What is the most reliable solid culture medium for tuberculosis treatment trials?


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ARTICLE INFO

Article history:
Received 2 June 2013
Received in revised form 25 February 2014
Accepted 2 March 2014

Keywords:
Solid media
Mycobacterium tuberculosis
Drug trials
LCA

SUMMARY

We conducted a prospective study to determine which solid medium is the most reliable overall and after two months of therapy to detect Mycobacterium tuberculosis complex (MTB). MTB isolation and contamination rates on LJ and Middlebrook 7H10 and 7H11 agar with and without selective antibiotics were examined in a single laboratory and compared against a constructed reference standard and MGIT 960 results. Of 50 smear positive adults with pulmonary TB enrolled, 45 successfully completed standard treatment. Two spot sputum specimens were collected before treatment and at week 8 and one spot specimen each at weeks 2, 4, 6, and 12. The MTB recovery rate among all solid media for pre-treatment specimens was similar. After 8 weeks, selective (S) 7H11 had the highest positivity rate. Latent class analysis was used to construct the primary reference standard. The 98.7% sensitivity of 7H11S (95% Wilson confidence interval 96.4%–99.6%) was highest among the 5 solid media (P = 0.003 by bootstrap); the 82.6% specificity of 7H10S (95% CI 75.7%–87.8%) was highest (P = 0.098). Our results support 7H11S as the medium of choice. Further studies in different areas where recovery and contamination are likely to vary, are recommended.

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1. Introduction

Various solid media were developed for the isolation of Mycobacterium tuberculosis complex (MTB) from clinical specimens. Egg-base media such as Lowenstein–Jensen (LJ) and Ogawa have been the primary culture media for both diagnostic specimens and specimens from tuberculosis (TB) patients receiving anti-TB therapy. More recently agar-base media such as Middlebrook 7H10 and 7H11 have been used instead. Advantages are agar-base media avoid the need for drug-free eggs and an inspissator, growth appears sooner, easy to visualize and separate individual colonies with a larger transparent surface, and their larger surface area makes it easier to recover MTB in the presence of contaminants. Furthermore, antibiotics which do not inhibit the growth of MTB can be incorporated into the agar media to suppress contaminant growth and make the media selective for MTB [1]. Microbiological and logistical differences influence the choice of medium for research and clinical purposes.

A brief review of the development of solid media helps to increase understanding of differences encountered in their use. Egg-base media contain homogenized whole eggs, organic and inorganic salts, asparagine, and glycerol [2]. A colored dye such as malachite green, which also inhibits many contaminating microorganisms, is usually added to the medium to allow the buff colored colonies of MTB to be seen more easily. The most frequently used
culture medium in TB laboratories worldwide and the medium used most commonly in earlier phase 3 TB drug trials is oplex LJ medium [3]. LJ medium is relatively inexpensive to prepare in local laboratories, is fairly resistant to contamination, and does not depend on incubation with supplemental carbon dioxide for mycobacterial recovery. 7H10 and 7H11 agar media were developed by Middlebrook [4,5] in the 1940s in an effort to culture more fastidious mycobacteria, to be able to detect growth more quickly by visualizing small early colonies, and to be able to identify colonial morphology more easily on transparent media. 7H10 and 7H11 media are supplemented with oleic acid, albumin, dextrose, and catalase (OADC). 7H11 differs from 7H10 by the addition of casein hydrolysate which improves recovery of some fastidious strains of MTB [6]. The most widely used selective media are 7H10 and 7H11 agar supplemented with polymyxin B, amphotericin B, carbenicillin, and trimethoprim, which has been used for sputum culture and recovery of MTB from contaminated cultures [1]. These selective media have little effect on recovery of MTB. Selective media are also suitable for performing quantitative colony counts and have been used for this purpose in phase 2 early bactericidal activity (EBA) trials of TB drugs and drug combinations [7]. There is no general agreement on which solid medium is most reliable for isolation of MTB, so the choice is based on personal experience, laboratory tradition, or both. The unpublished experience in some laboratories has been that some strains grow on one type of solid medium and not another. Before liquid culture was adopted for MTB isolation, some labs used both an egg-base and an agar-base medium to optimize recovery. Overall, egg-base media have been more popular because CO2 incubation is used with agar media and the OADC supplement adds to the cost.

