PLoS one

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

Zhenling Cui[1], Jie Wang[1], Changtai Zhu[1,3], Xiaochen Huang[1], Junmei Lu[1], Qing Wang[4], Zhongnan Chen[5], Junling Wang[6], Yan Zhang[7], Delin Gu[8], Lingjie Jing[1], Jin Chen[1], Ruijuan Zheng[1], Lianhua Qin[1], Hua Yang[1], Ruiliang Jin[1], Zhonghua Liu[1], Aixiao Bi[1], Jinming Liu[2]*, Zhongyi Hu[1]*

1 Shanghai Key Laboratory of Tuberculosis, Shanghai Pulmonary Hospital, Medical School, Tongji University, Shanghai, China, 2 Department of Respiratory Medicine, Shanghai Pulmonary Hospital, Medical School, Tongji University, Shanghai, China, 3 Department of Laboratory Medicine, Changzhou Tumor Hospital, Soochow University, Changzhou, China, 4 Anhui Province Chest Hospital, Hefei, China, 5 Hunan Province Chest Hospital, Changsha, China, 6 Shandong Province Chest Hospital, Jinan, China, 7 Hangzhou Red Cross Hospital, Hangzhou, China, 8 The Sixth People Hospital of Nantong, Nantong, China

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*.

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

* E-mail: shhuzy@163.com (ZH); jinmingliu2007@yahoo.com.cn (JL)

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 µL 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°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 37°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 µg/ml, 16 µg/ml, 4 µg/ml, and 4 µg/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 µg/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].
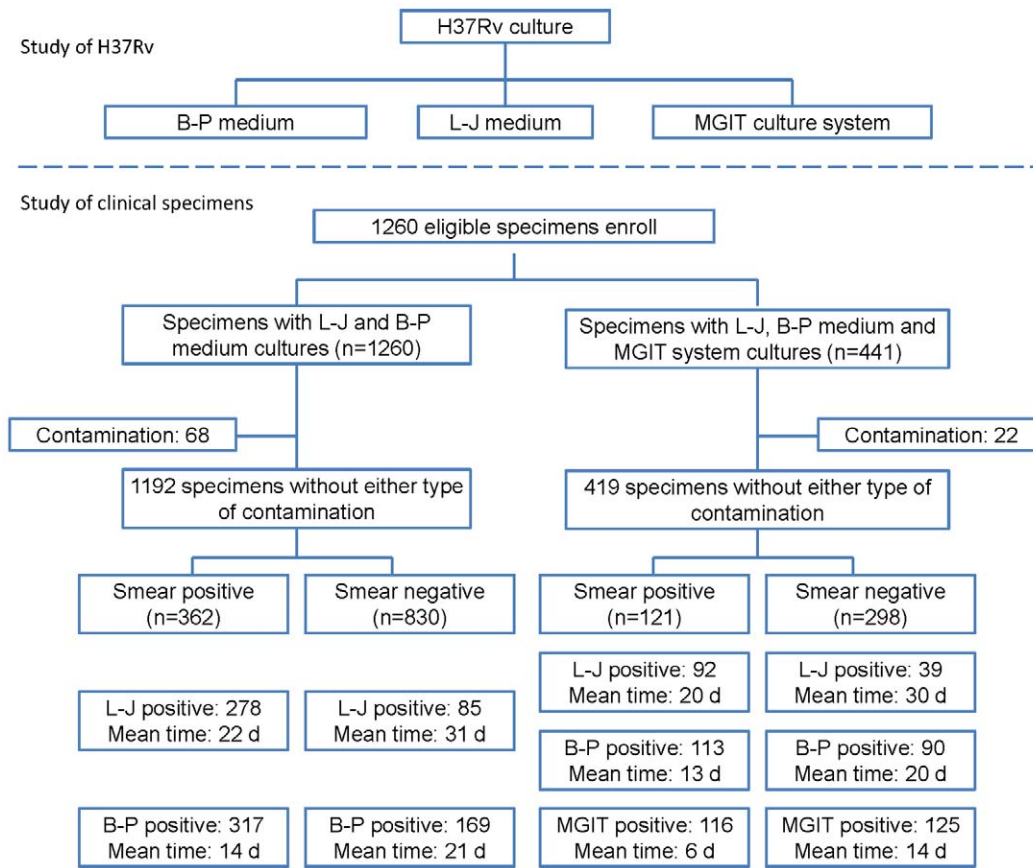
Figure 1. Flow chart. This study evaluated the biphasic medium (B–P medium) for the recovery of H37Rv and mycobacteria in clinical sputum specimens. A total of 1260 sputum specimens were decontaminated and cultured in the L-J slant and biphasic medium. Among them, the MGIT culture system was also used in 441 decontaminated specimens in addition to the presence of the L-J slant and biphasic medium.
doi:10.1371/journal.pone.0036331.g001

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.

