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An alternative method for sputum storage and transport for *Mycobacterium tuberculosis* drug resistance surveys

R. Lumb,* M. Ardian,†‡§ G. Waramori,§ H. Syahrial,¶ E. Tjitra,¶ G. P. Maguire,# N. M. Anstey,#†† P. M. Kelly#††**

*** Institute of Medical & Veterinary Science, Adelaide, South Australia, Australia; † District Ministry of Health, Timika, Papua Province, ‡ International SOS, Timika, Papua Province, § Public Health and Malaria Control Department, PT Freeport Indonesia, Timika, Papua Province, ¶ National Institute of Health Research and Development, Jakarta, Indonesia; # Menzies School of Health Research, Darwin, Northern Territory, ** Western Australian Country Health Services—Kimberley region, Broome, †† Institute of Advanced Studies, Charles Darwin University, Darwin, Australia**

SUMMARY

SETTING: A district level tuberculosis (TB) programme in Indonesia.

OBJECTIVE: To evaluate whether a single sputum specimen could be stored by refrigeration for an extended period of time, then transported to a reference laboratory and successfully cultured for *Mycobacterium tuberculosis*.

METHODS: Single sputum specimens were collected from newly diagnosed smear-positive pulmonary TB patients, refrigerated at the study site without addition of 1% cetylpyridinium chloride, batched and sent to the reference laboratory, where they were decontaminated and inoculated into BACTEC MGIT 960 liquid media.

RESULTS: One hundred and seven patients were enrolled. The median specimen storage time was 12 days (range 1–38) and median transportation time was 4 days (2–12). The median time from specimen collection until processing was 18 days (4–42). Only 4 (3.7%) specimens failed to grow *Mycobacterium* species and *M. tuberculosis* was isolated from 101 (94.4%) specimens. Six specimens with breakthrough contamination successfully grew *M. tuberculosis* after a second decontamination procedure.

CONCLUSIONS: Single sputum specimens collected at a remote setting, refrigerated for relatively long periods without preservatives and transported without refrigeration to a reference laboratory can yield a high positive culture rate. These findings offer potential logistic simplification and cost savings for drug resistance surveys in low-resource countries.

KEY WORDS: tuberculosis; drug resistance; survey; sputum; specimens

RESISTANCE of *Mycobacterium tuberculosis* (TB) to first-line anti-tuberculosis drugs is of global concern and a major threat to the DOTS strategy.[1] In particular, knowledge about the level of drug resistance in the 22 countries with the highest burden of TB has been identified as a high priority by the World Health Organization (WHO).[2] Guidelines for conducting drug resistance surveillance (DRS) are well established[3] and their utility has been confirmed.[4] However, the successful conduct of DRS is complex and expensive, requiring infrastructure, in-country expertise, quality assurance, logistics and sufficient funding to achieve a timely and meaningful outcome. Correct collection and transportation of sputum specimens from remote survey settings to a quality-controlled TB culture laboratory is crucial to ensure accurate results that can contribute to national and global surveillance of TB drug resistance.[3–5] Various factors contribute to the complexity and cost of DRS to conform to the current WHO protocol, especially in high-burden countries, where mycobacteriology laboratory facilities are often lacking. The WHO recommends that two sputum specimens be collected and transported to a designated reference laboratory without delay.[3] There may be a substantial cost associated with handling and transportation of specimens to a reference laboratory. In addition, sputum specimen preservatives, such as 0.6% cetylpyridinium bromide (CPB) or 1% cetylpyridinium chloride (CPC), are recommended. These preservatives must be removed by centrifugation prior to inoculation onto culture medium.[6] Unfortunately, many laboratories in high-burden countries do not have centrifuges capable of generating a sufficient relative centrifugal force capable of reliably sedimenting mycobacteria.[7]

A previous pilot study did not add CPC to sputum

Correspondence to: Assoc Prof Paul M Kelly, Menzies School of Health Research, P O Box 41096, Casuarina, Northern Territory, 0811, Australia. Tel: (+61) 8 8922 8837. Fax: (+61) 8 8927 5187. e-mail: paulk@menzies.edu.au

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samples prior to transport from the field site. Despite considerable transport delays, satisfactory culture results were obtained from both specimens in the study.[8] The preparation and transportation of a single sputum specimen for each patient without refrigerated transport or preservatives was therefore formally evaluated during a DRS survey in Mimika District, Papua Province, Republic of Indonesia.