Historically, most clinical trials of anti-TB drugs have been conducted with the widely used and readily available LJ medium [8]. Drug regimens were developed using two-month culture conversion on LJ media as a surrogate for response to anti-TB therapy [9]. Previous studies have compared different solid media for isolating MTB from sputum specimens collected at time of diagnosis; however, no reports compare recovery on different solid media during treatment. Detection of MTB in sputum after one and two months of treatment is a widely used endpoint in clinical trials [10]. However, the medium providing the highest sensitivity and specificity when the tubercle bacilli are under drug pressure should be the medium of choice for determining response to therapy and specifically the two-month sputum conversion rates.

When used in the diagnosis of TB and for monitoring the response to anti-TB therapy, egg-base and agar-base media have been assumed to be equivalent for isolating MTB, in the absence of published reports to the contrary [5]. Hence, culture data from different or multicenter studies using various solid media have been aggregated or compared without regard for the type of solid media used. Selecting the most reliable medium for standard use across all sites in multicenter studies may provide more reliable sputum culture results for future registration trials of new anti-TB drugs and regimens.

The objective of this study was to compare culture results of five types of solid media with (S) and without selective antibiotics: LJ, 7H10, 7H11, 7H10S, and 7H11S to determine which solid medium is the most reliable overall during the two months of anti-TB therapy. In addition to examining recovery and contamination rates, culture results for the five media were compared against a constructed reference standard. To assess the accuracy of each medium, we employed latent class analysis (LCA), which has often been used in medical settings to evaluate diagnostic tests in the absence of a gold standard [11,12]. LCA combines data from observed measures (manifest variables) to construct an unobserved reference standard for comparison (latent variable). This analysis focuses on relationships among solid media; however, as a secondary analysis, we compared results on solid media with the Mycobacteria Growth Indicator Tube (MGIT) 960 system — the most widely used liquid culture system. Liquid media, such as that used in the MGIT 960 system, are more sensitive than solid media for MTB culture [14]. Additionally, serial sputum collections from patients on anti-TB treatment remain culture-positive longer on liquid media than on solid media [13,15,16].

2. Methods

2.1. Study population

HIV-uninfected patients aged 18–60 years, with suspected cavity pulmonary TB, highly positive sputum smears for acid fast bacilli (AFB), and no history of previous TB treatment, presenting to the National TB Treatment Centre at Mulago Hospital in Kampala, Uganda between August 2009 and August 2010 were recruited into a prospective study to assess the performance of various solid media. The study protocol was approved by the Joint Clinical Research Centre in Kampala, University Hospitals Case Medical Center, and U.S. Centers for Disease Control and Prevention institutional review boards and the Ugandan National Council for Science and Technology. Informed consent for study participation and HIV testing was obtained from all patients. All patients received pre- and post-HIV test counseling.

At enrollment, medical history, physical examination, performance status (Karnofsky performance scale), posteroanterior chest radiograph, sputum AFB smears, complete blood count, serum aspartate aminotransferase, total bilirubin, and creatinine were recorded. Patients with a Karnofsky performance scale score over 50% [17] and a highly positive sputum AFB smear [grade 3+ or 4+] [18] were enrolled to maximize the duration of culture positivity and therefore to maximize the possible number of positive culture results.

2.2. Short-course chemotherapy

All patients were treated with an ATS/CDC/IDSA-recommended regimen consisting of two months of daily isoniazid, rifampicin, ethambutol, and pyrazinamide followed by four months of daily isoniazid and rifampicin with dosages adjusted for body weight [19]. Patients who were culture-positive after two months of treatment were treated with seven months of continuation phase therapy in accordance with these guidelines. All doses were supervised. Cultures from patients on study treatment were included in the study even if the patient’s treatment regimen subsequently was changed because of drug resistance or withdrawal from study. Anti-TB drugs were obtained from VersaPharm, Inc., Marietta, GA, USA (rifampin) and Svizera Europe BV, Almere, the Netherlands (isoniazid, ethambutol, and pyrazinamide), and manufactured under Good Manufacturing Practice.

