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Bleach sedimentation method for increased sensitivity of sputum smear microscopy: does it work?

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SUMMARY

SETTING: A non-governmental organisation (NGO) supported tuberculosis control programme in Bangladesh with good smear microscopy.

OBJECTIVE: To verify whether bleach sedimentation method increases the sensitivity of sputum smear microscopy for acid-fast bacilli (AFB), and if so, how.

DESIGN: Duplicate smears from successive routine specimens, peripheral centres examining direct smears, and blind examination of bleach sediment smears at central laboratories.

RESULTS: When all 3287 sputum samples were examined in duplicate and the International Union Against Tuberculosis and Lung Disease cut-off for positivity was applied, more positives were not found by bleach sedimentation. Using the much lower American Thoracic Society (ATS) threshold, the percentage positives rose slightly from 15.5% for direct smear to 16.6% after bleach. The gain was more evident when suspect examinations only were taken into consideration, as bleach missed many positives identified by direct follow-up smear. When patients rather than individual smears were counted, more suspects were detected by bleach (10% gain on average), but with considerable variation between the centres (range 6–16%). To arrive at this gain, the ATS cut-off was used, with corrections for false results. Under routine conditions, however, this threshold is too low in view of possible transfer of AFB.

CONCLUSIONS: Bleach sedimentation can increase the diagnostic yield, but only to a minor extent if all other factors have been optimised already; it is not a panacea. Precautions against false negatives as well as false positives should be taken, and the additional workload is not negligible.

KEY WORDS: tuberculosis; diagnosis; Ziehl-Neelsen smear; stains and staining; sensitivity and specificity

CONCENTRATION OF SPUTUM in preparation for microscopy or culture was used with many variations in the first half of the twentieth century.[1–3] The Clorox method was soon rated as being the best,[4] but was then again forgotten, perhaps because of later emphasis on fluorescence microscopy and culture. It has been the merit of Miörner et al. to have revived interest in these techniques in the context of developing countries.[5,6] However, the increase reported seems quite high—114% using centrifugation.[6,7] In a later report, the gain using bleach followed by overnight sedimentation was only 21–25%, though not statistically significant when compared to that obtained with bleach plus centrifugation.[8] The usefulness of bleach plus centrifugation has been contradicted by Wilkinson and Sturm,[9] who also reported a considerable proportion of false negatives compared to direct smear. On the other hand, trials in the Malawi National Tuberculosis Programme (NTP) reportedly gave excellent results using centrifugation.[10]

It thus seemed justified to undertake an extensive field-study in Bangladesh projects sponsored by the Damien Foundation, where with monitoring by continuous quality control, acid-fast bacilli (AFB) microscopy has been shown to be reasonably accurate.[11]

OBJECTIVES

The objectives of the study were 1) to determine the incremental yield of bleach digestion followed by overnight sedimentation in terms of smear positivity and also of cases detected, under routine conditions of a field project with already good microscopy, and 2) to acquire a better understanding of the mechanisms involved in the bleach sedimentation method.

METHODS

Four peripheral Damien Foundation Bangladesh diagnostic and treatment centres were chosen on the

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basis of good performance and accessibility. The study included all sputum samples handled over a period of 3 to 6 months. Besides the usual direct smear, centres prepared an additional smear according to the bleach method.[8] Briefly, specimens were left to react with an equal quantity of household bleach in the sputum containers for 15 minutes. The contents were then transferred to 15 ml plastic conical tubes, and distilled water was added to the top. The tubes were left to stand for sedimentation overnight, and next morning a small, thick smear ('bleach smear') was made from the sediment after decanting. For the whole study period, the tubes had to be re-used after thorough rinsing and scrubbing with a tube-brush and detergent.

A variation was used in two of the centres. Sputum was first left to stand overnight in its original pot without any additive, to become liquefied by autodigestion. Next day a small, thick smear was made ('homogenisation smear'), and the sputum was further processed with bleach as above. After another night, the bleach sediment smear was added to the same slide.

Only direct smears were examined at the centres as part of their routine work. Dried smears from processed sputum were fixed by heat and stored in slide boxes in the centres, inside a cupboard. About once a month they were transferred to two central laboratories to be stained and examined, i.e., with a delay of 1 to 2 months since smear preparation. All staining was according to Ziehl-Neelsen (ZN; hot method, using 1% fuchsin). Microscopes used were Olympus CHD binocular types, all in good condition.

