Evaluation of a Novel Biphasic Culture Medium for Recovery of Mycobacteria: A Multi-Center Study

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Abstract

Background: Mycobacterial culture and identification provide a definitive diagnosis of TB. Culture on Löwenstein-Jensen (L-J) medium is invariably delayed because of the slow growth of M. tuberculosis on L-J slants. Automated liquid culture systems are expensive. A low-cost culturing medium capable of rapidly indicating the presence of mycobacteria is needed. The aim of this study was to develop and evaluate a novel biphasic culture medium for the recovery of mycobacteria from clinical sputum specimens from suspected pulmonary tuberculosis patients.

Methods and Findings: The biphasic medium consisted of 7 ml units of L-J slant medium, 3 ml units of liquid culture medium, growth indicator and a mixture of antimicrobial agents. The decontamination sediments of sputum specimens were incubated in the biphasic culture medium at 37°C. Mycobacterial growth was determined based on the appearance of red granule sediments and the examination using acid-fast bacilli (AFB). The clinical sputum specimens were cultured in the biphasic medium, on L-J slants and in the Bactec MGIT 960 culture system. Among smear-positive specimens, the mycobacteria recovery rate of the biphasic medium was higher than that of the L-J slants (P<0.001) and similar to that of MGIT 960 (P>0.05). Among smear-negative specimens, the mycobacterial recovery rate of the biphasic medium was higher than that of L-J slants (P<0.001) and lower than that of MGIT 960 (P>0.05). The median times to detection of mycobacteria were 14 days, 20 days and 30 days for cultures grown in MGIT, in biphasic medium, on L-J slants for smear negative specimens, respectively (P<0.001).

Conclusions: The biphasic culture medium developed in this study is low-cost and suitable for mycobacterial recovery. It does not require any expensive detection instrumentation, decreases the time required for detection of M. tuberculosis complex, and increases the detection rate of M. tuberculosis complex.

Citation: Cui Z, Wang J, Zhu C, Huang X, Lu J, et al. (2012) Evaluation of a Novel Biphasic Culture Medium for Recovery of Mycobacteria: A Multi-Center Study. PLoS ONE 7(4): e36331. doi:10.1371/journal.pone.0036331

Editor: Joyoti Basu, Bose Institute, India

Received December 12, 2011; Accepted March 31, 2012; Published April 27, 2012

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Funding: This study was supported by grants from the National Key Project for Infectious Disease (No. 2008ZX10003-003-02). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

As a contagious disease, tuberculosis (TB) poses an increasing global risk to human health. According to the report of the World Health Organization, about 9.4 million cases (range: 8.9–9.9 million) occurred globally in 2009 and most of them were in Southeast Asia, Africa, and western Pacific [1]. There are two major reasons for the increasing incidence of TB: the increasing emergence and transmission of multi-drug-resistant and extensively drug-resistant M. tuberculosis strains and the increasing incidence of HIV globally [2–9]. Due to the fact that the symptoms of TB can vary greatly from one case to another, clinical diagnosis of TB is challenging. The creation of reliable laboratory testing tools is vital to TB diagnosis. Mycobacterial culture and identification can provide a definitive diagnosis of TB. Laboratory culture also provides the necessary isolates for conventional drug susceptibility testing. Löwenstein-Jensen (L-J) medium, a solid medium, is used extensively in many clinical laboratories for detecting the presence of M. tuberculosis. It is less expensive than the liquid media, but the results are invariably delayed because of the slow growth of M. tuberculosis on L-J slants [10]. Liquid media increase the case yield by 10% over solid media, and automated liquid culture systems can significantly reduce the diagnostic delay from weeks to days. However, automated liquid culture systems are expensive and difficult to operate [11]. A low-cost culturing medium that can rapidly detect the presence of M. tuberculosis is needed. In this study, we developed a novel biphasic medium for recovery of mycobacteria during tuberculosis diagnosis and evaluated its efficacy relative to L-J medium and the mycobacterial
growth indicator tube (MGIT) system using clinical sputum specimens obtained from six hospitals in China.