METHODS

Background and setting

The setting and background for this study are described elsewhere.[9] Briefly, all new, previously untreated, sputum smear-positive pulmonary TB (PTB) patients diagnosed in Mimika District between 7 July 2003 and 5 May 2004 who were notified in the district register were eligible for enrolment in the DRS survey. A diagnosis of smear-positive PTB was based on three sputum specimens.[10,11] Once diagnosed, consenting patients were enrolled into the DRS survey and a single additional sputum sample was collected prior to commencing TB treatment for transport to the reference laboratory.

Sputum specimens

The specimens were kept in a domestic refrigerator at +4°C in the TB clinic until being prepared for transport by air with a commercial courier via Darwin to the Institute of Medical and Veterinary Sciences (IMVS) Mycobacterium Reference Laboratory in Adelaide, Australia. Preparation and packaging of the specimens complied with International Air Transport Association (IATA) regulations. No decontamination procedure was performed prior to storage or transportation. Specimens were sent in batches at 3- to 6-week intervals during the study period. After leaving the TB clinic, specimens were kept at room temperature until arrival at the reference laboratory.

Reference laboratory methods

On arrival at the IMVS, sputum specimens were decontaminated using 2% sodium hydroxide and 0.5% N-acetyl-cysteine for 25 min, then neutralised to pH 7, concentrated by centrifugation (3000 × g for 15 min) and inoculated into a single Mycobacterium Growth Indicator Tube 960 (MGIT, Becton Dickinson Microbiology Systems, Sparks, MD, USA). Microscopy of sputum concentrates was performed using fluorochrome and Ziehl-Neelsen stains, and the results recorded using standard International Union Against Tuberculosis and Lung Disease (IUATLD) grading scales.[6] Contaminated specimens were re-decontaminated with 4% sulphuric acid. *M. tuberculosis* isolates were identified by ZN staining, hybridisation with commercial nucleic acid probes (Accuprobe, Gen-Probe, Inc, San Diego, CA), and biochemical investigations (e.g., nitrate reduction).[12,13] The IMVS successfully participates in external quality assessment programmes through the WHO Global Supranational Reference Laboratory Quality Control Network and the Centers for Disease Control and Prevention, Atlanta, GA, USA.

Data analysis

All laboratory data were entered into an MS Excel spreadsheet (Microsoft Corp, Redmond, WA, USA). STATA™ (Version 8, Stata Corp, College Station, TX, USA) was used for analysis. Some of the predictor variables and one outcome variable, most notably time measurements, were non-normal distributions and we have therefore reported summary statistics of median and range. A log-transformation of the time to positive culture outcome variable resulted in a normal distribution suitable for linear regression analysis. Transformation of predictive time variables did not significantly affect the relationship with outcomes. We used linear regression to analyse predictors of the other outcome of interest (successful culture). An extension of the Wilcoxon rank-sum test (nptrend) was used as a non-parametric test for trend across ordered groups. Statistical significance was reached if $P < 0.05$.

Ethics

Ethical permission for this study was obtained from the Ethics Committees of the National Institute of Health Research and Development in Jakarta, Indonesia, and the Menzies School of Health Research in Darwin, NT, Australia.

RESULTS

A total of 107 patients were enrolled in the study and all submitted a single sputum specimen prior to the beginning of treatment. Only 4/107 (3.7%) failed to grow *Mycobacterium* species within 6 weeks of incubation in a broth-based culture system. One hundred and one (98.1%) of the mycobacterial isolates were *M. tuberculosis*, and two were rapid growing non-tuberculous mycobacteria (Table 1). The four specimens that failed to grow had a low mycobacterial load as measured by sputum smear microscopy at arrival (two smear-negatives and two scanty positives). Both scanty positives had leaked badly during transit, and only a very small volume of sputum was available

Table 1 Results of culture of sputum specimens sent to reference laboratory

	n (%)	95%CI
Mycobacterium tuberculosis	101 (94.4)	88.2–97.9
Rapid growing mycobacteria*	2 (1.9)	0.2–6.6
No growth	4 (3.7)	1.0–9.3
Total	107 (100)	

* *M. mucogenicum* (1) and *M. fortuitum* complex (1).

for processing. The two smear-negative specimens were small in volume but otherwise satisfactory.

