Diagnosis of tuberculosis in developing countries in the era of high HIV transmission; alternative approaches
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Chapter 3

Efficacy and safety of short-term bleach-digestion of sputum in case-finding for pulmonary tuberculosis in Ethiopia.

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Efficacy and safety of short-term bleach digestion of sputum in case-finding for pulmonary tuberculosis in Ethiopia

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SUMMARY

BACKGROUND: In many settings, the diagnosis of pulmonary tuberculosis depends on sputum microscopy. However, this technique has low sensitivity. We studied the efficacy and safety of sputum digestion with bleach prior to Ziehl-Neelsen (ZN) staining.

METHODS: Positive sputum smears were assessed for staining quality and viability of mycobacteria after varying bleaching times. Two hundred smears were then prepared from the first, second and third sputum sample of tuberculosis suspects. Equal amounts of 5% bleach were added to the remaining first sputum and ZN smears were prepared.

FINDINGS: Optimal quality and staining was achieved with 30–45 min of bleaching. No growth was observed from positive samples after 15 min. Bleached specimens had 26% (52/200) positivity compared to 17.5% (35/200) of unbleached smears (P < 0.001). The bleached smears had 92.3% sensitivity, 93.4% specificity, 78.3% and 97.7% positive and negative predictive values, respectively, against a case definition. Ten patients failed to submit a second or third sputum. Six patients were positive on either the standard or bleach-digested smears, or both.

INTERPRETATION: Bleach digestion is simple, cheap and kills mycobacteria. Its positivity rate is as good as three standard smears. This method has the potential to improve over-burdened services in developing countries.

KEY WORDS: tuberculosis; diagnosis; bleach smears; safety; efficacy

NINETY-FIVE PER CENT of cases of tuberculosis and 98% of avoidable TB deaths occur in developing countries.1,2 TB control programmes in most resource-poor countries rely on smear microscopy for diagnosis of TB. International guidelines recommend that patients with clinical presentations suggestive of pulmonary TB should submit three sputum samples for direct smear preparation, appropriate staining and microscopy.3 However, the sensitivity of direct smear microscopy is low (8.8%–46.6% in most African laboratories) compared with mycobacterial culture.4 Moreover, most patients submit sputum specimens as out-patients, and significant numbers of patients may not be able to submit all recommended three samples.5 Concentration of sputum with centrifugation after digestion with household bleach (NaOCl) has been investigated in an attempt to improve sensitivity, with variable results.6–9 The need for electricity and centrifuges limits the use of the centrifugation method in most resource-poor settings, but overnight sedimentation of sputum with bleach appears to be no better than centrifugation,10 and may lead to further delays in availability of results and requirements for patients to make repeated visits.

Over-burdened smear microscopy services in developing countries urgently need methodologies that are safe, simple and give rapid results. This study therefore set out to assess the case-finding potential of short-term household bleach digestion of sputum samples prior to Ziehl-Neelsen (ZN) staining for diagnosis of pulmonary tuberculosis (PTB). We first assessed the optimum bleach (NaOCl) contact time for sputum digestion; we then went on to look at the first sputum specimens submitted and compare the smear positivity rate achieved after bleach digestion with that obtained by standard direct smear microscopy. Finally, we compared the smear positivity yield from bleach-digested first specimens against internationally accepted case definitions for smear-positive TB that require two or more positive direct smears.11,12

METHODS

Throughout the study, microscopy scalings were made in accordance with the International Union Against Tuberculosis and Lung Disease (IUATLD)13 and the American Thoracic Society (ATS)14 scales. Unless...
otherwise stated, all grades are reported in the text according to the IUATLD scale.

**Phase one**

The first part of the study was based in the Arrmauer Hansen Research Institute (AHRI), Addis Ababa, Ethiopia, where mycobacterial culture facilities are well established.