**Materials and Methods**

**Ethics Statement**

All participating patients were treated in accordance with the Declaration of Helsinki on the participation of human subjects in medical research. Ethical approval for this study was obtained from the Shanghai Pulmonary Hospital Ethics Committee, Hunan Provincial Chest Hospital Ethics Committee, Shandong Provincial Chest Hospital Ethics Committee, Anhui Provincial Chest Hospital Ethics Committee, the Sixth People’s Hospital of Nantong Ethics Committee, and Hangzhou Red Cross Hospital Ethics Committee. We obtained informed consent from the next of kin, caretakers, and guardians in the cases of the minor/child participants. Written informed consent was obtained from each participant.

**M. tuberculosis** strains and sputum specimens

*M. tuberculosis* strain (ATCC27294) was obtained from the National Tuberculosis Reference Laboratory (Beijing, China). Sputum specimens were collected from 1620 patients with suspected pulmonary tuberculosis. The study inclusion criteria were as follows: age $\geq 16$ years, cough lasting $\geq 3$ weeks, ability to provide informed consent, and ability to fit into one of the following categories: new suspected pulmonary TB patients; suspected treatment failure; suspected relapse; and treatment default [12].

Six clinical laboratories in the Shanghai Pulmonary Hospital, Hunan Provincial Chest Hospital, Shandong Provincial Chest Hospital, Anhui Provincial Chest Hospital, the Sixth People’s Hospital of Nantong, and Hangzhou Red Cross Hospital participated in this study. The research plans were carefully designed and the investigators from each hospital were trained. The sputum samples were tested immediately after being obtained from the patients. They were tested using smear microscopy, the biphasic medium, L-J slant, and the MGIT 960 automated culture system. The physicians were blind to the results of these assays and the laboratory staffs were blind to the diagnoses of the patients. A flow chart of this study is shown in Figure 1.

**Dilution of *M. tuberculosis* suspension (ATCC27294)**

*M. tuberculosis* (ATCC27294) suspension in log-phase growth was adjusted to an optical density of 1.0 McFarland in sterile saline, corresponding to a cell density of approximately $10^7$ colony forming units (CFU/ml) [13]. The suspension was then subjected to ten-fold serial dilutions to 1 CFU/ml in tubes with glass beads containing 4.5 ml sterile saline. Aliquots of 0.1 ml of each dilution were cultured in the biphasic medium, on L-J slants, and in the MGIT 960 automated system. In addition, Aliquots of 0.5 ml of each dilution were cultured in the biphasic medium and the MGIT 960 automated system.

**Sputum smear microscopy**

Sputum specimens were put onto slides for homogenous smear preparation. The smears were then stained with 0.1% auramine O using a standard protocol [14].

**Decontamination of sputum specimens**

Sputum specimens were digested and decontaminated using the N-acetyl-L-cysteine-sodium hydroxide method; the final sodium hydroxide concentration was 2% [15]. The processed sediment was washed one time using sterile phosphate buffered saline (PBS) solution and then resuspended in 2 ml PBS sterile solution. Aliquots of 100 $\mu$L processed suspension were inoculated into two petri dishes containing L-J slant medium and two petri dishes containing the biphasic medium. All specimens were tested using L-J medium, the biphasic medium, and the MGIT 960 automated system. The remaining suspension was stored at $-70\,^\circ$C.

**Culture on L-J slant medium**

The culturing of mycobacteria on L-J slant medium was performed as described previously [16]. Slants were incubated at $57\,^\circ$C and examined visually every 2 or 3 days for 6 weeks. When bacterial clones were observed on the slants, they were identified by staining with Ziehl-Neelsen or Auramine O. Positive staining of the cultures was considered indicative of the presence of mycobacteria.

**Culture in the biphasic medium**

The biphasic medium consisted of 7 ml L-J slant medium, 3 ml liquid culture medium, and growth indicator. Aliquots of 7 ml L-J slant medium were prepared in 25 ml sterile flasks. Before use, aliquots of 3 ml liquid culture medium (Middlebrook 7H9 liquid culture with 10% OADC enrichment [Becton Dickinson Co., MD, U.S.A.], the mixture of antimicrobial agents and growth indicator) were added to the culture flasks (Fig. 2). The mixture of antimicrobial agents included polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (Sigma-Aldrich Co., U.S.A.). The final concentrations of these agents in the liquid medium were 40 units/ml, 4 $\mu$g/ml, 16 $\mu$g/ml, 4 $\mu$g/ml, and 4 $\mu$g/ml, respectively. The growth indicator was 2,3,5-triphenyl-tetrazolium chloride (TTC, Sigma-Aldrich Co., U.S.) and the final concentration in the liquid medium was 10 $\mu$g/ml. TTC, a redox indicator, can be deoxidized to 1,3,5-triphenylformazan (TTF), which is bright red and water-fast [17]. TTC was added to the liquid culture before incubation. At the TTC concentrations used in this study, TTC turns from colorless to pink and granular in response to bacterial metabolic reduction. These pink granule sediments assembled around the bacteria. After the inoculation of specimen suspension in the biphasic medium, the culture media were observed once every 2 or 3 days. Any red sediment observed was tested by acid-fast staining (AFB) with Ziehl-Neelsen or Auramine O. The AFB-positive cultures were confirmed for the presence of mycobacteria.

