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Rate of Recovery of *Mycobacterium tuberculosis* from Frozen Acid-Fast-Bacillus Smear-Positive Sputum Samples Subjected to Long-Term Storage in Northwest Ethiopia[▽]

Belay Tessema,[1,2,3]* Joerg Beer,[2] Frank Emmrich,[3,4] Ulrich Sack,[3,4] and Arne C. Rodloff[2]

Department of Medical Laboratory Technology, College of Medicine and Health Sciences, University of Gondar, Gondar, Ethiopia[1]; Institute of Medical Microbiology and Epidemiology of Infectious Diseases, University Hospital, University of Leipzig, Leipzig, Germany[2]; Institute of Clinical Immunology, University Hospital, University of Leipzig, Leipzig, Germany[3]; and Fraunhofer Institute for Cell Therapy and Immunology, Leipzig, Germany[4]

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Tuberculosis is a major public health problem in Ethiopia. The diagnosis and treatment of drug-resistant tuberculosis remain a challenge in the country. This study aimed to assess whether single morning sputum samples could be stored at −20°C for extended periods of time at remote settings and then transported and successfully cultured for *Mycobacterium tuberculosis*. Single morning sputum samples were collected from all smear-positive tuberculosis patients diagnosed at Gondar Hospital, Gondar Health Center, Metemma Hospital, Bahir Dar Hospital, and Debre Markos Hospital in Northwest Ethiopia between March and July 2009. Specimens were stored at the study sites and sent to the mycobacteriology laboratory at the University Hospital, Leipzig, Germany, where specimens were processed and inoculated into the BacT/Alert 3D system and Lowenstein-Jensen and Gottsacker media. Ice packs were added in the package of the specimens during transport. A total of 319 patients were enrolled in this study. The median specimen storage time was 132 days (range, 16 to 180 days). Of all specimens, 283 (88.7%) were culture positive by any of the three culturing systems. *M. tuberculosis* isolates from four contaminated specimens in all culturing systems were successfully isolated on Middlebrook 7H10 agar; thereby, the recovery rate increased to 287 (90.0%). The length of time of sputum storage had no significant effect on the rate of recovery of *M. tuberculosis* in all culturing systems. In conclusion, single morning sputum specimens collected at remote settings stored at −20°C for long periods of time without the addition of preservatives can yield a high recovery rate. These findings suggest a simple and cost-effective alternative method of sputum storage for epidemiological and drug resistance studies in low-resource countries.

Tuberculosis (TB) remains a leading infectious cause of morbidity and mortality worldwide. In 2007, the World Health Organization (WHO) estimated 9.27 million new cases of TB, with 1.3 million deaths globally (22). Africa still carries a disproportionate burden of global TB cases, 29% of all TB cases (1). Ethiopia ranks seventh among the world's 22 high-burden tuberculosis countries. The country had 314,267 TB cases in 2007, with an estimated incidence rate of 378 cases per 100,000 people (22).

The nationwide anti-TB drug resistance survey in 2005 showed that the prevalences of multidrug-resistant TB (MDR-TB) were 1.6% among new cases and 11.8% among previously treated TB cases (21). However, the isolation and drug resistance testing of *Mycobacterium tuberculosis* remain a challenge in Ethiopia; so far, there are only two laboratories in the capital city, Addis Ababa, that perform culture and drug sensitivity testing for *M. tuberculosis*. The successful isolation of *M. tuberculosis* from remote settings requires proper collection, storage, and transportation of sputum specimens to TB laboratories (4, 19, 20).

* Corresponding author. Mailing address: Institute of Medical Microbiology and Epidemiology of Infectious Diseases, University Hospital, Liebigstrasse 21, 04103 Leipzig, Germany. Phone: 4917652670708. Fax: 493419715209. E-mail: bt1488@yahoo.com.
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The WHO recommends that two sputum specimens be collected from remote areas and transported to reference laboratories without delay (19). There may be a substantial cost associated with the handling, transportation, and processing of two sputum specimens from each patient without delay. In addition, sputum specimen preservatives, such as 0.6% cetylpyridinium bromide (CPB) or 1% cetylpyridinium chloride (CPC), are recommended when a delay is unavoidable. CPC is known to effectively liquefy and decontaminate sputum samples and keep tubercle bacilli viable for up to 8 days; it is still the most commonly used preservative for the storage and transport of sputum specimens (12, 15, 17, 18).

Once CPB or CPC has been added to a specimen, refrigeration as a storage method is no longer possible (19). Moreover, once preserved specimens have reached the culture laboratory, a centrifugation step without refrigeration is necessary to remove the preservative prior to culture (12). Previous studies have also shown the negative effects of CPC on microscopic examination and culture, including (i) a significant reduction in the positivity of acid-fast bacilli (AFB) with Ziehl-Neelsen staining (14, 16) and (ii) the inhibition of mycobacterial growth, especially when inoculated into culture media, including Middlebrook 7H9 and 7H10 media (17), and the Bactec MGIT 960 system (13). Palomino et al. also recommended that sputum samples should be transported rapidly to the reference laboratory to avoid overgrowth by other microorganisms: when

the transport or processing of the sputa is delayed, specimens should be stored for no more than 5 days at 4°C until they are transported or processed for culture (8).

