*Smears stained individually and well separated on a staining rack.
†Smears stained in batches by machine in staining jars, using the same solutions.

Table 2 Results of culture for microscopy-positive specimens only by staining process

Staining process	Total smear-positive *n*	Culture-positive *n* (%)	Culture-negative *n* (%)	Culture-contaminated *n* (%)	*M. tuberculosis* positive *n* (%)
Individual*	111	100 (90.1)	2 (1.8)	9 (8.1)	74 (66.7)
Bulk†	543	504 (92.8)	12 (2.2)	27 (5.0)	388 (71.5)

*Smears stained individually and well separated on a staining rack.
†Smears stained in batches by machine in staining jars, using the same solutions.

smears were stained individually, of which 111 (1.7%) were positive compared to 32 717 stained in bulk, resulting in 543 (1.7%) positives. The proportions of mycobacteria positive, negative or contaminated cultures were also very similar in both study arms; no significant difference could be detected despite the large numbers involved. Smear-negative specimens yielded respectively 6.0% and 6.3% culture-positives in the individual and bulk staining arms.

Table 2 shows the same analysis, focusing on smear-positive specimens only. Of the 111 positive individually stained smears, 100 (90.1%, 95% confidence interval [CI] 83.0–95.0) were also culture-positive, compared to 504/543 smear-positives using bulk staining (92.8%, 95%CI 90.6–95.0). The proportion of false-negative cultures (1.8%, 95%CI 0.2–6.4 using individual staining vs. 2.2%, 95%CI 1.1–3.8 using bulk staining) was almost the same for both arms. The proportions of *M. tuberculosis* isolates (66.7% vs. 71.5%) and contaminated cultures (8.1 vs. 5.0%) varied slightly more. None of the differences reached statistical significance. Of the specimens yielding false-negative cultures, 10 were 1+ on smear (two individually stained and eight bulk-stained), while only two showed scanty AFB and two more had 2+ grading, all in the bulk staining arm.

Individual staining required approximately 2–3 times more staining solutions and 5–7 times more working hours than bulk staining.

DISCUSSION

Our results, obtained in an extremely busy mycobacterium reference laboratory, demonstrate that the transfer of AFB from positive to negative smears due to bulk staining occurs rarely, if at all. Although the proportion of false-negative cultures was slightly higher, positive cultures were also more frequent with bulk staining, and the main difference was in the contamination rate. None of the parameters analysed differed significantly, implying that smears from both arms were comparable. The higher rate of smear and culture positivity after bulk staining suggests that the higher rate of smear-positive, culture-negatives can be explained by the lower contamination rate, rather than by a transfer of bacilli between smears. Moreover, the increase in false-negative cultures (smear-positive, but culture-negative), which might indicate AFB transfer during bulk staining, reached only 0.4%, a very small difference well compensated for by the resources saved in term of stains and manpower. This proportion of possible false-positives created is negligible compared to those reported for other standard diagnostic methods, such as chest radiography, particularly in the current context, where there is strong emphasis on increased case detection.[9] The identical proportion of smear-positive specimens suggests that the quality of staining and microscopy was also the same in both arms.

Our study has explored yet another line of evidence attempting to quantify the much-feared but never documented carry-over of AFB during the staining process. As we were unable to distinguish the phenomenon from other possible causes of smear-positive, culture-negative results in an analysis comprising 39 350 specimens processed by smear and culture, we can only agree with previous researchers that AFB transfer does not occur to a detectable extent. Most recently, Affolabi et al. did not report any evidence of AFB transfer in studies using several hundreds of duplicate smears stained either individually, in batches, or from one or two negative smears inserted in a batch of about 20 heavy positives and all stained in one batch.[10] In an earlier report, Fodor studied positive bulk-stained smears for their positivity in culture, complemented by further smears, history or even autopsy evidence, and could confirm all positives from 118 batches of bulk staining.[11] Mitchison et al. defended bulk machine staining of sputum smears because of its better quality, the lower cost, and the lack of any detectable AFB transfer using duplicate smears stained by either method.[5] Finally, while the risk of AFB transfer has been repeatedly mentioned as an important source of false-positive smears in reputed guides and handbooks,[8,12] to the best of our knowledge no study has ever proven its occurrence, let alone at a frequency sufficiently high to justify prohibiting this simplification.

Our study was limited by the relatively low number of smear-positive cases detected, particularly when using individual staining, in spite of the large number of suspects screened. With these numbers, significance would have been reached only by an excess of at least 5% false-negative cultures. However, this can not be a serious argument against the validity of our study, considering that the trend observed for

bulk staining was towards not fewer but more true positives (smear- and culture-positive specimens).

