

## Viability of stored sputum specimens for smear microscopy and culture

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### SUMMARY

A laboratory study was performed to determine how long sputum specimens from smear-positive tuberculosis patients can be stored at room temperature or in the refrigerator and retain a positive acid-fast bacilli (AFB) smear or a positive mycobacterial culture. Sputum samples from 30 patients were examined up to 4 weeks and samples from 13 patients examined up to 8 weeks. Provided samples had not dried out, all sputum smears

remained AFB positive up to 4 and 8 weeks. In both patient groups, at 4 weeks 37–39% of specimens at room temperature grew mycobacteria compared with 54–67% of specimens stored in the refrigerator. These results have implications for tuberculosis programme policy.

**KEY WORDS:** sputum; smear microscopy; mycobacterial culture

IN MALAWI, sputum specimens submitted at rural health centres are transported to hospital laboratories for smear examination. Logistical difficulties can lead to delays in getting sputum examined. Long sputum storage times may result in false-negative sputum smears.<sup>1–3</sup> Sputum specimens are also submitted to the central mycobacterial reference laboratory for culture for patients with previously treated smear-positive pulmonary tuberculosis (PTB) in order to determine drug sensitivity patterns which may influence the choice of drugs for the continuation phase of treatment. There are often delays in transporting specimens from district hospitals to the central reference laboratory, and this can lead to loss of culture viability.<sup>2–5</sup>

We designed two studies to examine how long sputum specimens can be stored, either at room temperature or in the refrigerator, before losing their acid-fastness for smear microscopy or their culture viability.

### METHODS

#### *Patients and study methodology*

Patients with smear-positive PTB registered at Queen Elizabeth Central Hospital, Blantyre, were asked to submit two further sputum specimens into containers before commencing treatment. These specimens were processed on the day of submission (day 0). Thereafter, one sputum container was stored in the refrigerator at 4°C and one at room temperature (20°–25°C). In the first study (Study 1), the refrigerated and room-temperature sputum specimens were processed

for smear microscopy and mycobacterial culture on days 2, 4, 7, 9, 11, 14, 21 and 28. When results were obtained for Study 1, a second study (Study 2) was conducted with smaller numbers of patients in which duration of sputum storage was extended up to 8 weeks, with smear and culture examinations performed on a weekly basis.

#### *Smear and culture examination*

Sputum smears were examined for acid-fast bacteria (AFB) using Ziehl-Neelsen stain.<sup>6</sup> For mycobacterial culture, each specimen was decontaminated by sodium hydroxide before inoculation onto Löwenstein-Jensen (L-J) slopes: examination was at weekly intervals for up to 8 weeks.<sup>7</sup>

#### *Observer variability*

There were two technicians involved in the laboratory. Observer variability in smear microscopy and culture was tested using nine portions of sputum from each of three patients (AFB 3+, AFB 2+ and AFB-negative). There was no intra- or inter-observer variability in smear or culture results.

#### *Statistical analysis*

Differences in culture viability at defined times and between refrigerated and room-temperature specimens were assessed using  $\chi^2$  test with Yates correction and  $\chi^2$  test for trend. Differences at the 5% level were regarded as significant. Odds ratios (OR), 95% confidence intervals (CI) and *P* values were also calculated.

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**Table 1** Sputum cultures up to 28 days after sputum submission

| Initial AFB smear result         | Storage of sputum | No. of specimens | Positive sputum cultures for <i>M. tuberculosis</i> |                       |                        |                        |                        |
|----------------------------------|-------------------|------------------|-----------------------------------------------------|-----------------------|------------------------|------------------------|------------------------|
|                                  |                   |                  | Day 0<br><i>n</i> (%)                               | Day 7<br><i>n</i> (%) | Day 14<br><i>n</i> (%) | Day 21<br><i>n</i> (%) | Day 28<br><i>n</i> (%) |
| 3+                               | Room              | 10               | 10                                                  | 9                     | 4                      | 2                      | 3                      |
|                                  | Refrigerator      | 10               | 10                                                  | 10                    | 8                      | 8                      | 9                      |
| 2+                               | Room              | 10               | 10                                                  | 9                     | 5                      | 7                      | 5                      |
|                                  | Refrigerator      | 10               | 10                                                  | 10                    | 9                      | 8                      | 7                      |
| 1+                               | Room              | 10               | 10                                                  | 8                     | 6                      | 3                      | 3                      |
|                                  | Refrigerator      | 10               | 9                                                   | 9                     | 7                      | 4                      | 4                      |
| Combined AFB smears (3+, 2+, 1+) | Room              | 30               | 30 (100)                                            | 26 (87)               | 15 (50)                | 12 (40)                | 11 (37)                |
|                                  | Refrigerator      | 30               | 29 (97)                                             | 29 (97)               | 24 (80)                | 20 (67)                | 20 (67)                |

Two of 540 sputum cultures became contaminated.

## RESULTS

### Study 1

There were 30 patients: 10 with 3+ AFB, 10 with 2+ AFB and 10 with 1+ AFB. If specimens did not dry out, all those that could be processed remained smear-positive on each examination day from the day of submission up to 28 days of storage, either at room temperature or in the refrigerator. Results of sputum culture-positivity are shown in Table 1. There was a gradual decline in culture-positivity up to 28 days of storage for both room-temperature and refrigerated specimens. A significantly higher proportion of refrigerated specimens were culture-positive at 14 days (OR = 4.0, 95%CI 1.1–4.9,  $P = 0.03$ ) and culture-positive at 28 days (OR = 3.4, 95%CI 1.1–11.6,  $P = 0.04$ ) compared with room-temperature specimens.