**Culture using the MGIT 960 automated system**

Cultures were prepared according to the manufacturer’s instructions using a MGIT 960 automated system (Becton Dickinson Co., MD, U.S.A.). For clinical specimens, the inoculation volume of each specimen sediment was 0.5 ml per flask [18]. The time required for detection of mycobacteria in the MGIT 960 system was read automatically by the system, expressed in days, and rounded to the nearest integer. The positive culture organisms were tested using acid-fast staining (AFB) with Ziehl-Neelsen or Auramine O. The AFB-positive cultures were confirmed for the presence of mycobacteria.

**Identification of the *M. tuberculosis* complex**

All positive cultures generated on biphasic medium, L-J slant medium, and the MGIT 960 automated system were tested using elective inhibition assay with p-nitrobenzoic acid to identify *M. tuberculosis* complex [19].
Quality control
Each new batch of medium for each of the assays (biphasic, L-J, and MGIT 960) was tested for sterility and for growth of *M. tuberculosis*.

Statistical analysis
Comparisons of the time to detection of mycobacteria between any two different culture systems were performed using a McNemar test and comparison of the times to detection of mycobacteria among any three different culture systems were performed using an analysis of variance (ANOVA). Comparisons of the rates of positive cultures for mycobacteria and comparisons of the rates of contaminated cultures for different culture systems were performed using a Chi-square test. Concordance of culture results was determined using sensitivity, specificity, and positive and negative predictive values with 95% confidence intervals (CIs) and kappa values.
Results

Growth results of the different concentration dilutions of *M. tuberculosis* (ATCC27294) cultured in different media are shown in Figure 2. After incubation, red granules were usually observed first at the bottom of the liquid culture. The longer the incubation time, the more red granules were observed. The bacterial colonies with pink granule sediment were clearly observable with the naked eye. The greater the quantity of inoculum introduced to the bi-phasic medium, the sooner the red granules appeared, and they appeared in greater numbers. The culture results of *M. tuberculosis* with the inocula ranging from 10^7 CFU to 1 CFU in the biphasic medium were shown in Figure 3. In the same inocula, the time to detection of *M. tuberculosis* in the biphasic medium was significant less than on L-J slants \( (P<0.05) \) and more than that in the MGIT 960 system \( (P<0.05) \). In the same inocula, the time to detection of *M. tuberculosis* with the 0.5 ml volume was significantly less than with the 0.1 ml volume \( (P<0.005 \) for biphasic medium and \( P<0.05 \) for MGIT 960 system).

A total of 1260 sputum specimens were collected for a comparison of the mycobacterial growth in the biphasic medium and other systems. 210 specimens were from Hunan, 212 from Shandong, 212 from Anhui, 90 from Nantong, 95 from Hangzhou, and 441 from Shanghai. The number of contaminated cultures was 50 (4.0%) on L-J slants and 58 (4.6%) in biphasic medium. The difference between the number of contaminated cultures on L-J slants and in the biphasic medium was not statistically significant \( (P>0.05) \). Of 441 specimens from Shanghai, the number of contaminated cultures were 19 (4.3%), 19 (4.3%), and 17 (3.8%) on L-J slants, in the biphasic medium, and in MGIT culture system, respectively. These numbers of contaminated cultures were not found to be significantly different \( (P>0.05) \).