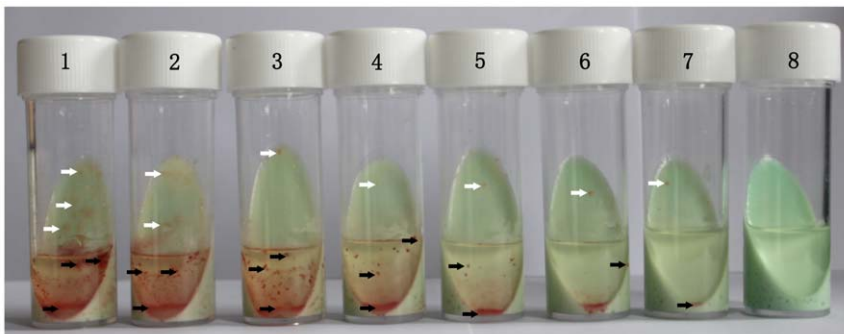**Figure 2. H37Rv culture in biphasic medium.** H37Rv ranging in concentration from 10^7 cfu to 1 cfu per vial were incubated in biphasic medium at 37°C for 2 weeks. The numbers of H37Rv were 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10, 1, and 0 CFU/ml in tubes 1 through 8. Both white and black arrows indicate the locations of positive colonies of mycobacteria.
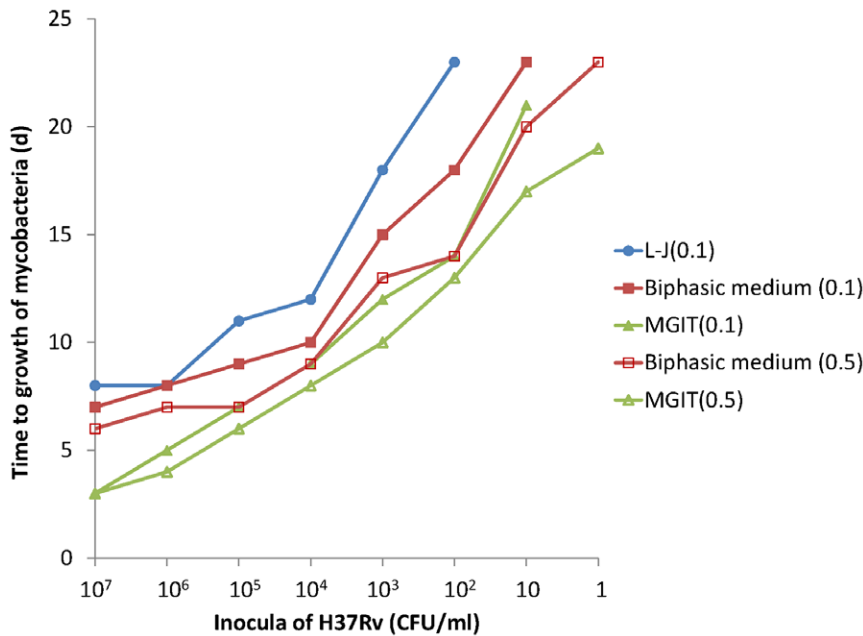doi:10.1371/journal.pone.0036331.g002

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

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 slant ($P<0.05$) 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%

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	46.28	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

doi:10.1371/journal.pone.0036331.t001

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 medium	1192	486 (40.8)	456 (38.3)	30 (2.5)
L-J medium	1192	363 (30.5)	337 (28.3)	26 (2.2)
Biphasic medium	419	203 (48.4)	187 (44.6)	16 (3.8)
L-J medium	419	131 (31.2)	118 (28.2)	13 (3.1)
MGIT 960	419	234 (55.8)	217 (51.8)	17 (4.1)

doi:10.1371/journal.pone.0036331.t002

(337/363) of mycobacteria were positive for *M. tuberculosis*. About 90% of mycobacteria from each medium were *M. tuberculosis complex*. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of biphasic medium for the recovery of mycobacteria were 98.3%, 84.4%, 73.5%, and 99.1% those of L-J medium, respectively (Table 3). The kappa value was 0.76, indicating substantial agreement between the two media systems.