2.3. Sputum collection and processing

Two spot, deep-cough sputum specimens were collected prior to initiation of treatment and at week 8. One spot specimen was obtained at each of weeks 2, 4, 6, and 12. Spot specimens were also collected monthly during the remainder of treatment but are not part of the present analysis. When more than one specimen was collected at a time point, both were included in this analysis. Graphic presentations of change over time show only one specimen per time point, using the first of two collected at baseline and week 8. All specimens were collected in the clinic setting. If transport was delayed for more than two hours, specimens were refrigerated at 2–8 °C. Specimens were processed within 24 h of collection. Spuva were decontaminated using a standard NALC-sodium
hydroxide—sodium citrate method with a 1.5% final NaOH concentration [18]. Following centrifugation, sediments were reconstituted with 2.5 ml of phosphate buffer, pH 6.8. Sputum smear slides for AFB examination were prepared from these reconstituted sediments.

2.4. Solid media culture

Commercially prepared LJ slants (Becton Dickinson, Sparks, Maryland) were used. Middlebrook 7H11 and 7H10 media were prepared from the base (DIFCO, BD Franklin Lakes, NJ, USA), supplemented with OADC (BBL, BD Franklin Lakes, NJ, USA) following standard operating procedures. 7H11 medium is 7H10 with the addition of 10 g/L pancreatic digest of casein, which stimulates more luxuriant growth. The media were made selective by the addition of final concentrations of 200 U/ml of polymyxin B, 50 g/mL of carbenicillin, 20 µg/mL of trimethoprim, and 10 µg/mL of amphotericin B [1]. All drugs were from Sigma—Aldrich, St. Louis, MO, USA. Every batch of media was prepared after the addition of a gas-permeable tape was incubated in presence of 5% CO2. All media were examined for colonies of typical MTB morphology even in the presence of contaminants. There was no classification of the contaminant growth.

2.5. MGIT 960 liquid culture

MGIT tubes supplemented with final concentrations of 12.5 µU/ml polymyxin B, 1.25 µg/ml amphotericin B, 5 µg/ml nalidixic acid, 1.25 µg/ml trimethoprim and 1.25 µg/ml azlocillin (PANTA) were inoculated with 0.5 ml of the reconstituted sediment and placed in the MGIT 960 system (Becton, Dickinson and Company, Franklin Lakes, NJ). The tubes were automatically and continuously monitored for growth and remained in the instrument until it signaled “positive” for growth or “negative” at the end of the 42-day incubation. All positive cultures were stained with the Ziehl—Neelsen method to confirm the presence of AFB and inoculated on blood agar plates to detect contaminants.

2.6. Identification

Positive cultures were confirmed as MTB using the Capilia (Tauns, Numazu, Japan) lateral flow MPB64 antigen detection method. We categorized each culture result as MTB positive, contaminated, or MTB negative. If a culture showed both MTB growth and contamination we categorized it as MTB positive. We categorized cultures with contamination and no detectable MTB growth as contaminated. Since the end point was MTB recovery or not and we had multiple cultures from the same patient in case a pure isolate was required, contaminated specimens were not reprocessed.

2.7. Statistical analysis

Details of the statistical analysis will be reported in a separate paper and are summarized here.

2.7.1. Primary analysis

All analyses were performed in SAS (v9.2) using an LCA procedure created by The Methodology Center at Pennsylvania State University [20] in SAS v9.2 (SAS Institute, Cary NC, USA), and packages polCA [21] and randomLCA [22] in R (R Development Core Team, Vienna, Austria). All culture results of specimens collected from baseline through week 12 from each patient were included in the LCA. We used the observed results on the five solid media as manifest variables in the latent class model to indirectly measure the latent variable, which we defined as the reference MTB status of a specimen on an ideal solid medium. We tested multiple models with varying numbers of latent classes (subgroups of the latent variable) and chose to use a model with two latent classes, based on goodness of fit and interpretability, to construct the reference standard. After interpreting the model parameters, we concluded that the two latent classes dichotomized the latent variable into either reference MTB positive or reference MTB negative on an ideal solid medium. Results from the LCA allowed us to assign each specimen to one of these classes (reference MTB positive or reference MTB negative) based on its response pattern, in other words, the pattern of culture results over the five solid media. This class assignment will hereafter be referred to as the latent class construct. Sensitivities and specificities of each solid medium were calculated by comparing the observed culture results of each single specimen against the latent class construct. The sensitivity of a medium is the proportion of cultures (or, equivalently in this case, specimens) that are deemed to be culture-positive among those that are deemed positive by the latent class construct. The specificity of a medium is the proportion of cultures (or, equivalently in this case, specimens) that are deemed to be culture-negative among those that are deemed negative by the latent class construct.