The median time for sputum specimens stored without preservatives and under refrigeration before transportation was 12 days (range 1–38 days). Twenty-nine (27.1%) of the specimens were refrigerated for more than 3 weeks prior to shipment. Specimen transportation from Timika occurred without refrigeration with a median time of 4 days (range 2–12 days) to reach the laboratory. Only 12 (11.2%) of the specimens took more than 1 week to be processed from the time of shipment. Overall, the median time from specimen collection until processing was 18 days (range 4–42 days). Low sputum specimen volume (<2 ml) was present in 33/107 (30.8%) specimens, and some specimens had as little as 200 μl. The total time delay between sputum specimen collection and inoculation into the culture medium had no significant effect on the time to positive culture (Table 2).

Of 107 sputum smears examined at the reference laboratory, 101 (94.4%) were smear-positive for acid-fast bacilli (AFB), of which 75/101 (74.3%) had an AFB burden of at least 2+. Only six (5.6%) had breakthrough contamination with non-mycobacterial organisms. All contaminated specimens were re-decontaminated and *M. tuberculosis* was successfully cultured. The total time delay between specimen collection and inoculation into MGIT tubes had no significant effect on the time to positive culture (see Table 2). The extent of smear positivity on arrival at the reference laboratory was highly predictive of a positive culture result (odds ratio [OR] 6.30, 95% confidence interval [CI] 1.64–24.18, $P = 0.007$). The length of time specimens were kept in refrigeration prior to transport had no effect on the proportion of positive culture results. After adjustment for the (unrefrigerated) transit time, smear positivity at the clinic remained statistically significantly associated with a positive culture result (OR 8.44, 95%CI 1.47–48.29, $P = 0.017$). Sputum smear positivity was inversely related to the time interval from MGIT tube inoculation to positive culture (see Table 3). This relationship was statistically significant, and unaffected by duration of refrigeration or time in transit to the laboratory.

Table 2 Effect of time delay to process sputum specimens compared with time to recovery of *Mycobacterium tuberculosis*

Days*	Patients *n* (%)	Time to positive culture Median (range)
1–7	9 (9)	13.0 (7–23)
8–14	21 (21)	13.0 (7–24)
15–21	28 (28)	11.5 (7–24)
22–28	25 (25)	13.0 (6–23)
29–42	18 (18)	12.5 (9–25)
Total	101 (100)	13.0 (6–25)

* Total days from specimen collection to inoculation on culture medium.
P (trend) = 0.12.

Table 3 Smear positivity and time to isolation of *Mycobacterium tuberculosis*

Smear microscopy	Patients *n* (%)	Time to culture Median (range)
Negative	2 (2)	21 (19–23)
Scanty	5 (5)	16 (16–21)
1+	19 (19)	15 (10–25)
2+	28 (28)	13 (6–19)
3+	47 (47)	11 (6–24)
Total	101 (100)	13 (6–25)

P (trend) <0.001.

DISCUSSION

Despite an often considerable delay between collection of specimens and processing in a reference laboratory, plus the lack of chemical preservation and the collection of only one sputum specimen per patient, the yield of positive *M. tuberculosis* cultures in this study was very high. These findings suggest that in some settings, a simpler and cheaper alternative to the current WHO recommendations for conducting drug resistance studies in low-income settings is possible.

The WHO protocol recommends the collection of two sputum specimens to ensure a high yield of positive cultures.[3] DRS studies are expensive and technically demanding, in part due to this requirement. These recommendations have a considerable impact, as they necessitate two clinic attendances for the patient, added to which are the logistics of storage, transportation and dual laboratory processing. Our results demonstrate an acceptably high yield of positive cultures from a single specimen, with considerable cost savings.

When specimens are likely to be exposed to room temperature for >48 h, the WHO recommends that an equal volume of either 0.6% CPB or 1% CPC should be added to homogenise and decontaminate the sample.[14,15] Studies suggest that CPC is preferred because of its superior solubility in viscous sputum specimens and CPC's lower toxicity to tubercle bacilli.[14] However, once CPB or CPC has been added to a specimen, refrigeration as a storage method is no longer possible, as these preservatives are likely to crystallise and become inactive.[3] Once preserved specimens have reached the culture laboratory, a centrifugation step (without refrigeration) is necessary to remove the preservative prior to culture.[6] Furthermore, once CPB or CPC has been added, subsequent decontamination using the Petroff method is not advised due to the adverse consequences upon the mycobacteria.[3] Our study demonstrates that contamination is rare and easily corrected in the reference laboratory without a decontamination step prior to transport.