The results of the peripheral centres and central laboratories were compared by a co-ordinator. For specimens with discordant results, records were checked to exclude clerical errors, and a selection of these slides was reread by the senior laboratory technician. As culture is not available at or near these centres, it was thus not possible to use this as a reference method.

All results were entered and analysed in an Epi-Info 6.01 computer file. This software was also used to grade results as positive, negative or scanty according to the three main quantification scales: American Thoracic Society (ATS),[12] World Health Organization (WHO)[13] and International Union Against Tuberculosis and Lung Disease (IUATLD)[14] (Table 1). Pearson's χ^2 test was used to compare the positivity rates.

RESULTS

For more clarity, only the results of tests using the more sensitive ATS scale are mentioned here and in the Tables.

Comparison of overall positivity rates of individual smears, irrespective of type of specimen

Bleach sedimentation yielded 16.6% positives against 15.5% with direct smear (Table 2), a gain of 7% (not statistically significant, NS). Forty positive direct smears (8% of the total) were missed with bleach.

By contrast, the positivity of the homogenisation smears was 17.2% compared to 15.8% with direct smears, an increment of 9% (Table 3).

Comparison of positivity rates considering suspect and follow-up smears separately (Table 4)

Bleach sedimentation showed a slightly higher positivity rate in suspect smears: 17.6 versus 16.1%, or a gain of 9% (NS). Especially clear, although not significant because of the small numbers involved, was the 27% loss of positivity in follow-up smears: 7.2% for bleach versus 9.9% for direct smears.

This opposite effect on follow-up smears was not seen with homogenisation smears: the same number of positives (9, 5.7%) was found as with direct smear. Comparing positivity rates for bleach and homogenisation smears showed virtually the same result for suspects (both at 18.4–18.5% positivity). However, homogenisation identified nine positive follow-up smears against only five using the bleach method.

Comparison of number of patients detected and its variation between the diagnostic centres

For the analysis shown in Table 5, individual patients rather than smears are considered (as identified by at least one positive out of a series of three suspect

Table 1 Quantification scales and thresholds

Quantified result	Interpretation ATS scale (threshold at 1/100 fields)	Interpretation WHO scale (threshold at 4/100 fields)	Interpretation IUATLD scale (threshold at 1/10 fields)
Negative	Negative	Negative	Negative
1–2 AFB per 300 fields	Doubtful	Doubtful negative	Doubtful
1–3 AFB per 100 fields	Positive 1+	Doubtful negative	Doubtful
4–9 AFB per 100 fields	Positive 1+	Doubtful positive	Doubtful
1–9 AFB per 10 fields	Positive 2+	Positive 1+	Positive 1+
1–9 AFB per field	Positive 3+	Positive 2+	Positive 2+
10 or more AFB per field	Positive 4+	Positive 3+	Positive 3+

ATS = American Thoracic Society; WHO = World Health Organization; IUATLD = International Union Against Tuberculosis and Lung Disease.

Table 2 Bleach sedimentation method versus direct, individual smears. ATS cut-off for positivity

Direct smear	Bleach sedimentation method			
	Positive	Negative	Scanty	Total (%)
Positive	466	41	3	510 (15.5)
Negative	76	2684	7	2767 (84.2)
Scanty	2	7	1	10 (0.3)
Total (%)	544 (16.6)	2732 (83.1)	11 (0.3)	3287

All centres, suspect and follow-up smears mixed.
Scanty = <1 AFB / 100 oil immersion fields; AFB = acid-fast bacilli.

smears). Furthermore, results are also shown separately for each centre.

The gain using bleach method now reaches 11% compared to direct smear, still a non-significant difference. There is also some variation between the different centres—the gain ranges from 8 to 16% (also NS).

However, the results in Table 5 were obtained after corrections. Some false negative readings were proven for all types of smears by rereading, and in these cases the correct result has been used for the analysis. Furthermore, transfer of AFB from positive to negative sedimentation specimens was strongly suggested by the finding of isolated low scanty results, exclusively among bleach smears, and clustered in time. As far as could be traced from records, the same sedimentation tube had contained a high positive sputum the last time it had been used. Before these corrections, the average gain was 17%, with a range between centres of from 4 to 26% (data not shown).