2.7.2. Secondary analysis

Solid media culture results were also compared to MGIT results as a secondary analysis. Sensitivities and specificities were calculated with MGIT as the reference standard.

We performed bootstrap analyses with 50,000 bootstrap replicates to evaluate statistical variability of each individual sensitivity and specificity estimate. We report confidence intervals calculated using the Wilson score method, as they were the same as bootstrap confidence intervals to >3 significant digits. We report bootstrap P-values for how often each solid medium would be the most sensitive or specific in repeated sampling.

3. Results

Fifty patients were enrolled in the study. Baseline characteristics of these patients are summarized in Table 1. Out of the 50 patients, 45 (90%) successfully completed treatment and follow up. Five patients were withdrawn from study treatment due to: drug resistance, 2; lost to follow up, 1; death, 1; ineligibility, 1. However, the culture results from these five patients were included in the analysis.

Culture results were analyzed for 393 specimens from 50 patients. Growth from all positive cultures was identified as MTB. All isolates were fully susceptible to first-line TB drugs, except those from the two patients having drug-resistant TB. Specific culture findings for each time point are shown in a Supplemental Table. The recovery rate of MTB from sputum specimens collected pre-treatment (day 0) was high and comparable among all solid media and MGIT (Figure 1). The isolation of MTB from serially collected specimens decreased during treatment. Recovery rates among the various media varied more at weeks 8 and 12 than at previous times. Selective 7H11 had the highest 8-week positivity rate (30%) among the five solid media, while 53% were positive on MGIT. No contamination was observed in the 7H10S, 7H11S, and MGIT cultures at day 0 (Figure 2). Baseline contamination rates of LJ, 7H10, and 7H11 were 6–8%. The amount of contamination was higher with all media at eight weeks. Contamination rates of LJ, 7H10, 7H11, and 7H11S were higher than the widely accepted range of 3–5% for solid media for culture of diagnostic specimens [18]; however, there are
no previously published contamination rates for specimens from patients on treatment. The frequency that cultures were both contaminated and MTB positive varied among the different media. The percentage of positive cultures having contaminant growth and MTB was: 26%, 7H11; 10%, 7H10; 7%, LJ; 6%, 7H11S; 4%, 7H10S%; and 26%, MGIT. The sequence in which the media were inoculated had no effect on the isolation and contamination rates of the respective media.

### 3.1. Primary analysis

Among all 393 culture results, the sensitivity of the five solid media relative to the latent class construct ranged from 78% to 99%, and specificity ranged from 52% to 83% (Table 2). The selective media were more sensitive and specific for the detection of MTB than the other solid media. When each medium is considered separately, 7H11S had the highest sensitivity (bootstrap $P = 0.003$) while 7H10S had the highest specificity (bootstrap $P = 0.098$) in magnitude.

### 4. Discussion

In our study we compared the performance of five solid media in recovery of MTB in patients on standard short course treatment. In order to determine which solid medium is most reliable without using any prior assumptions, a reference standard based purely on the solid media data was constructed using LCA. We found that, over the first 12 weeks of therapy, 7H11S had the greatest sensitivity and 7H10S the greatest specificity among the tested solid media. In particular, at 8 weeks, when culture status is often assessed as a phase 2 clinical trial endpoint, 7H11S had the greatest sensitivity and 7H10S had the greatest specificity. When liquid MGIT 960 was used as a reference, the two selective agar-based media (7H11S and 7H10S) had the most reliable performance in comparison to the