Refrigeration prior to transport is the agreed international protocol. In one study, increasing storage time at room temperature resulted in reduced recovery of *M. tuberculosis* and rising contamination rates.[16] Pardini et al. found that sputum specimens stored and

transported to an international reference laboratory took a mean of 20 days (range 7–36 days) to arrive.[17] Specimens were either preserved using CPC or left untreated and stored at +4°C. Recovery of *M. tuberculosis* from solid culture media (for example Löwenstein-Jensen [LJ] or 7H10) was significantly increased in CPC treated specimens compared with untreated specimens, and contamination rates were considerably lower.[17] These findings are in conflict with our study, with the most likely explanation being the selection of patients. In Timika, all were newly diagnosed smear-positive patients, while in Pardini et al.'s study some patients were already on anti-tuberculosis treatment. It is not clear if smear-negative patients were also included in the sample. Interestingly, in Pardini et al.'s study, specimen smear positivity was a factor in the recovery of *M. tuberculosis*. There was no significant difference in successful culture between the CPC and untreated groups when sputum smear microscopy results were 1+ or greater. Another study found that preserved specimens left at room temperature (25–35°C) for 5–18 days before being decontaminated with NALC-NaOH yielded a superior recovery of *M. tuberculosis* from untreated specimens also left at room temperature.[18] However, the result was highly dependent on the culture medium used. For LJ, the CPC method gave superior results, but for MGIT 960, the culture yield was not significantly different.[18] Our study demonstrated that prolonged storage of sputum specimens under refrigeration and without preservatives had little detrimental effect upon the recovery of *M. tuberculosis*.

Once the sample size for a DRS survey has been determined, WHO recommends that it be increased by 5–20% to account for expected losses including those due to contamination or no growth and non-interpretable drug susceptibility tests.[3] The present study determined that 94.4% of single sputum specimens submitted for culture grew *M. tuberculosis*, with a low contamination rate. The IMVS laboratory used the same standard decontamination protocol for processing Timika specimens as it did for routine diagnostic specimens. The breakthrough contamination rate of 5.6% for Timika specimens was similar to that for diagnostic specimens collected locally and processed by the IMVS laboratory, and was at the lower end of reported culture contamination rates for the BACTEC MGIT 960 system.[17–19] None of the 107 specimens processed for mycobacterial culture were lost due to an overwhelming contamination event. These results suggest that a reduced number of specimens collected in DRS surveys may be contemplated, based on collection of a single rather than multiple sputum specimens, thus saving time and expense.

There are several limitations to the present study. In a low-income setting, refrigerators are generally not present in TB diagnostic centres. However, it is feasible that, in the context of a DRS survey, a refrigerator could be provided at a district or provincial laboratory level to act as a staging point where specimens can be referred to and stored under refrigeration prior to ongoing transportation (within 6 weeks) to a reference laboratory. Using our method, even with the provision of a number of refrigerators, the cost savings would still be substantial. In the present study, one contributor to the successful recovery of *M. tuberculosis* from single specimens may be the use of the automated BACTEC MGIT 960 system rather than solid media. Current WHO recommendations are to use conventional solid media such as LJ, modified Ogawa or Stonebrink.[3,6] The newer, more expensive, broth-based culture systems such as BACTEC MGIT 960 or radiometric BACTEC 460TB have a reduced time to culture positivity and improved recovery of *M. tuberculosis* when compared with solid media.[19–21] Also, the CPC method is incompatible with the BACTEC 460 TB system,[22] and most likely with the MGIT 960 system. Unfortunately, such broth-based culture systems are expensive and require a dedicated infrastructure to perform optimally. In the present study, 74.3% of specimens were 2+ smear-positive or greater, and this raises the question of whether the findings are generalisable to other settings. However, a recent review of the TB laboratory network in another province of Indonesia found that 73.3% of smears were 2+ or greater (R Lumb, personal observations, 2005) suggesting that the present study is applicable at least to other provinces in Indonesia.