There were 1192 specimens that did not show any contamination in any culture medium. Of these, 199 (16.7%) were from Hunan, 200 (16.8%) from Shandong, 200 (16.8%) from Anhui, 85 (7.1%) from Nantong, 89 (7.5%) from Hangzhou, and 419 (35.2%) from Shanghai. The characteristics of the 1192 participants are summarized in Table 1. The numbers of isolates of mycobacteria recovered by biphasic medium, L-J medium and MGIT culture system are presented in table 2. Of the 1192 specimens, 486 and 363 were culture-positive for mycobacteria on both biphasic medium and L-J medium, in which 93.8% (456/486) and 92.8%

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**Table 1. Characteristics of the 1192 participants.**

| Characteristics | Sub-category | Hunan (n = 199) | Shandong (n = 200) | Anhui (n = 200) | Hangzhou (n = 89) | Nantong (n = 85) | Shanghai (n = 419) |
|-----------------|--------------|----------------|-------------------|----------------|------------------|-----------------|-------------------|
| Age, mean       |              | 47.4           | 46.26             | 48.0           | 50.3             | 53.4            | 49.4              |
| (SD)            |              | (15.7)         | (20.76)           | (16.3)         | (15.6)           | (14.0)          | (15.3)            |
| Sex             | Male         | 151            | 136               | 166            | 51               | 65              | 281               |
|                 | Female       | 48             | 64                | 34             | 38               | 20              | 138               |
| Smear           | Positive     | 26             | 128               | 30             | 32               | 25              | 121               |
|                 | Negative     | 173            | 72                | 170            | 67               | 60              | 298               |

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Figure 3. Growth of H37Rv culture on and in different culture media. For L-J (0.1), biphasic medium (0.1), and MGIT (0.1), a mean 0.1 ml of inocula were incubated. For biphasic medium (0.5) and MGIT (0.5), a mean 0.5 ml of inocula were incubated.

doi:10.1371/journal.pone.0036331.g003
Table 2. Rates of recovery of mycobacteria by biphasic medium, L-J medium and MGIT 960 culture system.

| Medium      | All specimens | No. (%) of isolates recovered | Mycobacteria | MTBC | NTM |
|-------------|---------------|-------------------------------|--------------|------|-----|
|             |               |                               |              |      |     |
| Biphasic    | 1192          | 486 (40.8)                    | 456 (38.3)   | 30   | 2.5 |
| medium      |               | 363 (30.5)                    | 337 (28.3)   | 26   | 2.2 |
| L-J medium  |               | 203 (48.4)                    | 187 (44.6)   | 16   | 3.8 |
| Biphasic    | 419           | 131 (31.2)                    | 118 (28.2)   | 13   | 3.1 |
| medium      |               | 234 (55.8)                    | 217 (51.8)   | 17   | 4.1 |
| MGIT 960    | 419           | 357 (85.8)                    | 337 (80.8)   | 26   | 2.5 |

For comparison, the medium culture recovery was also performed in the MGIT 960 culture system. Of the 1192 specimens, there were 362 smear-positive specimens and 830 smear-negative specimens. Among the smear-positive specimens, 317 (87.6%) were culture-positive for mycobacteria in the biphasic medium and 278 (76.8%) on L-J slants. The rate of positive cultures in the biphasic medium was higher than that on L-J culture slants among smear-positive specimens. The rate of positive cultures in the biphasic medium was 98.3%, 84.4%, 73.5%, and 99.1% of smear-positive specimens, 317 (87.6%) were culture-positive for mycobacteria in the biphasic medium, 39 (13.1%) on L-J slants, and 118 (36.3%) in the MGIT system. For smear-negative specimens, the median time to detection of mycobacteria were 6 days (interquartile range [IQR], 4–10 days), 13 days (IQR, 11–18 days), and 20 days (IQR, 15–23 days) for cultures grown in the MGIT system, in the biphasic medium, and on L-J slants, respectively (F = 140.143, P < 0.001 by ANOVA). Among smear-negative specimens, the median times to detection of mycobacteria were 14 days (IQR, 10–16 days), 20 days (IQR, 15–23 days), and 30 days (IQR, 25–36 days) for cultures in the MGIT system, in the biphasic medium, and on L-J slants, respectively (F = 105.261, P < 0.001 by ANOVA).

Figure 5 shows the time to detection of mycobacteria as determined by the AFB smear status of the source specimen for the 419 specimens from the Shanghai Pulmonary Hospital that were positive for mycobacteria grown on L-J slants, in the biphasic medium, and in the MGIT system. Among smear-positive specimens, the median times to detection of mycobacteria were 14 days (IQR, 10–16 days), 20 days (IQR, 15–23 days), and 30 days (IQR, 25–36 days) for cultures in the MGIT system, in the biphasic medium, and on L-J slants, respectively (F = 105.261, P < 0.001 by ANOVA).