DISCUSSION

Widely diverging experiences have been reported for the bleach method. They range from more than double positivity rates in the hands of its recent promoters and using centrifugation,[6,7] over still excellent results in Malawi [10] and Zambia,[15] to no gain at all in South Africa.[9] The use of sedimentation overnight as an alternative to centrifugation was reported to yield almost the same number of positive results, although the gain for either compared to direct was much less than in earlier reports.[8]

We applied the method under routine field conditions and using different readers for both direct and bleach smears, to see if it could improve the sensitivity of AFB microscopy in an already good service, and to understand how it works. We used overnight sedimentation and not centrifugation, as the use of good centrifuges was considered unrealistic under field conditions. This might partly explain the rather modest results, although sedimentation and centrifugation have been shown to yield almost the same numbers of positives (although with more AFB after centrifugation[8]).

Simply cross-tabulating all results of the 3287 sputum samples examined by both the direct and bleach sedimentation method showed little difference. Using the ATS cut-off, there was a gain in positivity rate of only 7% using the bleach method, but none at all when the IUATLD cut-off was used, and very little (2%) for the WHO scale (data not shown). Indeed, with these scales the increment occurred in the group of scanties. Choosing the appropriate cut-off and scale may be important, to avoid excessive false positives. As reported earlier, we have had bad experiences with the most 'rewarding' threshold of the ATS.[11] Furthermore, in this study it would have resulted in false positive cases, as will be discussed further on.

The cross-tabulation also showed that many (8%) of the positives in direct smear had been missed by bleach smears, as has already been reported by Wilkinson et al.[9] Considering follow-up examinations

Table 3 Homogenisation method versus direct, individual smears. ATS cut-off for positivity

Direct smears	Homogenisation smears			
	Positive	Negative	Scanty	Total (%)
Positive	239	9	0	248 (15.8)
Negative	30	1286	0	1316 (83.9)
Scanty	1	3	0	4 (0.3)
Total (%)	270 (17.2)	1298 (82.8)	0	1568

All centres, suspect and follow-up smears mixed.
Scanty = <1 AFB / 100 oil immersion fields. AFB = acid-fast bacilli.

Table 4 Positive smears and rates with different methods, considering suspect and follow-up smears separately. ATS scale

	No. of positive suspect smears (%)	No. of positive follow-up smears (%)
Bleach smear vs direct*	520 vs 477 (17.6 vs 16.1)	24 vs 33 (7.2 vs 9.9)
Homogenisation smear vs direct†	261 vs 239 (18.5 vs 16.9)	9 vs 9 (5.7 vs 5.7)
Bleach smear vs homogenisation smear†	260 vs 261 (18.4 vs 18.5)	5 vs 9 (3.2 vs 5.7)

* Bleach vs direct: $n = 2954$ suspect and 333 follow-up specimens.
†Homogenisation vs direct or bleach: $n = 1411$ suspect and 157 follow-up specimens. Only two of four centres prepared homogenisation smears as well as bleach sediment smears.

Table 5 Case-detection: numbers of smear-positive patients (ATS scale) and percentage gain by bleach method compared to direct smear by centre

Centre	Direct smear	Bleach sedimentation	Percent gain for bleach
I	50	54	8%
II	80	86	8%
III	31	36	16%
IV	75	85	13%
Sum of all	236	261	11%

Numbers represent cases identified by at least one positive smear. Corrected according to rereading results and with omission of false positives caused by transferred AFB.
AFB = acid-fast bacilli.

only, positives missed by bleach were especially frequent, reaching one quarter of direct smear positives. This may have to do with the mechanism of the method: digestion and flocculation of substances (which may have been due to proteins from pus) present in the sputum that presumably make the AFB co-precipitate during centrifugation or even sedimentation. The result is a real concentration of AFB at low gravitational force, convincingly demonstrated by Gebre et al.,[7] and quite obvious from viewing some of these smears. Some samples form hardly any sediment, however, and flocculation fails, as has also been reported by Hanks et al. for the alum method.[1] Assuming that this is caused by the nature of the sample, positive follow-up specimens might be expected to remain undetected more often because of their on average lower numbers of AFB and more watery consistency. Without flocculation, the already rare AFB will actually have been diluted in the bleach method.