CONCLUSIONS

Considering the many difficulties encountered in AFB microscopy laboratories operating under field conditions, all possible efforts should be made to remove the real obstacles. Conversely, it would be a serious error to impose unjustified and unnecessary restrictions. It has always been universally accepted that high quality microscopy is impossible once numbers exceed 25–30 AFB smears per day, but strangely, for staining, the same principle seems to have been applied only in industrialised countries. Current and past evidence shows that, with a higher workload, bulk staining of AFB smears can also be practised universally without a significant risk of increased false-positives.

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RÉSUMÉ

CONTEXTE : Un laboratoire à haut débit pratiquant en routine l'examen microscopique par fluorescence à la recherche des bacilles acido-résistants (BAAR) dans les frottis avec une coloration de groupe de lames automatisée.
OBJECTIFS : Dans les frottis de crachats provenant de colorations de groupes de lames, déterminer le risque de bacilloscopies faussement positives se présentant sous forme d'échantillons à bacilloscopie positive et à culture négative ou sous forme d'une diminution des positifs au frottis et à la culture.
SCHÉMA : On a pratiqué en routine un frottis direct des BAAR et une culture de Löwenstein-Jensen sur un total de 39 350 échantillons en routine. Parmi ceux-ci, 6633 ont été sélectionnés au hasard pour une coloration individuelle des BAAR, alors que les 32 717 restants ont été traités par une coloration de groupe de lames automatisée. On a comparé les résultats positifs en culture et en frottis.
RÉSULTATS : Dans les frottis colorés individuellement, il y a eu 111 échantillons positifs, parmi lesquels 100 (90,1%) ont été également positifs à la culture (IC95% 83,0–95,0) ; d'autre part, parmi les 543 frottis positifs après coloration des lames groupées, 504 (92,8%, IC95% 90,6–95,0) étaient également positifs à la culture. Les proportions d'échantillons à bacilloscopie positive, culture négative ont été de 1,8% après coloration individuelle vs. 2,2% pour les colorations des lames groupées et les proportions d'échantillons à bacilloscopie positive, culture positive de 90,1% pour les colorations individuelles vs. 92,8% pour les colorations des lames groupées (non significatif).
CONCLUSIONS : Le risque de transfert de BAAR d'un frottis positif à un frottis négatif au cours des colorations de lames en groupe pour BAAR est négligeable, s'il existe. La coloration de lames en groupe ne doit pas être découragée, puisque même dans les pays à faible revenus, cette méthode permettra d'épargner une importante quantité de ressources, particulièrement sur le plan humain, et d'améliorer les résultats des colorations dans les laboratoires où la charge de travail est élevée.

RESUMEN

MARCO DE REFERENCIA : Un laboratorio de tuberculosis de alto rendimiento, donde se practica en forma corriente la microscopia de fluorescencia para bacilos acidorresistentes (BAAR) usando un sistema automatizado de tinción múltiple.

OBJETIVOS : Determinar el riesgo de obtener resultados positivos falsos en las baciloscopias de esputo preparadas por tinción múltiple ; este riesgo se puede evaluar en función del número de muestras con baciloscopia positiva y cultivo negativo o de una disminución de la proporción de baciloscopias positivas con cultivo positivo.

MÉTODOS : Se practicaron BAAR directa y cultivo en medio de Löwenstein-Jensen de 39 350 muestras sistemáticas de esputo. De estas muestras, 6633 escogidas aleatoriamente se tiñeron en forma individual y las 32 717 restantes se procesaron en tinción múltiple automatizada. Se compararon los resultados positivos de las baciloscopias y los cultivos.

RESULTADOS : Se obtuvieron 111 baciloscopias positivas con las muestras teñidas individualmente, de las cuales 100 (90,1% ; IC95% 83,0–95,0) presentaron también cultivo positivo. De las 543 muestras con baciloscopia positiva en la tinción múltiple, 504 presentaron cultivo positivo (92,8% ; IC95% 90,6–95,0). La proporción de muestras con baciloscopia positiva y cultivo negativo fue 1,8% en la tinción individual y 2,2% con la tinción automatizada ; la proporción de baciloscopias positivas con cultivo positivo fue 90,1% en la tinción individual y 92,8% en la tinción automatizada (diferencia no significativa).

CONCLUSIONES : El riesgo de transferencia de BAAR de los frotis positivos a los negativos durante la tinción múltiple es insignificante, si acaso existe. No habría razón para desaconsejar este método de tinción, pues incluso en países de bajos ingresos esta técnica ahorra recursos de manera considerable, sobre todo en materia de mano de obra y además mejora los resultados de la tinción en los laboratorios con alto rendimiento.