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 ($\chi^2 = 14.347$, $P<0.001$). Among smear-negative specimens, 169 (20.4%) were culture-positive for mycobacteria in the biphasic medium and 85 (10.2%) were culture-positive for mycobacteria on L-J slants. The rate of positive cultures in the biphasic medium was higher than that observed on the L-J slants among smear-positive specimens ($\chi^2 = 32.798$, $P<0.001$). The culture results of each hospital are shown in Table 4. Among the 419 specimens from the Shanghai Pulmonary Hospital, 113 (93.4%) of smear-positive specimens were culture-positive for mycobacteria in the biphasic medium, 92 (76.0%) on L-J slants, and 116 (95.9%) in the MGIT system. For smear-negative specimens, 90 (30.2%) were found culture-positive for mycobacteria in the biphasic medium, 39 (13.1%) on L-J slants, and 118 (39.6%) in the MGIT system. The rate of positive cultures in the biphasic medium was similar to that of MGIT 960 among smear-

positive specimens ($\chi^2 = 0.732$, $P>0.05$) and lower than that of MGIT 960 among smear-negative specimens ($\chi^2 = 5.790$, $P<0.05$).

Figure 4 shows the median times to recoveries of mycobacteria 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 6 days (interquartile range [IQR], 4–10 days), 13 days (IQR, 11–18 days), and 20 days (IQR, 15–41 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 1192 specimens positive for mycobacteria both on L-J slants and in the biphasic medium. For smear-positive specimens, the median time to detection of mycobacteria were 14 days (IQR, 10–20 days) for cultures in the biphasic medium and 22 days (IQR, 16–29 days) for cultures in L-J slants. For smear-negative specimens, the median times to detection of mycobacteria were 21 days (IQR, 16–28 days) for cultures in the biphasic medium and 31 days (IQR, 24–38 days) for cultures on L-J slants.

Eighteen random frozen backup specimen sediments found positive for mycobacteria in the MGIT system were re-cultured in

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	Neg	Pos	Neg	Pos	Neg	Pos	Neg
Pos	357	129	19	20	104	14	48	10	33	5	23	7	130	73
Neg	6	699	3	157	0	81	0	142	0	51	2	53	1	215
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 (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

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

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 significant 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
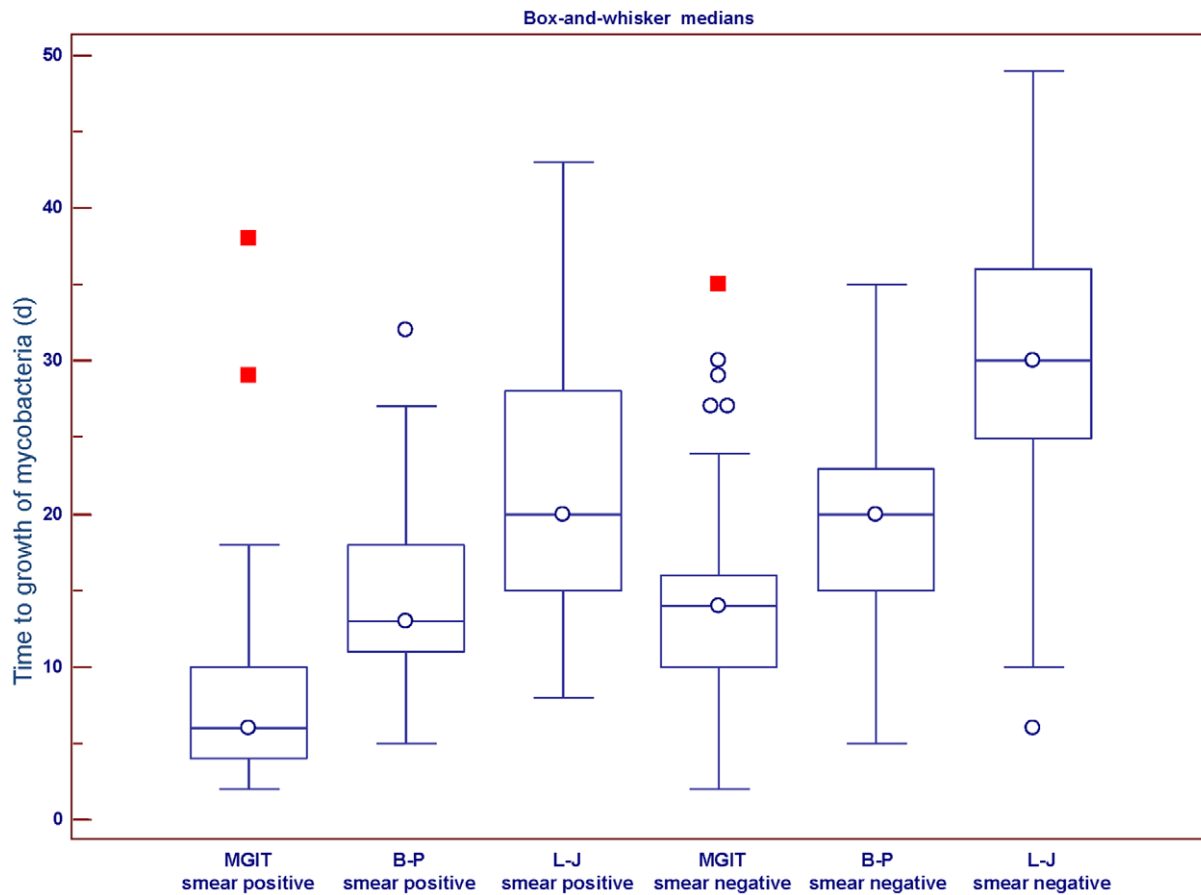