On the other hand, the rudimentary homogenisation used in parallel for part of the samples in our study also resulted in a 9% gain, comparable to that of the bleach method. This was also lost applying the IUATLD scale, but stayed almost the same (8%) using the WHO cut-off, indicating that the gain was not only due to low scanty results (data not shown). Besides, fewer (4%) direct smear positives had been missed with homogenisation smears, and these were not follow-up specimens. We assume that here the mechanism may be one of dispersion of AFB throughout the specimen and the breaking up of AFB clumps, and that this is also part of the effect of bleach preparation. In their elaborate multi-centre trials, Slosarek et al.[16] found mechanical declumping to be one of the few manipulations resulting in increased sensitivity of smear microscopy. This may mean that homogenisation of the specimen in practice often works better than the 'careful selection of a purulent particle', though the latter technique is the most efficient in principle, and should continue to be taught.

The biggest gain using the bleach method was observed when identification of positive patients instead of samples was considered, but only in some of the centres. Before correction for various errors, the average incremental yield from bleach reached 17% (ATS scale), with wide variations between centres (4–26%).

However, quality control rereadings showed errors in some of the centres as well as in some of the central laboratories. Frequent errors were found to be correlated with relatively busy periods or clear overload. Secondly, transfer of AFB to negative specimens had happened, as discussed hereafter. Because of these findings, we decided to recalculate the yield as positive patients per centre, disregarding isolated low scanty results, and correcting for quality control false negatives. In that way, bleach gain varied from 4 to 11% according to the scale used, and gains were not observed in all centres: 11% (range 8–16%) using the ATS scale, or 9% (range 4–12%, data not shown) with the WHO scale, and 4% (range −2–10%, data not shown) with the IUATLD scale. Also, homogenisation resulted in a gain when patient detection was considered, but now it attained only about half of the bleach gain (data not shown).

Individual performance of technicians has previously been reported to be the main factor responsible for differences in AFB smear sensitivity.[16,17] Our results seem to confirm this finding, and we think that this may explain the extremely variable yield of the method in different settings. Gebre et al. found over 100% gain by bleach, but the extremely low rate (30.8%) of direct smear-positives compared to culture-positives mentioned in their paper puts the performance of their technicians in direct smear very low on the scale as reviewed by Urbanczik.[18] In the Zambia experience,[15] direct ZN identified only 44%, against ZN of centrifuged bleach sediments identifying 77% of positives by fluorescence microscopy on direct smear, the reference method. These are very unusual proportions: typically a fluorescence technique increases the number of positives by 10 to 20%,[19–21] but not 110%, as in this report from Zambia. A problem with the ZN staining and/or its reading might be suspected as the underlying reason. Similarly, in our centre IV (Table 5), the considerable bleach gain was made entirely during the first half of the trial period, when workload at the centre was extremely high and routine quality control revealed errors. Not one more positive was found later on, coinciding with a greatly reduced workload in the centre and excellent results in routine cross-checking.

Scrutinising our data for an explanation for these individual differences, we observed that there had very probably also been a transfer of AFB between some bleach samples. Clustered in time, several suspects showed a few AFB in only one out of all six to nine smears, and this was always a bleach smear. In our setting, this is a very rare finding. A strong indication that transfer of AFB happened via re-used tubes came from the finding that in the centre where this information could be traced, the former sample in the same tube had been a high positive. Under routine conditions the continuous supply of distilled water for dilution needed with bleach method would be a problem. Saprophytic AFB from tap water used for dilution in case of stock interruptions should then also be suspected as a source of contamination.

The difference when positive patients were considered rather than smears was partly caused by another draw-back of the bleach smears—their fragility. Quite a few were badly damaged during staining, which must have contributed to false negative bleach results. This had not always been noted by the technicians, and besides we thought it closer to reality not to attempt the exclusion of these samples.

CONCLUSIONS

Ziehl-Neelsen staining of sputum smears is well known for its widely variable sensitivity.[18] Several technical requirements have to be fulfilled in order to reach the high end of this spectrum. Pre-concentrating the sputum, such as by means of the bleach method, can help, but it can not compensate for whatever technical deficiency. And the gain it may offer will only be minimal if all other factors have been optimised already. AFB become easier to find because of their increased numbers per field as well as the improved background, but this makes less difference to a more careful technician.

