Diagnosis of tuberculosis in developing countries in the era of high HIV transmission; alternative approaches

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Chapter 5

Microbiological validation of smear microscopy after sputum digestion with bleach; a step closer to a one-stop diagnosis of pulmonary tuberculosis.

Tuberculosis 2005, in press
Microbiological validation of smear microscopy after sputum digestion with bleach; a step closer to a one-stop diagnosis of pulmonary tuberculosis.

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**Short title:** Sputum digestion and PTB 

**Keywords:** Tuberculosis, diagnosis, Ziehl-Neelsen, sputum, smear, digestion, bleach, Nigeria.

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ABSTRACT

Background: Although direct smear microscopy is relatively insensitive for the diagnosis of TB, simple digestion of sputum with household bleach prior to smear preparation has been reported to improve its sensitivity. This method has not been validated against culture.

Methods: 756 consecutive patients with symptoms suggestive of pulmonary TB (PTB) were asked to submit 3 sputum specimens to prepare direct smears. One specimen was selected at random for culture and another specimen was digested to prepare a further smear.

Findings: 455 (60%) patients were culture positive. 235 (31%) had “definite” PTB based on the WHO case definition for PTB (≥2 positive direct smears or one positive smear and positive culture). A further 223 (29%) patients were “very likely” to have PTB (positive culture but three negative direct smears). The WHO case definition identified 51% (235/458) of the patients with “definite” or “very likely” PTB. One digested smear detected 219 (93%) of the 235 patients with “definite” PTB and 10 patients with “very likely” PTB and had a sensitivity and specificity (95%CI) of 50% (229/458; 45%-55%) and 99% (97%-100%) respectively. The positive and negative predictive values for one digested smear were 98% (95%-99%) and 56% (52%-60%) respectively, which were not different (p>0.5) to the values for the WHO case definition (100% and 57% respectively).

Interpretation: A single, bleach-digested smear is as sensitive and specific as 3 direct smears for the diagnosis of PTB. The method has the potential to improve access to a quality-assured TB diagnosis, particularly for poorer patients.
INTRODUCTION

Globally, tuberculosis (TB) causes more adult deaths than any other single infectious disease. Approximately 95% of cases and 98% of deaths occur in the developing world. DOTS, the WHO's global strategy for controlling the TB epidemic, relies upon direct sputum smear microscopy for detecting infectious cases. This usually involves patients submitting 3 sputum specimens over the course of 2 days. The internationally recommended definition of a smear-positive TB case requires at least 2 positive smears or, where facilities are available, one positive smear together with one culture positive for *Mycobacterium tuberculosis*.

Direct smear microscopy is an inherently insensitive technique since large numbers of organisms have to be present in the sputum to be detectable by direct examination. The sensitivity of the technique is further undermined in areas with high HIV prevalence since HIV co-infection, probably through modifying the TB disease process, increases the proportion of patients with culture-positive, smear negative TB. Furthermore, the standard diagnostic process is associated with high drop out rates, since patients need to make repeated visits to the health facility to submit specimens and obtain results. The requirement to process and examine 3 specimens from each TB suspect can create heavy workloads for laboratories, which may impact upon the quality of the service.

The global targets for TB control under DOTS include detecting 70% of new smear positive cases by 2005 (case detection). Current estimates suggest the global case detection rate is about 37% and that this target will not be achieved. It is recognised that health system weaknesses present challenges to improving case detection. These include health service staffing crises in several countries, and a more general lack of access to TB diagnostic services, particularly for poor people.

Various groups have attempted to modify the smear diagnostic process to make it more sensitive, or to reduce the number of smears required for diagnosis. Methods aiming to concentrate the TB bacilli in sputum smears by digestion of the sputum with bleach and subsequent concentration of sputum (by centrifugation or overnight sedimentation) have received much attention.