### Table 1

Baseline characteristics of patients with smear positive pulmonary TB enrolled in the study ($N = 50$).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>$n$ (%)</th>
</tr>
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<tbody>
<tr>
<td>Age, years (median, IQR)</td>
<td>27 (22–30)</td>
</tr>
<tr>
<td>Male sex (%)</td>
<td>38 (76)</td>
</tr>
<tr>
<td>Body mass index (kg/m²) (median, IQR)</td>
<td>18.6 (16.9–20.0)</td>
</tr>
<tr>
<td># days of TB therapy before study (%)</td>
<td>0 (66)</td>
</tr>
<tr>
<td></td>
<td>3 or 4 days (34)</td>
</tr>
<tr>
<td>Bilateral cavitary disease</td>
<td>6 (12)</td>
</tr>
<tr>
<td>Cavitary disease</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>1 (2)</td>
</tr>
<tr>
<td>$&lt;$ 4 cm total diameter</td>
<td>18 (36)</td>
</tr>
<tr>
<td>$\geq$ 4 cm total diameter</td>
<td>31 (62)</td>
</tr>
<tr>
<td>Extent of disease</td>
<td></td>
</tr>
<tr>
<td>$25$ to $50%$ of lungs involved by disease</td>
<td>19 (38)</td>
</tr>
<tr>
<td>More than $50%$ of lungs involved by disease</td>
<td>31 (62)</td>
</tr>
</tbody>
</table>

### Table 2

Sensitivities and specificities of the five solid media were calculated relative to two reference standards: the latent class construct (LCC) and MGIT.

<table>
<thead>
<tr>
<th>Method</th>
<th>Latent class construct</th>
<th>MGIT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity</td>
<td>Specificity</td>
</tr>
<tr>
<td>LJ</td>
<td>77.9 (72.1, 82.7)</td>
<td>60.4 (52.1, 68.2)</td>
</tr>
<tr>
<td>7H10</td>
<td>81.6 (76.2, 85.9)</td>
<td>61.1 (53.1, 68.5)</td>
</tr>
<tr>
<td>7H11</td>
<td>93.8 (89.9, 96.2)</td>
<td>52.0 (44.0, 59.9)</td>
</tr>
<tr>
<td>7H10S</td>
<td>93.9 (90.1, 96.2)</td>
<td>82.6 (75.7, 87.8)</td>
</tr>
<tr>
<td>7H11S</td>
<td>98.7 (96.4, 99.6)</td>
<td>76.7 (69.2, 82.8)</td>
</tr>
</tbody>
</table>

Sputum specimens were collected over the course of the first 12 weeks of tuberculosis treatment from 50 Ugandan participants.

LJ: Lowenstein–Jensen culture medium; 7H10 and 7H11: non-selective Middlebrook agar media; 7H10S and 7H11S: selective Middlebrook agar media.

* $95\%$ confidence intervals using the Wilson score method.
non-selective agar and LJ media. Thus the two analyses produced the same conclusion. We observed that differences between the media increase with time on therapy. By combining time points the comparisons are stronger, than if we had evaluated time points separately, and the effects on culture results of declining bacterial burden and increasing bacterial injury are captured as time on therapy increases.

The findings that selective media were both more sensitive and more specific than their non-selective counterparts were surprising. It is generally believed that antibiotics such as those used to make the media selective have some inhibitory effect on MTB that results in delayed growth on agar-base media [23]. In their original description Mitchison et al. indicated that selective media has little effect on the recovery of MTB [1]. Thus one would expect the non-selective and selective media to be similar. One possible explanation for the enhanced sensitivity of selective media is improved isolation of MTB on media that inhibits faster growing bacteria and fungi. In the absence of the antibiotics the contaminants would outgrow MTB. In our study, this reasoning is supported by the fact that contamination was higher on non-selective media.

The superior sensitivity of 7H11S over 7H10S is noteworthy. Both media types are commonly used by laboratories using an agar medium for isolation. Middlebrook 7H11 is preferred for isolation and agar proportion drug susceptibility testing because it supports the growth of MTB better (often numbers and size of colonies are larger and colonies appear sooner) than 7H10 does [6]. Recovery rates of the two media were comparable with pre-treatment specimens, but, once patients were on therapy, isolation of MTB was higher on 7H11. This suggests that the casein in 7H11 provides an advantage for tubercle bacilli exposed to anti-TB drugs. These two media are distinguished by the addition of an enzymatic digest of casein in the 7H11 base. The casein digest provides nitrogen, vitamins, and amino acids and is reported to support the growth of some fastidious MTB that grow poorly or not at all on 7H10 [6]. There have been no previous comparative studies comparing 7H10 with 7H11 except the 1968 report on 96 cultures from Cohn [6], in which 13/96 (13.5%) cultures were problematic.