Figure 4. Box plot of median time required for growth of mycobacteria in each culture medium. Box lengths indicate interquartile range. Horizontal lines in each box represent the median of the distribution. Whiskers extend from the normal minimum value to the normal maximum value. For L-J, biphasic medium (B–P), and MGIT, the results of 121 smear positive sputum specimens and 298 smear negative sputum specimens are shown.
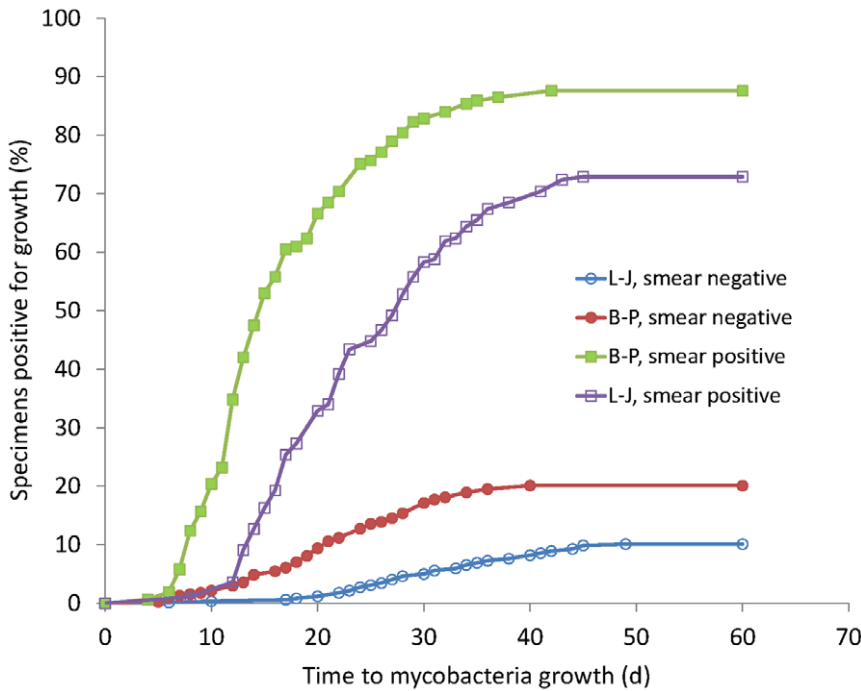doi:10.1371/journal.pone.0036331.g004

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
	8	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 mycobaterial 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 JLW YZ DLG LJJ JC RJZ LHQ HY RLJ ZHL AXB. Analyzed the data: ZLC CTZ. Contributed reagents/materials/analysis tools: ZYH J. Liu. Wrote the paper: ZLC CTZ ZYH.

References

1. World Health Organization (2010) Global tuberculosis control - surveillance, planning, financing. World Health Organization. WHO/HTM/TB/2008.393.

2. World Health Organization (2010) Multidrug and extensively drug-resistant TB (M/XDR-TB): 2010 global report on surveillance and response. World Health Organization. WHO/HTM/TB/2010.3.

3. World Health Organization (2010) Anti-tuberculosis drug resistance in the world (Fourth Global Report). World Health Organization. WHO/HTM/TB/2008.394.

4. Keshavjee S, Gelmanova IY, Farmer PE, Mishustin SP, Strelis AK, et al. (2008) Treatment of extensively drug-resistant tuberculosis in Tomsk, Russia: a retrospective cohort study. The Lancet 372: 1403–1409.

5. Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, et al. (2006) Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. The Lancet 368: 1575–1580.

6. Liberato IR, de Albuquerque MF, Campelo AR, de Melo HR (2004) Characteristics of pulmonary tuberculosis in HIV seropositive and seronegative patients in a Northeastern region of Brazil. Rev Soc Bras Med Trop 37: 46–50.