Previous studies have shown that digestion of sputum with household bleach prior to smear preparation (without a concentration step) can also improve the perform-
ance of sputum microscopy. Studies carried out in Ethiopia have reported that a single digested smear can equal the sensitivity of 3 direct smears. These, entirely smear-based, studies reported patients with positive digested smears but 3 negative direct smears, suggesting that the digestion method was associated with either decreased specificity or increased sensitivity. Mycobacterial culture is a more sensitive method than direct sputum smear microscopy for diagnosing pulmonary TB (PTB). We therefore conducted a study comparing a bleach digested smear with direct smear microscopy and culture in patients presenting with symptoms suggestive of PTB to district hospitals in Abuja.

If a single digested sputum smear could replace the examination of 3 direct smears, and provide a one-stop diagnosis, it could make a significant contribution to improving case detection rates while reducing costs and workload.
MATERIAL AND METHODS

This was a cross-sectional, prospective study of patients (aged >15 years) presenting to 8 district hospitals in Abuja, Nigeria, with symptoms suggestive of pulmonary tuberculosis and no previous history of TB treatment. All consenting patients were asked to submit 3 sputum samples over 2 days (first on-the-spot, early morning and second on-the-spot specimens) as recommended by the International Union Against Tuberculosis and Lung Diseases (IUATLD)\(^1\), and adopted by the national TB and Leprosy Control Programme (NTLCP) of Nigeria \(^1\).

All samples were transported to the TB Research Laboratory at Zankli Medical Centre and processed within 24 hours. Direct smears were prepared from each specimen and stained using the hot Ziehl-Neelsen (ZN) method\(^1\). Subsequently, one of the 3 specimens was randomly selected and used to prepare a digested smear. A second randomly selected specimen was used for mycobacterial culture.

Sputum was digested by adding an equal volume of locally-obtained household bleach (Jik, 3.5% NaOCl, Reckitt Benkiser (Nig) Ltd, Agbara) to the sputum specimen in the original container. The container was slanted at an angle of 45° on the laboratory bench and left for 30-45 minutes as described by Yassin et al\(^10\). A drop of the digested material was taken from the bottom of the container by pastette, smeared on a new glass slide and stained using the hot ZN method (as above). All smears (including direct smears within each set) were read blind to the other results. All smears were re-examined by a second laboratory technician, unaware of the results of the first technician. A third technician compiled the slide readings, reviewed slides with discrepant results and discussed results with the laboratory team to reach a consensus. The IUATLD grading system was used throughout for reporting both direct and digested smear results. For the purposes of this study, smears with less than 10 acid-fast bacilli (AFB) per 100 fields were grouped together under the description "scanty" and considered as positive\(^11\). Where results of direct or digested smears were unavailable for analysis (due to non-submission of specimen, insufficient specimen, or mishap during laboratory processing) the results were considered as negatives in the analysis.

The BACTEC 960 system was used for mycobacterial culture after specimens had been decontaminated using Petroff's method. Cultures were incubated at 37°C for up to 42 days. Facilities were not available for definitive confirmation of isolates as
members of the *M. tuberculosis* complex (MtbC), therefore the term “positive culture” here refers to the growth of AFB in culture.

The categorisation of patients for the analysis was guided by internationally-accepted case definitions. The WHO considers any patient culture-positive for MtbC as a definite case. A “definite” sputum smear positive (SS+) PTB case is defined as: one with 2 or more initial sputum smear examinations positive for AFB; or one sputum smear examination positive for AFB plus sputum culture positive for MtbC. Patients with negative smear microscopy but positive culture were considered to be ‘very likely’ to have PTB, but would be considered to be sputum smear negative (SS-). Patients with only one positive direct smear but negative culture were considered to be ‘less likely’ to have PTB and those with negative direct microscopy and negative culture were considered to be ‘unlikely’ to have PTB.

Diagnosis based on a single digested smear was compared with the case definitions and the sensitivity and specificity, positive and negative predictive values (PPV and NPV) were calculated. The proportion of cases identified by direct microscopy and a single digested smear were tested using Chi-squares. The agreements of the digested smears with the first on the spot, early morning and second on the spot smears were compared using Kappa statistics.