Eighteen random frozen backup specimen sediments found positive for mycobacteria in the MGIT system were re-cultured in positive specimens (χ² = 0.732, P > 0.05) and lower than that of MGIT 960 among smear-negative specimens (χ² = 5.790, P < 0.05).

Table 3. Comparison of culture in biphasic medium to culture in L-J medium for detection of mycobacteria.

| Culture in biphasic medium | Culture on L-J medium |
|----------------------------|-----------------------|
|                           | Total | Hunan | Shandong | Anhui | Hangzhou | Nantong | Shanghai |
|                           | Pos   | Neg   | Pos     | Neg   | Pos      | Neg     | Pos      |
| Pos                       | 357   | 129   | 19      | 20    | 104      | 14      | 48       |
| 14                         | 10    | 33     | 5       | 23    | 7        | 130     | 73       |
| Kappa value                | 0.76  | 0.56   | 0.86    | 0.87  | 0.88     | 0.76    | 0.64     |
| Sensitivity (%; 95% CI)    | 98.3 (96.4–99.2) | 86.4 (66.7–95.3) | 100 (96.4–100) | 100 (96.4–100) | 100 (92.6–100) | 100 (89.6–100) | 92.0 (75.0–97.8) | 99.2 (95.8–99.9) |
| Specificity (%; 95% CI)    | 84.4 (81.8–86.7) | 88.7 (83.2–92.6) | 85.3 (76.8–91.0) | 93.4 (88.3–96.4) | 91.1 (80.7–96.1) | 88.3 (77.8–94.2) | 74.7 (69.3–79.3) |
| PPV (%; 95% CI)            | 73.5 (69.4–77.2) | 48.7 (33.9–63.8) | 81.3 (81.1–92.8) | 82.8 (71.1–90.1) | 86.8 (72.7–94.3) | 76.7 (59.1–88.2) | 64.0 (57.2–70.3) |
| NPV (%; 95% CI)            | 99.1 (98.2–99.6) | 98.1 (94.6–99.4) | 100 (95.5–100) | 100 (97.4–100) | 100 (93.0–100) | 96.4 (87.7–99.0) | 99.5 (97.4–99.9) |

Pos: positive. Neg: negative. CI: confidence interval.

doi:10.1371/journal.pone.0036331.t003

doi:10.1371/journal.pone.0036331.t002
the biphasic medium, in the MGIT system, and on L-J slants. Of these, 9 samples were from smear-negative specimens and 9 from smear-positive specimens. The effects of different inoculation volumes are shown in Table 5. For the same sample at the same concentration, the time to detection of mycobacteria in the biphasic medium was significantly less than that on L-J slants ($P<0.001$) and significantly more than in the MGIT culture system ($P<0.001$). For the same sample, the time to detection of mycobacteria at an initial inoculation volume of 0.5 ml was significant less than with 0.1 ml ($P<0.005$ for biphasic medium and $P<0.05$ for MGIT system). The median time to detection of mycobacteria in the biphasic medium was longer than in the MGIT system by 2–3 d.

**Discussion**

Rapid diagnosis of tuberculosis is critical to control of the disease; therefore, use of the most rapid methods available for

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**Table 4. Culture results by method among specimens from each hospital.**

| Characteristics | Smear | Hunan (n = 199) | Shandong (n = 200) | Anhui (n = 200) | Hangzhou (n = 89) | Nantong (n = 85) | Shanghai (n = 419) | All (n = 1192) |
|-----------------|-------|-----------------|-------------------|-----------------|------------------|-----------------|-------------------|---------------|
| Biphasic medium | Positive | 17/26 | 110/128 | 26/30 | 28/32 | 23/25 | 113/121 | 317/362 |
|                 | Negative | 22/173 | 8/72 | 32/170 | 10/57 | 7/60 | 90/298 | 169/830 |
| L-J medium      | Positive | 12/26 | 100/128 | 26/30 | 27/32 | 21/25 | 92/121 | 278/362 |
|                 | Negative | 10/173 | 4/72 | 22/170 | 6/57 | 4/60 | 39/298 | 85/830 |
| MGIT 960        | Positive | No | No | No | No | No | 116/121 | No |
|                 | Negative | No | No | No | No | No | 118/298 | No |