Although both selective Middlebrook media showed statistically significant advantages over the other three solid media, there is reason to exercise caution in generalizing from this study, especially because the sample contains only 50 participants. Another limitation is that laboratory-prepared LJ medium was not evaluated as commercially prepared LJ was used. Since media formulations and sterilization methods used to prepare LJ often differ between laboratories and access to fresh, antibiotic-free eggs is unreliable, LJ is likely to exhibit bigger variations in performance between batches and between laboratories, which was not evaluated in this study. Strengths of our study include performing sputum cultures simultaneously on five solid and one enriched liquid media using standard procedures at a single quality-controlled laboratory at multiple time points during directly supervised treatment and careful follow up of a prospective cohort of well characterized adults with culture-confirmed tuberculosis. Contemporary registration TB drug trials usually include cultures on both solid and liquid media and the highly sensitive MGIT system is the most widely used liquid culture medium.

Combining microbiology data from multiple laboratories for use in drug trials requires some degree of standardization among the laboratory procedures used. One approach is to standardize the media used for culturing sputum specimens. With the widespread adoption of the MGIT 960 system and the fact that it is a standardized commercial system, the most frequently used liquid culture system in recent phase 2 studies has been MGIT. The decision as to which solid medium to use has been debatable due to the lack of comparative studies and the historical use of LJ. Preparing Middlebrook agar media is simpler and more likely to result in a consistent product, whether it is “homemade” or commercially prepared. Furthermore, using Selectatabs (MAST, UK), tablets containing accurately assayed drug concentrations, instead of preparing drug solutions when making selective media, provides uniformity to the selective media. Microbiologists with clinical trials experience agree that Middlebrook agar is preferable to LJ (TB Clinical Trials Consultants Meeting, St. George’s, University of London, Nov, 2011) and that 7H11 medium is preferable to 7H10. Our finding of a higher sensitivity of 7H11S over 7H10S is consistent with this preference. To increase specificity, one could include 7H10S and use a bi-plate containing both selective media. However, this would double the cost for the antibiotics, necessitate two types of agar base, and require the two media to be analyzed separately for clinical trial results.

Due to high sensitivity of MGIT and yet similar contamination rate to selective Middlebrook media (Figure 2) one would argue whether there is a need to combine a solid culture medium with MGIT in clinical trials. For now there are reasons to use both. Culture conversion on solid media at 2 months remains a critical marker of response to therapy. A similar marker is yet to be defined for MGIT. Combining a solid and MGIT improves recovery of isolates incase MGIT is contaminated and solid media is not.

To validate the results of this analysis, studies should be performed in different geographic locations where the recovery and contamination rates, as well as the distribution of MTB sub-lineages and strains, are likely to vary. At a minimum, these studies should analyze culture results at 8 weeks, which is currently the most common interim endpoint in phase 2 treatment trials, and should compare at least binary outcomes on LJ, 7H11, and 7H11S media. Definitive validation studies, however, would evaluate isolation and contamination rates at time points through the full duration of therapy and during follow up for up to two years to ascertain cure, through absence of treatment failure and relapse.

**Acknowledgment**

Thank you to all the patient participants in this study and to the Tuberculosis Research Unit/Joint Clinical Research Center clinical and laboratory staff for their participation and their diligent work.

**Ethical approval:** The study protocol was approved by the institutional review boards of the Joint Clinical Research Centre in Kampala; the University Hospitals Case Medical Center; and the U.S. Centers for Disease Control and Prevention and the Ugandan National Council for Science and Technology. Informed consent for study participation and HIV testing was obtained from all patients. All patients received pre- and post-HIV test counseling.

**Funding:** This work was supported by the Tuberculosis Trials Consortium (sponsored by the Centers for Disease Control and Prevention under contract 200-2009-32598) and the Tuberculosis Research Unit at Case Western Reserve University (established with funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health and Human Services under contract HHSN266200700022C/N01-Al-70022). The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the funders (CDC and TB RU).

**Competing interests:** None declared.

**Appendix A. Supplementary data**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2014.03.002.
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