Ethical approval was obtained from the Research Ethics Committees of the Liverpool School of Tropical Medicine, UK; the Gwagwalada Specialist Hospital and the Department of Health Services of the Federal Capital Development Authority, Abuja, Nigeria.
RESULTS

A total of 2251 (99% of a potential 2268 smears) direct smears, 736 (97%) bleached smears and 756 BACTEC cultures were prepared from 756 patients attending the 8 district hospitals between September 2003 and June 2004. The results of seventeen direct sputum smears and 20 digested smears were not available because of non-submission or insufficient volume of specimen or mishaps during laboratory processing. Four hundred and fifty five (60%) patients were culture-positive and 301 culture-negative.

The results of the sputum smears are shown in Table 1. In the cases of 455 culture-positive patients, 222 (49%) of the first on the spot specimens, 232 (51%) of the morning and 224 (50%) of the second on the spot specimens were positive by direct smear microscopy. Smears prepared from early morning sputum were more likely to be graded as "+++" than on-the spot smears (p<0.01 and p<0.02 for the first and second spot smears respectively). Two hundred and twenty six (51%) of the 455 culture-positive patients had positive digested smears. Digested smears had slightly lower grades (scanty or "+") than the direct smears consistent with the small dilution effect of the digestion process. The number of digested smears labelled as negative however was similar to the direct smears. Of the 301 culture-negative patients, 3 (1%) of the first on the spot and 4 (1%) of the morning and second on the spot direct smears were positive. In comparison, 7 (2%) of the digested smears of these patients were positive. Most of these positive digested smears were graded as scanty. The agreements between the results of the digested and direct smears are shown in table 2. The Kappa index of agreement was very high between digested and direct smears with Kappa = 0.92, and 0.94 for the two on the spot and morning specimens respectively.

In accordance with the study definitions described above, a total of 235 (31%) patients were considered "definite" SS+ PTB cases (Table 3). All of these were diagnosed on the basis of 2 or more positive direct smears (there were no cases of a singleton positive smear confirmed by culture). A further 223 (29%) patients were "very likely" to have SS+ PTB as they had positive culture but negative direct smear microscopy. Two patients had one positive direct smear but negative culture (considered to be "less likely" to have PTB) and 296 patients had 3 negative direct smears and negative culture. The WHO case definitions therefore identified 51% of the cases with PTB (i.e. "definite" PTB plus "very likely" PTB). In comparison, a single
digested smear identified 219 (93%) of the 235 patients with “definite” SS+ PTB and a further 10 who were “very likely” to have SS- PTB (positive culture but negative direct smear microscopy). A single digested smear therefore identified 229 (50%) of the cases with PTB. The number of patients with PTB identified by a single digested smear is essentially the same as that identified by a strategy employing 3 direct smears with confirmatory culture of singleton positives and has the same specificity (294/298; 99%; 95%CI 97%-100%). The positive and negative predictive values for the combined WHO case definitions for PTB were 100% (99%-100%) and 57% (53%-61%) respectively. In comparison, a single digested smear had a PPV and NPV of 98% (95%-99%) and 56% (52%-60%) respectively (p>0.5).
DISCUSSION

Direct sputum smear microscopy is considered the cornerstone of TB case-finding in resource-poor countries. It is relatively cheap and simple and identifies the most infectious cases amongst those presenting to health care facilities (i.e. those whose sputum contains the most TB bacilli). However, the requirement for repeated visits to submit specimens and receive a diagnosis is associated with considerable drop-out from the diagnostic process, particularly of poorer patients. Furthermore, health service resources (including human resources) for TB control are increasingly constrained in many parts of the world, particularly those areas with high rates of HIV/TB dual infection. Sputum smear microscopy is considered one of the critical services provided by district health laboratories in Africa. Work in Malawi has shown that sputum smear microscopy using the 3-smear strategy can consume more than 40% of total staff time and, with the exception of blood transfusion services, consumes more of the annual laboratory budget than any other investigation. The large numbers of sputum smears being examined in high prevalence settings also makes it difficult to adequately assess the quality of the service. Large samples of smears need to be rechecked (blind) by quality assessors if an assurance of minimum quality standards is to be statistically valid. Consequently, few national external quality assessment schemes function adequately in high prevalence countries.