CONCLUSION

In a remote setting, successful recovery of *M. tuberculosis* from single sputum specimens, even after prolonged delay and without using chemical preservatives or decontamination prior to transport, is achievable. The present study challenges the findings of previous reports and the current WHO protocols for TB DRS. This alternative, simpler method of sputum specimen storage and transport warrants further investigation as a potential cost-saving measure for future DRS surveys in low-income countries. Future studies in field conditions are required to confirm these findings in other settings. These should specifically include the use of solid culture media.

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RÉSUMÉ

CONTEXTE : Un programme de tuberculose (TB) au niveau du district en Indonésie.

OBJECTIF : Evaluer dans quelle mesure un seul échantillon d'expectoration pourrait être conservé par réfrigération pendant une longue période de temps et transporté ensuite vers un laboratoire de référence et mis avec succès en culture pour *Mycobacterium tuberculosis*.

MÉTHODES : Des échantillons uniques d'expectoration ont été recueillis chez des patients atteints de TB pulmonaire à bacilloscopie positive récemment diagnostiquée. Ils ont été réfrigérés au site de l'étude sans addition de chlorure de cétylpyridinium à 1%, étiquetés et envoyés au laboratoire de référence où ils ont été décontaminés et inoculés dans un milieu liquide BACTEC MGIT 960.

RÉSULTATS : On a enrôlé 107 patients. La durée médiane de conservation des échantillons a été de 12 jours (extrêmes 1–38), la durée de transport de 4 jours (2–12). La durée médiane entre le recueil des échantillons et leur traitement a été de 18 jours (4–42). Seulement quatre échantillons (3,7%) n'ont pas permis la culture d'une espèce de mycobactérie. *M. tuberculosis* a été isolé dans 101 (94,4%) échantillons. Six échantillons avec contamination massive ont permis le développement de *M. tuberculosis* après une deuxième procédure de décontamination.

CONCLUSIONS : Des échantillons uniques d'expectoration prélevés dans un contexte périphérique, conservés au réfrigérateur pour des périodes relativement longues sans produit préservateur et transportés sans réfrigération vers un laboratoire de référence peuvent donner un taux élevé de cultures positives. Ces observations ouvrent la voie à des simplifications logistiques potentielles ainsi qu'à des économies pour les enquêtes de résistance aux médicaments dans les pays à faibles ressources.

RESUMEN

MARCO DE REFERENCIA: Un programa de lucha contra la tuberculosis (TB) a nivel de distrito en Indonesia.

OBJETIVO: Evaluar la posibilidad de tener éxito en el cultivo de *Mycobacterium tuberculosis* tras el almacenamiento local de muestras únicas de esputo en medio refrigerado durante un período prolongado y su posterior transporte al laboratorio de referencia.

MÉTODOS: Se recogieron muestras únicas de pacientes con diagnóstico reciente de TB pulmonar con baciloscopia positiva, se almacenaron localmente en medio refrigerado sin adición de cloruro de cetilpiridinio y se agruparon luego para enviarlas al laboratorio de referencia donde se descontaminaron y se sembraron en medio líquido en el sistema automatizado BACTEC MGIT 960.

RESULTADOS: Participaron en el estudio 107 pacientes. La mediana del tiempo de almacenamiento fue 12 días (entre 1 y 38) y la mediana del tiempo de transporte fue 4 días (entre 2 y 12). La mediana del lapso entre la recogida de la muestra y su procesamiento fue 18 días (entre 4 y 42). Se obtuvieron cepas de *M. tuberculosis* de 101 muestras (94,4%); sólo en cuatro muestras (3,7%) no pudo aislarse ninguna especie de micobacteria. En seis muestras con contaminación intercurrente se aisló con éxito *M. tuberculosis* después de la segunda descontaminación.

CONCLUSIONES: Es posible obtener una alta tasa de cultivos positivos partiendo de muestras únicas de esputo recogidas en un sitio remoto, almacenadas sin conservantes durante períodos relativamente prolongados y transportadas luego sin refrigeración a un laboratorio de referencia. Estos resultados ofrecen una simplificación logística viable con el fin de disminuir los costos de los estudios de farmacorresistencia en países con escasos recursos.