Sputum samples from eight patients, in which acid-fast bacilli (AFB) were detected by standard direct ZN staining, were collected from the city's TB Centre. Equal amounts of a locally purchased 5% NaOCl (Chorra Chemical Mnf. Ltd, Addis Ababa, Ethiopia) were added to each flat-bottomed sputum container, shaken by hand for 20 sec, and placed at 45°C to allow sedimentation into the dependent corner of the container. One drop was transferred from the digested sediment using a 2-ml Pastette (Alpha Laboratory Ltd/LW4040 Addis Ababa, Ethiopia) onto a new glass slide at 15-min intervals up to 120 min. Slides were stained and graded independently by two microscopists (MAY and HG). Where there was a discrepancy in grading, slides were re-examined by both readers together and a consensus grade was reached.

The quality of the fields and the morphology of AFB were graded on a pre-determined scale from 6 (excellent) to 1 (no AFB). A positive standard ZN-stained slide was used as a reference. The time category scoring the highest grade was taken as the optimum bleach contact time and was used during the second phase of the study (Table 1).

Thirty min after the addition of household bleach to sputum specimens, all the macroscopic changes were complete. The sputum samples became lighter and watery, except in cases of blood-stained sputum, where the mixture was shaken by hand for 20 sec and left to stand for 30–45 min. A drop from the dependent part of the digested specimen was transferred using a 2 ml pastette onto a new slide, as described in the first phase of the study. All slides were left to air dry prior to staining by the ZN hot staining method. The direct smears were examined and graded by the technician as part of routine laboratory practice. The bleach-digested smears were examined and graded by one of the investigators (MAY). The two examiners read the slides independently and were blinded to each other’s results.

TB suspects who did not complete the submission of the recommended three samples were registered as defaulters.

**Phase two**

The second phase of the study was conducted in Bushullo Major Health Centre (BMHC), Awassa, in the Southern Region of Ethiopia. BMHC is a missionary hospital, is relatively well equipped and locally known for the quality of its laboratory service, but mycobacterial culture facilities are not available. Sample size calculation was based on kappa statistic to identify differences in the yield of smear positivity among test procedures with a power of 80%.

Two hundred consecutive PTB suspects attending the centre for diagnosis were recruited between April and May 2000 and asked to submit sputum samples as recommended by the IUATLD and the National TB/Leprosy Control Programme of Ethiopia. Each patient was given a sputum container to submit the first specimen at the time of the initial clinical consultation (‘first on-spot’ specimen). After submission of this specimen, a labelled container was issued and patients were instructed to bring a sample of the first sputum expectorated on the morning of the next working day (‘overnight’ specimen). When the overnight specimen was delivered to the laboratory a third container was issued to each patient with instructions to submit the final specimen on the spot (‘second on-spot’ specimen). A record of whether sputum was collected from in- or out-patients was made.

**Sputum handling**

New twin-frosted labelled slides were used throughout the study. Standard, direct smears were prepared from ‘first on-spot’ specimens in accordance with routine laboratory practice, and an equal amount of 5% NaOCl (the same batch as that used for the initial phase) was added to the remaining sputum specimen. The mixture was shaken by hand for 20 sec and left to stand for 30–45 min. A drop from the dependent part of the digested specimen was transferred using a 2 ml pastette onto a new slide, as described in the first phase of the study. All slides were left to air dry prior to staining by the ZN hot staining method. The direct smears were examined and graded by the technician as part of routine laboratory practice. The bleach-digested smears were examined and graded by one of the investigators (MAY). The two examiners read the slides independently and were blinded to each other’s results.

TB suspects who did not complete the submission of the recommended three samples were registered as defaulters.

Treatment decisions were based on the results of standard smear microscopy of the three sputum samples. In routine practice, a patient is considered smear-

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Optimum bleach digestion time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Scale</td>
</tr>
<tr>
<td>1</td>
<td>Indet</td>
</tr>
<tr>
<td>2</td>
<td>Indet</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>++</td>
</tr>
<tr>
<td>8</td>
<td>++</td>
</tr>
<tr>
<td>Total</td>
<td></td>
</tr>
</tbody>
</table>

Scale for the clarity of the smear and for morphology of mycobacterium:

*Scale after re-examination by the two readers.