The results presented here confirm the relatively low sensitivity of direct sputum smear microscopy: it identified 51% of the patients who were culture positive. Moreover, the sensitivity of the direct smear in this study is likely to be higher than that seen in most settings since all smears were examined twice in a research laboratory, and all slides with 1-9AFB per 100 fields were considered positive. The IUATLD/WHO grading system recommends a threshold of 10 AFB per 100 fields before a slide is regarded as a positive (i.e. only grades 1+ and above are considered positive). Recent work investigating the impact of lowering the positivity threshold in order to increase sensitivity of direct smear microscopy recommended a threshold of 4 or even 1 AFB per 100 fields as most of these smears are likely to be true positives in areas of high TB prevalence. Most programmes require the collection of sputum specimens over 2 days to ensure the examination of an early morning specimen (the specimen considered most likely to be positive). Our findings indicate that, although morning samples from TB suspects are more likely to have higher smear grades, the proportion of slides classified as negative remains the same independently of whether the sample was collected as an on the spot or as a morn-
ing sputum. Although this may be setting specific and be associated with later presentation and more advanced disease in our study population, such observations have prompted studies to examine the number of sputum specimens that are required to diagnose a patient as smear-positive in other locations.  

This study confirms our earlier work in Ethiopia showing that a single digested smear is as sensitive as 3 direct sputum smears for the diagnosis of new cases of PTB. The inclusion of mycobacterial culture in this study provides the validation of smear results, absent in previous work, and strengthens the evidence. Furthermore, since this study was conducted in an area of Nigeria where approximately 50% of PTB cases are HIV seropositive it can be expected that the digested smear is appropriate for settings with high dual HIV/TB infection rates (Lawson et al, manuscript in preparation). We believe that the method works, not so much through concentration of the TB bacilli in the specimen, but through the digestion of the material that forms the background of the microscopic field. The methylene blue-stained background of the microscopic field appears paler and less distracting, and AFB are seen more clearly against it (Figure 1).

The digestion method may benefit from some further development. During this study a number of digested smears were unavailable due to laboratory mishap. In every case this mishap was the observed loss of the entire (heat-fixed) smear during the staining process. This is probably not an all-or-nothing phenomenon and it may be expected that all the digested smears are prone to some loss of material. It is possible that the bleach digests the proteinaceous material required for adherence of the clinical material to the glass slide. Bovine serum albumin has been used to promote adherence of sodium hydroxide treated sputum to microscope slides, but is likely to be a prohibitively expensive option for resource-poor settings. The use of alternatives such as sterile skimmed milk may be worth investigating. The effects of age and storage conditions of bleach on the performance of the method also need to be explored.

The advantages of using a single digested smear in place of the conventional 3 direct smears on specimens collected over 2 days includes the potential of a same-day diagnosis, reducing the drop-out rate during the diagnostic process (due to an anticipated reduction in patient costs and time involved) and easier and safer preparation of smears, since digested sputum is easier to manipulate and is sterilised by the bleach treatment. This approach also reduces the laboratory load and costs as fewer speci-
mens need to be processed resulting in a reduction in time spent on clerking specimens and writing reports, reduction in smears being fixed, stained and examined and the clearer background to the microscopic field may be expected to make the screening of negative smears faster. Angeby et al recently reviewed studies of all methods using bleach for sputum digestion/concentration aimed at improving sputum smear microscopy. The reviewers concluded that there is enough evidence and enough local concern to promote the introduction of a bleach method as a part of the DOTS strategy in countries where culture is not performed routinely, but did not advocate any particular method of those reviewed.

This study provides evidence to support implementation, in an operational research setting, of a digested smear method for diagnosing new cases of TB in resource-poor countries. Household bleach is an almost universally available product even in rural areas of developing countries. A single digested smear equals the sensitivity and specificity of the 3 direct smear strategy. It can be expected to improve case-finding and lead to considerable savings in time and expenditure for health services and patients. Future work should focus on documenting and quantifying these savings.
References


Table 1. Microscopy results of standard direct smears and bleach digested smears by culture.