### Study 2

There were 13 patients: six with 3+ AFB, five with 2+ AFB and two with scanty AFB. Results for smear-positivity and culture-positivity for all patients combined are shown in Table 2. Sputum smears were all positive up to 4 weeks. By 8 weeks three specimens had dried out but the remaining 10 specimens were still smear-positive. There was a general decline in sputum culture-positivity, similar to that found in Study 1. At 4 weeks, positive cultures were found in five (39%) room-temperature specimens and seven (54%) refrigerated specimens. At 8 weeks, positive cultures

were found in three (23%) room-temperature specimens and six (46%) refrigerated specimens. These differences were not statistically significant.

## DISCUSSION

Results show that in a laboratory environment in Malawi all sputum specimens (whether stored at room temperature or in the refrigerator) that could be processed remained smear-positive for AFB up to 4 weeks in the first and second studies, and up to 8 weeks in the second study. The major threat to smear microscopy was in the sputum specimen drying out, and this may have been a result of the study design which involved opening and shutting sputum containers on prescribed days in order to obtain portions of sputum for examination.

Mycobacterial culture viability declined with time, although the ability to grow mycobacteria was better for specimens kept in the refrigerator than at room temperature. In both studies, at 4 weeks between 37–39% of specimens kept at room temperature could grow mycobacteria compared with 54–67% of specimens stored in the refrigerator. At 8 weeks, the ability to grow mycobacteria had declined to less than 25% for room-temperature specimens and less than 50% for refrigerated specimens. The decline in mycobacterial culture viability was not constant, particularly in Study 2, which probably re-

**Table 2** Sputum smear and sputum culture results up to 8 weeks after sputum submission

| Type of specimen                                    | Storage of sputum | Weeks from submission of sputum specimen |    |    |    |    |    |    |    |    |  |
|-----------------------------------------------------|-------------------|------------------------------------------|----|----|----|----|----|----|----|----|--|
|                                                     |                   | 0                                        | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  |  |
| Sputum smears positive for AFB                      | Room              | 13                                       | 13 | 13 | 12 | 13 | 12 | 12 | 12 | 10 |  |
|                                                     | Refrigerator      | 13                                       | 13 | 13 | 13 | 13 | 12 | 12 | 12 | 10 |  |
| Sputum cultures positive for <i>M. tuberculosis</i> | Room              | 10                                       | 9  | 8  | 5  | 5  | 2  | 2  | 6  | 3  |  |
|                                                     | Refrigerator      | 8                                        | 10 | 10 | 6  | 7  | 6  | 5  | 8  | 6  |  |

Sputum specimens were examined for 13 patients: 1 sputum specimen became dry at 6 weeks, 3 sputum specimens became dry at 8 weeks, 5 of 234 sputum cultures became contaminated.  
AFB = acid-fast bacilli.

flects observer variability in sampling portions of sputum and in decontaminating specimens. In both studies, the proportion of contaminated cultures was small, probably reflecting a rather harsh decontamination process.

This study has implications for the Malawi National Tuberculosis Control Programme. First, specimens for smear microscopy which arrive late in hospital laboratories from health centres should not be automatically discarded by laboratory technicians. Smears can still be positive even if sputum has been stored for up to 8 weeks. Second, sputum specimens sent to the mycobacterial central reference unit for culture and sensitivity should be kept in a refrigerator prior to transportation. While it is better to avoid delay, at least 50% of refrigerated specimens may show mycobacterial growth even if the delay is up to 4 weeks.

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#### RÉSUMÉ

Une étude de laboratoire a été conduite pour déterminer pendant combien de temps les échantillons d'expectoration provenant de patients atteints de tuberculose à bacilloscopie positive peuvent être conservés à la température ambiante ou au réfrigérateur tout en conservant une bacilloscopie positive pour les bacilles acido-résistants ou en donnant lieu à une culture positive pour les mycobactéries. On a examiné les expectorations de 30 patients pendant des durées allant jusque 4 semaines et

celles de 13 patients jusqu'à 8 semaines. Si les expectorations fournies ne s'étaient pas desséchées, tous les frottis d'expectoration restaient positifs jusqu'à 4 et 8 semaines. Dans les deux groupes de patients, après 4 semaines, 37 à 39% des spécimens conservés à température ambiante continuaient à donner des mycobactéries cultivables par rapport à 54 à 67% des échantillons conservés au réfrigérateur. Ces résultats ont des implications pour la tactique du programme de tuberculose.

#### RESUMEN

Se efectuó un estudio de laboratorio para determinar cuánto tiempo las muestras de esputos de los pacientes tuberculosos con baciloscopia positiva pueden almacenarse a la temperatura ambiente o en un refrigerador y conservar la baciloscopia positiva o el cultivo positivo. Se examinaron esputos de 30 pacientes durante 4 semanas y esputos de 13 pacientes durante 8 semanas. Mien-

tras los esputos no se secaban, todos conservaron la baciloscopia positiva durante 4 y 8 semanas. En ambos grupos de pacientes, a las 4 semanas, 37–39% de las muestras a temperatura ambiente mostraron crecimiento de micobacterias, comparadas con 54–67% de las muestras refrigeradas. Estos resultados tienen consecuencias para los programas de tuberculosis.