Indet = indeterminate; 1 = nothing is seen; 2 = very poor; 3 = poor; 4 = good; 5 = very good; 6 = excellent.
positive if at least two samples are smear-positive (+, ++ or ++++).12

Quality control
All smears prepared from 'first on-spot' specimens (both bleach-digested and direct) were retained. An arbitrary 10% of positive smears and 5% of negative smears were selected at random and re-examined by an experienced microscopy team at the Liverpool School of Tropical Medicine (LSTM). These microscopists were blinded to the initial results.

Two slides in each of the following ATS categories (4+, 3+, 1+), four slides of 2+ and 15 negative slides from both direct and bleach-digested smears were re-read independently. The quality control readings corroborated the initial results in all cases except one bleach-digested smear, where the initial grade was negative and the consensus of the team was that this was 1+ positive.

Statistics
Data were double-entered into Epi Info version 6.04 (Centers for Disease Control and Prevention, Atlanta, GA). McNemar’s \( \chi^2 \) test was used for dichotomous data and kappa statistics was used to assess the agreement of tests.13 Ninety-five per cent confidence intervals (CI) were calculated for sensitivity, specificity and positive and negative predictive values (PPV and NPV).

Approval
This study was approved by the Research Ethics Committee of the LSTM and by the Southern Region Health Bureau, Awassa, Ethiopia.

RESULTS
Smear positivity of 'first on-spot' specimens by standard and bleach-digested smear microscopy
Bleach digestion enhanced the detection of smears which would be graded as 'scanty' by the IUATLD scale but '1 + ' by the ATS scale. For clarity, therefore, only an analysis of results using the ATS scale is presented for this part of the study. Thirty-five (17.5%) of the 200 standard 'first on-spot' smears were graded as positive; 52 (26%) were positive after bleach digestion (\( \chi^2 = 15, P < 0.001 \)). The kappa test for agreement between the two tests was 0.75 with a standard error (SE) of 0.26. All smears that were positive by the standard preparations were also positive after bleach digestion.

Smear positivity of 'first on-spot' bleach-digested smears by standard case definitions for smear-positive pulmonary tuberculosis
Ten patients failed to submit the requested second and/or third sputum samples; six of them were positive by either the standard or bleach-digested first smear or both. Therefore, of the 10 suspects who did not complete the submission of three specimens, six were positive by the first-on-spot specimen. The remaining 190 suspects delivered three specimens each (four smears) for analysis.

The standard case definition (requiring at least two positive standard smears) yielded 39/190 (20.5%) positive patients and the bleach-digested 'first on-spot' yielded 46/190 (24.2%). The bleach-digested 'first on-spot' smear missed three cases (1.6%) that were positive by standard 'overnight' and 'second on-spot' smears. The standard case definition missed 10 cases (5.3%) that were positive by the bleach-digested, 'first on-spot' smears. The difference in yield was not statistically significant \( (P > 0.09) \) (Table 3).

The sensitivity (95%CI) of the bleach-digested, 'first on-spot' smear, taking the case definition as the true positivity, was 92.3% (80.5–98%), a specificity of 93.4% (88.5–96.6%), a PPV of 78.3% (64.7–88.4%) and a NPV of 97.7% (94.3–99.5%). The kappa was 0.8 (SE = 0.26). The bleach-digested 'first on-spot' smear had the highest positivity rate (26%). Using the ATS scale, the bleach-digested 'first on-spot' smears resulted in an increase in a smear positivity rate of 32.7% (17/52) compared to the yield from the direct smears of the same specimen, and a 15.2% (7/46) increase compared to the results of three direct smears.