The bleach method can increase the yield of positive suspects. On the other hand, however, some patients identifiable by direct smear will be missed, and it does not work with follow-up sputum. There is a definite danger of false positive results by transfer of AFB via conical tubes or by contamination with dilution water. Since this will be very difficult to avoid under field conditions, the threshold for positivity should not be set too low. The IUATLD or WHO scale should be preferred, which means that the gain to be expected becomes fairly modest. Finally, the additional work of specimen preparation is not negligible. Depicting the method as a remedy in case of overload or poor motivation may not be entirely justified. Thus the most that can probably be achieved is a small gain in centres that are already doing very well; and even then it must be used in a sound case-finding system, with built-in safeguards against its draw-backs.

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RÉSUMÉ

CADRE : Au Bangladesh, Programme de Lutte Antituberculeuse soutenu par une organisme non-gouvernemental et bénéficiant d'une bacilloscopie de bonne qualité.

OBJECTIFS : Vérifier dans quelle mesure et comment la méthode lessive-sédimentation augmente la sensibilité de l'examen microscopique des frottis pour recherche des bacilles acido-résistants (BAAR).

SCHÉMA : Deux frottis étalés à partir de chaque échantillon successif de routine ; examen direct des frottis par les centres périphériques et examen aveugle des frottis après lessive-sédimentation dans les laboratoires centraux.

RESULTATS : Dans les 3287 expectorations examinées à deux reprises, et après application des limites de positivité de l'UICTMR, on n'a pas trouvé davantage de positifs par la méthode lessive-sédimentation. Si l'on applique le seuil beaucoup plus bas de l'ATS, le pourcentage de positifs augmente légèrement de 15,5 à 16,6% par le passage du frottis direct au frottis après lessive. L'avantage est plus net si l'on ne prend en compte que les suspects, car l'examen après lessive a été négatif chez beaucoup de positifs en examen de suivi identifiés par les frottis directs. Si l'on fait le décompte des patients plutôt que des frottis individuels, on détecte plus de suspects par la méthode lessive (gain moyen 10%), mais avec des variations considérables entre les centres (extrêmes 6 à 16%). Pour arriver à ce gain, le seuil de positivité de l'ATS a été appliqué avec les corrections pour les résultats faux. Mais dans les conditions de routine, ce seuil est trop bas, vu la possibilité de transfert de BAAR.
CONCLUSION : La méthode de sédimentation à la lessive peut augmenter le rendement diagnostique, mais seulement dans une faible mesure, et si tous les autres facteurs ont déjà été optimalisés ; elle n'est pas une panacée. Des précautions doivent être prises tant pour les faux négatifs que pour les faux positifs, et la charge de travail supplémentaire n'est pas négligeable.

RESUMEN

MARCO DE REFERENCIA : Programa de control de la TB apoyado por una ONG en Bangladesh con buen sistema de baciloscopia.
OBJETIVO : Verificar si el método de sedimentación en lejía aumenta y de qué manera la sensibilidad de la microscopía directa del esputo para bacilos ácido-resistentes (BAR).
MÉTODO : Láminas duplicadas de muestras rutinarias de esputo sucesivas, con exámenes directos en los centros periféricos y examen de láminas con sedimentación en lejía, realizado a ciegas, en el laboratorio central.
RESULTADOS : Sobre 3287 esputos examinados en duplicado y aplicando el umbral de la UICTER para positividad, no se hallaron más casos positivos con la sedimentación en lejía. Utilizando el umbral más bajo de la ATS, el porcentaje de positivos aumentó discretamente de 15,5 en el esputo directo a 16,6% en lejía. La ventaja fue más clara considerando solamente los exámenes sospechosos, ya que el tratamiento con lejía pasó por alto muchos positivos identificados por baciloscopia de seguimiento. Si se contaban los pacientes en lugar de contar los esputos, se detectaron más sospechosos con la lejía (ganancia promedio 10%), pero con variaciones considerables entre los diferentes centros (rango 6 a 16%). Para alcanzar esta ventaja se utilizó el umbral de la ATS con correcciones para resultados falsos. Pero en condiciones de rutina este umbral es demasiado bajo en vista de posibles transferencias de BAR.
CONCLUSIONES : La sedimentación en lejía puede aumentar el rendimiento del diagnóstico, pero sólo en una pequeña proporción si todos los otros factores han sido optimizados, pero no es una panacea. Se deben tomar precauciones contra falsos negativos y contra falsos positivos, y la carga de trabajo adicional no es despreciable.