7. Caws M, Thwaites G, Stepniewska K, Nguyen TN, Nguyen TH, et al. (2006) Beijing genotype of Mycobacterium tuberculosis is significantly associated with human immunodeficiency virus infection and multidrug resistance in cases of tuberculous meningitis. J Clin Microbiol 44: 3934–3939.

8. World Health Organization (2009) A guide to monitoring and evaluation for collaborative TB/HIV activities. World Health Organization. WHO/HTM/TB/2009.414.

9. Raviglione MC, Narain JP, Kochi A (1992) HIV-associated tuberculosis in developing countries: clinical features, diagnosis, and treatment. Bull WHO 70: 515–526.

10. World Health Organization (1998) Laboratory services in TB control. Part III: Culture. World Health Organization. WHO/TB/98.258.

11. Bemer P, Palicova F, Rusch-Gerdes S, Drugeon HB, Pfyffer GE (2002) Multicenter evaluation of fully automated BACTEC Mycobacteria Growth Indicator Tube 960 system for susceptibility testing of Mycobacterium tuberculosis. J Clin Microbiol 40: 150–154.

12. World Health Organization (2009) Treatment of tuberculosis: guidelines – 4th ed. World Health Organization. WHO/HTM/TB/2009.420.

13. Greco S, Rulli M, Girardi E, Piersimoni C, Saltini C (2009) Diagnostic accuracy of in-house PCR for pulmonary tuberculosis in smear-positive patients: meta-analysis and metaregression. J Clin Microbiol 47: 569–576.

14. International Union Against Tuberculosis and Lung Disease (2000) Technical guide. Sputum examination for tuberculosis by direct microscopy in low-income countries. Paris: International Union Against Tuberculosis and Lung Disease.

15. Pfyffer GE, Brown-Elliot BA, Wallace RJ (2003) General characteristics, isolation, and staining procedures. In Manual of Clinical Microbiology. 8th edition. Washington, DC: ASM Press. pp 532–559.

16. Rieder HL, Van Deun A, Kam KM, Kim SJ, Chonde TM, et al. (2007) Priorities for Tuberculosis Bacteriology Services in Low-Income Countries International Union Against Tuberculosis and Lung Disease ISBN: 2-914365-29-2.

17. Tengerdy RP, Nagy JG, Martin B (1967) Quantitative measurement of bacterial growth by the reduction of tetrazolium salts. Appl Microbiol 15: 954–955.

18. Hanna BA, Ebrahimzadeh A, Elliott LB, Morgan MA, Novak SM, et al. (1999) Multicenter evaluation of the BACTEC MGIT 960 system for recovery of mycobacteria. J Clin Microbiol 37: 748–752.

19. Giampaglia CM, Martins MC, Inumaru VT, Butuem IV, Telles MA (2005) Evaluation of a rapid differentiation test for the Mycobacterium tuberculosis complex by selective inhibition with rho-nitrobenzoic acid and thiophene-2-carboxylic acid hydrazide. Int J Tuberc Lung Dis 9: 206–209.

20. Angeby KA, Klintz L, Hoffner SE (2002) Rapid and inexpensive drug susceptibility testing of Mycobacterium tuberculosis with a nitrate reductase assay. J Clin Microbiol 40: 553–555.

21. Kumar M, Khan IA, Verma V, Kalyan N, Qazi GN (2005) Rapid, inexpensive MIC determination of Mycobacterium tuberculosis isolates by using microplate nitrate reductase assay. Diagn Microbiol Infect Dis 53: 121–124.

22. Affolabi D, Odoun M, Sanoussi N, Martin A, Palomino JC, et al. (2008) Rapid and inexpensive detection of multidrug-resistant Mycobacterium tuberculosis with the nitrate reductase assay using liquid medium and direct application to sputum samples. J Clin Microbiol 46: 3243–3245.

23. Collins L, Franzblau SG (1997) Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against Mycobacterium tuberculosis and Mycobacterium avium. Antimicrob Agents Chemother 41: 1004–1009.

24. Jadaun GP, Agarwal C, Sharma H, Ahmed Z, Upadhyay P, et al. (2007) Determination of ethambutol MICs for Mycobacterium tuberculosis and Mycobacterium avium isolates by resazurin microtitre assay. J Antimicrob Chemother 60: 152–155.

25. Mirovic V, Lepsanovic Z (2002) Evaluation of the MB/BacT system for recovery of mycobacteria from clinical specimens in comparison to Lowenstein-Jensen medium. Clin Microbiol Infect 8: 709–714.