DISCUSSION
Identifying smear-positive TB cases is a core component of TB control programmes. Smear microscopy

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Smear positivity among the 'first on-spot' specimens by direct microscopy and after bleach digestion (ATS scale)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleach-digested</td>
<td>Direct (standard) smears</td>
</tr>
<tr>
<td>smears</td>
<td>Negative</td>
</tr>
<tr>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>Negative</td>
<td>148</td>
</tr>
<tr>
<td>+</td>
<td>11</td>
</tr>
<tr>
<td>++</td>
<td>5</td>
</tr>
<tr>
<td>+++</td>
<td>1</td>
</tr>
<tr>
<td>++++</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>165</td>
</tr>
</tbody>
</table>

\( \chi^2 \) test = 15, \( P \) value < 0.001.
ATS = American Thoracic Society.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Smear positivity by bleach digestion compared with standard case definition (at least two positive direct ZN smears)</th>
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</thead>
<tbody>
<tr>
<td>Bleach-digested</td>
<td>Case definition</td>
</tr>
<tr>
<td>'first on-spot'</td>
<td>ZN smear</td>
</tr>
<tr>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td>Positive</td>
<td>36</td>
</tr>
<tr>
<td>Negative</td>
<td>3</td>
</tr>
<tr>
<td>Total (%)</td>
<td>39 (20.5)</td>
</tr>
</tbody>
</table>

\( \chi^2 \) test = 2.77, \( P \) values = 0.096.
ZN = Ziehl-Neelsen.
remains the method of choice for screening suspects and identifying the most infectious cases for treatment. The probability of detecting bacilli by direct microscopy increases with increasing density of bacilli; this probability appears to be in the neighborhood of 60% with 1000 bacilli and 95% with 10 000 per ml of sputum. For this reason, smear microscopy is a good test for identifying the most infectious cases; it is not, however, a sensitive test for TB diagnosis. Improvement in the sensitivity of smear microscopy is important in resource-poor countries where other alternative diagnostic procedures are not sustainable. Mycobacterial culture in particular is regarded as the gold standard, but is too slow and cumbersome to be used in routine clinical practice in resource-poor settings.

Digestion of sputum with an equal amount of 5% bleach for 30–45 min resulted in easy detection of bacilli against a clear background. However, as smears prepared from digested sputum were thin and not easily visible to the naked eye, extra care was required in labelling and staining the correct side of the slides. These complexities may result in greater inter-observer variation than standard direct microscopy and further studies are required to assess its reliability in various settings.

Handling sputum specimens poses a risk of infection to health personnel. This risk is mostly associated with culture and drug susceptibility testing procedures. The use of bleach digestion may make laboratory workers feel safe from laboratory infection even if direct smear preparation may not pose a great risk of infection.

Using a scale with a sensitive threshold for smear positivity (ATS), ZN stains of the bleach digested ‘first on-spot’ smears resulted in an increase in the smear positivity rate compared to the yield from the standard smears of the same specimen, and compared to the results of three standard smears. The majority of the gain (76%, 13/17) was from the group of patients excreting few bacilli (1+). Although the final result of this group could not be validated by culture, these cases are likely to be true TB cases in a country like Ethiopia, where the annual risk of infection is expected to be greater than 2%. According to a study by Levy et al. in South Africa, only 6.7% (5/75) of specimens categorised as ‘scanty-positive’ by the IUATLD scale were deemed false-positive.

All of our ‘first on-spot’ direct smears positive on standard direct microscopy were also positive by the bleach method, which supports the validity of the bleach-digested smears. Furthermore, three of the 11 suspects categorised as scanty by the bleach method but negative on the ‘first on-spot’ standard smear were subsequently identified as positive on the ‘overnight’ and ‘second on-spot’ standard smears.

Although the sensitivity of a single bleach-digested smear against the standard case definition is high, it also missed three of the 39 suspects who fulfilled the case definition. Further studies should investigate whether this can be improved by using two or more bleached smears or a combination of bleach and standard techniques.

In our study, six smear-positive patients from the ‘first on-spot’ specimens were lost through the process of submission of the recommended three sputum specimens. However, only five additional patients were identified by the overnight and third specimen. This suggests that there may be no additional gain of smear-positive cases from repeated sputum submissions over the detection rate achieved with a single, bleach-digested smear.