<table>
<thead>
<tr>
<th></th>
<th>Unavailable</th>
<th>Negative</th>
<th>Scanty</th>
<th>+</th>
<th>++</th>
<th>+++</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Positive culture</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st on the spot</td>
<td>3 (1%)</td>
<td>230 (51%)</td>
<td>36 (8%)</td>
<td>83 (18%)</td>
<td>58 (13%)</td>
<td>45 (10%)</td>
</tr>
<tr>
<td>Morning</td>
<td>3 (1%)</td>
<td>220 (49%)</td>
<td>28 (6%)</td>
<td>72 (16%)</td>
<td>59 (13%)</td>
<td>73 (16%)</td>
</tr>
<tr>
<td>2nd on the spot</td>
<td>9 (1%)</td>
<td>222 (49%)</td>
<td>34 (8%)</td>
<td>72 (16%)</td>
<td>69 (15%)</td>
<td>49 (11%)</td>
</tr>
<tr>
<td>Bleach</td>
<td>16 (4%)</td>
<td>213 (47%)</td>
<td>30 (7%)</td>
<td>98 (22%)</td>
<td>55 (12%)</td>
<td>43 (10%)</td>
</tr>
<tr>
<td><strong>Negative culture</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st on the spot</td>
<td>1 (0.3%)</td>
<td>297 (99%)</td>
<td>0 (0%)</td>
<td>2 (0.7%)</td>
<td>1 (0.3%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Morning</td>
<td>1 (0.3%)</td>
<td>296 (99%)</td>
<td>1 (0.3%)</td>
<td>1 (0.3%)</td>
<td>1 (0.3%)</td>
<td>1 (0.3%)</td>
</tr>
<tr>
<td>2nd on the spot</td>
<td>-</td>
<td>296 (99%)</td>
<td>2 (0.7%)</td>
<td>0 (0%)</td>
<td>1 (0.3%)</td>
<td>1 (0.3%)</td>
</tr>
<tr>
<td>Bleach</td>
<td>4 (1%)</td>
<td>290 (96%)</td>
<td>4 (1%)</td>
<td>1 (0.3%)</td>
<td>1 (0.3%)</td>
<td>1 (0.3%)</td>
</tr>
</tbody>
</table>
### Table 2: Agreement between the bleach digested and direct smears.

<table>
<thead>
<tr>
<th>Direct smear</th>
<th>Bleach digested smear</th>
<th>Kappa (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>1st on-the-spot</td>
<td>Positive</td>
<td>211</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>233 (32%)</td>
</tr>
<tr>
<td>Morning</td>
<td>Positive</td>
<td>219</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>233 (32%)</td>
</tr>
<tr>
<td>2nd on-the-spot</td>
<td>Positive</td>
<td>212</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>233 (32%)</td>
</tr>
</tbody>
</table>

95% CI = 95% confidence interval.
<table>
<thead>
<tr>
<th>Certainty of PTB</th>
<th>N</th>
<th>Bleach digested smears</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unavailable</td>
<td>Negative</td>
<td>Scanty</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Definite</td>
<td>235</td>
<td>12 (5%)</td>
<td>4 (2%)</td>
<td>28 (12%)</td>
<td>91 (39%)</td>
<td>56 (24%)</td>
</tr>
<tr>
<td>Very likely</td>
<td>223</td>
<td>4 (2%)</td>
<td>209 (94%)</td>
<td>3 (1%)</td>
<td>7 (3%)</td>
<td>0</td>
</tr>
<tr>
<td>Less likely</td>
<td>2</td>
<td>0 (0%)</td>
<td>2 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Unlikely</td>
<td>296</td>
<td>4 (1%)</td>
<td>288 (98%)</td>
<td>3 (1%)</td>
<td>1 (0%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

1 Case definitions: Definite: ≥ positive direct smears (positive/negative culture) or one positive direct smear plus positive culture; Very likely: three negative direct smears but positive culture; Less likely: only one positive direct smear and negative culture; Unlikely: three negative direct smears and negative culture.
Figure 1. Direct (A) and bleach digested (B) hot ZN smears of the same sputum specimen. The arrows point to acid fast bacilli.
List of figures and tables

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Table 1. Microscopy results of standard direct smears and bleach digested smears by culture.

Table 2: Agreement between the bleach digested and direct smears.

Table 3. Bleach digested smears results by the case definitions'.