No means no detection by MGIT 960 culture system.

doi:10.1371/journal.pone.0036331.t004

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**Figure 4. Box plot of median time required for growth of mycobacteria in each culture medium.** Box lengths indicate interquartile range. Horizontal lines in each box represent the median of the distribution. Whiskers extend from the normal minimum value to the normal maximum value. For L-J, biphasic medium (B–P), and MGIT, the results of 121 smear positive sputum specimens and 298 smear negative sputum specimens are shown.

doi:10.1371/journal.pone.0036331.g004
Figure 5. Growth of mycobacteria in L-J and biphasic medium scenarios with acid-fast bacilli (AFB) smear microscopy and status of the source respiratory specimen. Results are shown for 830 AFB smear-negative respiratory specimens and 362 AFB smear-positive respiratory specimens that were found positive for *M. tuberculosis* by both L-J and biphasic medium (B–P). L-J, culture on L-J medium; B–P, culture in biphasic medium.

doi:10.1371/journal.pone.0036331.g005

Table 5. Growth time of mycobacteria in sputum culture and in different culture media.

| Smear       | No. of samples | Time (d) |
|-------------|----------------|----------|
|             |                | 0.1 ml of inoculum | 0.5 ml of inoculum |
|             |                | L-J medium | Biphasic medium | MGIT | Biphasic medium | MGIT |
| Negative    |                |            |                  |      |                  |      |
| 1           | 28             | 15         | 9                | 8    | 8                |
| 2           | 19             | 12         | 9                | 8    | 7                |
| 3           | 21             | 16         | 12               | 14   | 9                |
| 4           | 26             | 16         | 14               | 15   | 12               |
| 5           | 21             | 14         | 6                | 12   | 5                |
| 6           | 26             | 16         | 14               | 13   | 4                |
| 7           | 19             | 14         | 10               | 12   | 9                |
| 81          | 41             | No         | 18               | 19   | N                |
| 9           | No             | No         | No               | 16   | 30               |
| Positive    |                |            |                  |      |                  |      |
| 10          | 25             | 16         | 12               | 16   | 10               |
| 11          | 20             | 13         | 8                | 11   | 3                |
| 12          | 20             | 15         | 18               | 7    | 4                |
| 13          | 25             | 15         | 8                | 11   | 5                |
| 14          | 20             | 11         | 7                | 11   | 4                |
| 15          | 20             | 11         | 10               | 7    | 6                |
| 16          | 18             | 11         | 9                | 11   | 6                |
| 17          | 18             | 11         | 8                | 11   | 8                |
| 18          | 20             | 11         | 13               | 7    | 5                |

No indicates that no mycobacteria grew.

doi:10.1371/journal.pone.0036331.t005
culture and identification of *M. tuberculosis complex* (MTBC) is advocated. L-J medium is a widely used growth medium for the culturing of mycobacteria. When grown on L-J slants, *M. tuberculosis* appears in the form of brown, granular colonies. Due to the slow doubling time of *M. tuberculosis*, the culture must be incubated for 4–8 weeks. To reduce detection time and improve detection sensitivity, we developed a novel mycobacterial culture medium, biphasic medium, based on the traditional L-J slant medium for tuberculosis diagnosis. This new medium combines the characteristics of liquid culture medium and L-J slant. We performed a multi-center evaluation of the feasibility of this biphasic medium for use in the detection of mycobacteria in sputum specimens of suspected pulmonary tuberculosis patients. Compared to L-J medium, the biphasic medium provided more nutrition through the addition of liquid culture medium and it was found to be more conducive to the growth of mycobacteria. In order to prevent contamination by non-mycobacteria, a mixture of antibiotic ingredients was added to the liquid medium. The results showed that the contamination rate of the cultures in the biphasic medium was still slightly higher than in the L-J medium. However, the difference between the two contamination rates was not statistically significant. This suggests that the decontamination of specimens to culture in biphasic culture medium does not require more processing than the L-J medium. In fact, contaminated biphasic medium was significantly different from the normal biphasic medium. When the biphasic medium was contaminated by non-mycobacteria or fungi, the color of the solid medium would change from yellow-green to emerald green, the liquid medium would become turbid before shaking, and the solid medium would finally rot.