In one study from Lilongwe, Malawi, 37% of the patients who were suspected to have PTB were subsequently lost to the system because they did not submit further specimens, failed to collect results or were not registered for treatment. The comparatively low defaulter rate of 5% (10 patients) observed in our study could be attributed to the provision of results the day after submission of the ‘first on-spot’ specimens. In most government health institutions in Ethiopia, greater delays in provision of results are the norm. Salaniponi et al. identified that almost all TB suspects who submitted sputum to the laboratory in Malawi received their smear results after a median length of 4 days.

The additional cost of household bleach digestion to current costs for smear microscopy is likely to be negligible. Five hundred ml, which is enough for digestion of 100 samples, costs the equivalent of 50 UK pence in Ethiopia.

Considering the multifaceted advantages of bleach digestion and the dropout rate due to submission of multiple sputum samples, a single bleach-digested smear from the ‘first on-spot’ sample may be an appropriate approach for screening TB suspects in resource-poor countries. Suspects found to have smear-positive PTB could start their anti-tuberculosis treatment within one hospital/health centre visit. From the patient’s perspective, the additional costs of repeated health facility visits and lost time will be saved.

Cutting down the number of specimens recommended for diagnosis of PTB to two or possibly to one could be a relief for laboratories, which are usually overburdened and run by a single technician. The laboratory technicians could take adequate time to examine each slide and it is possible that sensitivity could further be improved. The cost for laboratory consumables could also be reduced by more than half. This is especially important in poor countries where laboratory supplies are frequently inadequate and inconsistent.

We believe that TB programmes, especially in resource-poor countries, should consider assessing the use of a single bleach-digested smear, in their case-finding activities. It may be that this approach is what is needed to make smear microscopy safe, simple and user-friendly to patients.
Acknowledgements

The authors would like to thank the Armauer Hansen Research Institute of Addis Ababa for their collaboration with the first part of the study, Southern Region Health Bureau and Bishoftu Major Health Centre for their generous support during the study. We appreciate and thank the support of the laboratory staff of BMHC; Kenene Fuffa and his colleagues for their involvement during data collection. We would like to thank the LSTM laboratory team for collection and voluntary re-reading of slides.

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References

El diagnóstico de la tuberculosis pulmonar se basa en el examen microscópico de la expectoración. Sin embargo, esta técnica tiene una baja sensibilidad. Se estudió la eficacia y la seguridad de la digestión de la expectoración con cloro antes de la tinción Ziehl-Neelsen (ZN).

MÉTODO: Se estudiaron los frotis positivos de esputo para evaluar la calidad de la tinción y la viabilidad de las micobacterias después de diversas duraciones de tratamiento con cloro. Enseguida, se prepararon 200 frotis a partir de la primera, segunda y tercera muestra de expectoración de los sujetos sospechosos de tuberculosis. Se agregaron cantidades iguales de cloro al 5% al resto de la primera expectoración y se prepararon frotis para la tinción ZN.

RESULTADOS: La calidad y la tinción óptimas se obtuvieron con tratamiento con cloro de 30 a 45 minutos. No se observó crecimiento a partir de las muestras positivas después de un tratamiento de 15 minutos. Las muestras tratadas con cloro tuvieron un 26% de positividad (52/200), en comparación con 17,5% (35/200) en las no tratadas ($P < 0,001$). Las muestras tratadas tuvieron una sensibilidad de 92,3%, una especificidad de 93,4% y valores predictivos positivo y negativo de 78,3% y de 97,7%, respectivamente. En 10 pacientes no se obtuvo la segunda o la tercera muestra de expectoración. Seis de ellos eran positivos ya sea al frotis estándar, al frotis tratado con cloro o a ambos.

INTERPRETACIÓN: La digestión con cloro es simple, barata y mata las micobacterias. Su tasa de positividad es tan buena como la de tres frotis estándar. Este método tiene la potencialidad de mejorar los servicios sobrecargados en los países en desarrollo.