In this study, a growth indicator was added to the biphasic medium so that we could detect mycobacterial growth using the color change. The determination of the results is more objective than with other methods. In this study, a new growth indicator, TTC, was used. It is different from previously used indicators. In a nitrate reductase assay (NRA), in both liquid and solid cultures, reagents such as hydrochloric acid, sulphanilic acid, and 1-naphthylamine must be added to the culture for some time after incubation [20–22]. This increases the risk that the mycobacteria will escape. NRA does not reflect the mycobacterial growth in real time. Other growth indicators such as alamar blue and resazurin require more mycobacteria to produce any colorimetric change, and the transition from one color to the next can be difficult to determine [23,24]. Rapid culture systems, such as Bactec MGIT and Bac Alert 3D, require specific, sophisticated detection equipment to evaluate the results. As such, they can only be used in certain laboratories [18,25]. In this study, only a small amount of mycobacteria is needed in the biphasic medium. The bacterial colonies have visible pink granule sediments that are detectable with the naked eye. The biphasic medium cannot provide quantitative data as accurately as other culture media, alamar blue assay, or the rapid culture systems. However, it can provide a rough estimate in a much more timely manner.

In this study, tests performed at six hospitals showed that biphasic medium was more sensitive but less specific than L-J medium. The reason for this lower specificity was that some culture-negative specimens on L-J medium were culture-positive in biphasic medium. Our results showed the recovery rate of mycobacteria in the biphasic medium was significant higher than on L-J slants. For both smear-positive and smear-negative specimens, the time to detection of mycobacteria in biphasic medium was significantly shorter, by one week, than that on L-J slants.

The contamination rate of the cultures in the biphasic medium was a slightly higher than in the MGIT system, probably because the solid part of the biphasic medium was sterilized by steam at 83°C, which may not have killed all the bacteria. Surviving bacteria grew in the biphasic medium’s nutrient-rich liquid. The time to detection of mycobacteria in the biphasic medium was longer than in the MGIT system. There are two possible reasons for this. First, the sensitivity of the MGIT system is higher. The second reason is the difference in incubation volume between the biphasic medium and MGIT system. Among the 419 specimens cultured side-by-side in the biphasic medium and MGIT systems, the incubation volumes were typically 0.1 ml for the biphasic medium and 0.5 ml for the MGIT system. During the latter part of this study, a small number of sputum specimens were inoculated in the biphasic medium and the MGIT system using the same incubation volume. The results showed that the time to detection of *M. tuberculosis* with 0.1 ml initial incubation volume was significantly longer than with 0.5 ml. When the incubation volumes were the same, the time to detection of *M. tuberculosis* in the biphasic medium was still longer, by 2–3 d, than in the MGIT system. For the smear-positive specimens, the culture-positive rates of both media were similar. For smear-negative specimens, due to the low mycobacterial concentration in the sample, the volume of inoculum was increased, and the culture-positive rate was higher. This was probably due to uneven distribution of mycobacteria in the sample. The same volume of inoculum sometimes contained mycobacteria and sometimes did not.

Our primary aim is to established biphasic medium for the diagnosis of TB by increasing the rate of recovery of mycobacteria. The results of our evaluation (Table 2) showed that about 90% of the isolated mycobacteria from suspected pulmonary tuberculosis patients were *M. tuberculosis complex*. Biphasic medium showed a higher mycobacterial recovery rate of than L-J medium. The biphasic medium has an added advantage in that it does not require as expensive detection instrumentation as the automated liquid culture systems do.

In conclusion, we found the biphasic culture medium to be low-cost and suitable for mycobacterial recovery in sputum specimens from suspected pulmonary tuberculosis patients, offering a shorter time to recovery of *M. tuberculosis* and an increase in the recovery rate. The biphasic culture medium is expected be a compelling alternative for mycobacterial culture, especially in hospitals that lack auto-culture equipment. With further study, a new type of drug susceptibility testing medium, based on the biphasic medium, may be developed.

**Author Contributions**

Conceived and designed the experiments: ZYH ZLC J. Liu. Performed the experiments: JW XCH J. Lu QW ZNC JLL JC RJZ LHQ HY R1J ZHL AXB. Analyzed the data: ZLC CTZ. Contributed reagents/materials/analysis tools: ZYH J. Liu. Wrote the paper: ZLC CTZ ZYH.

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