A simple inexpensive method for sputum storage and transport that preserves the viability of tubercle bacilli for long periods of time will be useful for epidemiological and drug resistance studies in remote settings. Therefore, the present study was undertaken to assess the rate of recovery of *M. tuberculosis* from single morning sputum specimens subjected to long-term storage at −20°C without the addition of chemical preservatives.

MATERIALS AND METHODS

Study design, area, and study period. All smear-positive pulmonary tuberculosis (PTB) patients diagnosed at Gondar Hospital, Gondar Health Center, Metemma Hospital, Bahir Dar Hospital, and Debre Markos Hospital in Northwest Ethiopia between 1 March 2009 and 15 July 2009 were included in this study. The diagnosis of smear-positive PTB was based on national guidelines for the microscopic examination of tuberculosis (7); direct smears were prepared from three sputum specimens. The first specimen was collected on the spot when the patient presented at the diagnostic center. The patient was then given another sputum container and instructed to collect an early-morning specimen on the next day and return to the clinic. A third specimen was collected when the early-morning specimen was delivered to the laboratory. Smears were stained by a Ziehl-Neelsen staining technique for microscopic examination without grading smear positivity. Once diagnosed, informed consent was obtained from the study subjects, and the single morning sputum sample was collected for storage and transport to the mycobacteriology laboratory in Leipzig for culturing. Institutional ethical clearance was obtained from the research and publication committee of Gondar University, Gondar, Ethiopia.

Storage and transport of sputum specimens. The specimens were kept at −20°C, without the addition of chemical preservatives, in the TB laboratory of each hospital until they were prepared for transport to the mycobacteriology laboratory at the Institute of Medical Microbiology and Epidemiology of Infectious Diseases, University Hospital Leipzig. The preparation and packaging of the specimens complied with International Air Transport Association (IATA) regulations (http://www.iata.org/ads/issg.htm). No decontamination procedure was performed prior to storage or transportation. Specimens were transported in one batch after the data collection period. Ice packs were added to the package of the specimens during transport, and specimens were stored at −80°C after arrival at the mycobacteriology laboratory in Leipzig until they were processed for culture.

Specimen processing and culturing methods. Specimens were processed for culture from 27 July to 6 September 2009 according to Deutsches Institut für Normung (DIN) recommendations for the detection of mycobacteria by culture methods (2). In brief, sputum samples were transferred into centrifuge tubes with a capacity of 50 ml, distilled water was added up to 10 ml, and the same volume (10 ml) of an *N*-acetyl–L cysteine (NALC)–NaOH solution (4% NaOH, 2.9% sodium citrate, and 0.5% NALC) was added and incubated at room temperature on the shaker for 20 min and then neutralized with phosphate buffer (pH 6.8) and concentrated by centrifugation (3,300 × *g* for 20 min). From the sputum concentrates, 0.5 ml was used for inoculation into BacT/Alert MP bottles supplemented with antibiotics according to the manufacturer's instructions (bioMérieux SA, France), 0.2 ml was inoculated into Lowenstein-Jensen (L-J) medium, and the same amount was inoculated into Gottsacker medium. Both L-J and Gottsacker media (Artelt-ENCLIT GmbH, Wyhra, Germany) were supplemented with polymyxin B (200,000 IU/liter), amphotericin B (10 mg/liter), carbenicillin (50 mg/liter), and trimethoprim (10 mg/liter) (PACT). Sputum concentrates were also used to prepare smears for the grading of the smear positivity of the sputa. The remaining sputum concentrates were stored at −80°C for further investigation by PCR.

BacT/Alert MP bottles were loaded and incubated into the BacT/Alert 3D system incubator cabinet for a maximum of 56 days at 36°C. The L-J and Gottsacker tubes were read twice on the first week and weekly thereafter for a total of 8 weeks of incubation at 36°C. Microscopy of sputum concentrates was performed by using an auramine-rhodamine stain, and the results were recorded by using standard grading scales (3). Positive bottles and tubes were sampled for acid-fast bacillus (AFB) staining by the Kinyoun staining method and inoculated onto a blood agar plate (overnight incubation) to confirm culture purity. Contaminated fluid cultures were inoculated onto Middlebrook 7H10 agar (BD Diagnostics, Heidelberg, Germany) without decontamination for the isolation of pure mycobacterial colonies. Samples that were culture negative by both solid and fluid culture media and samples that were contaminated in fluid cultures but negative in solid media were examined by PCR (Cobas Amplicor analyzer; Roche Diagnostics, Indianapolis, IN) for *M. tuberculosis* complex organisms.

Identification of mycobacteria. Isolates with the typical morphology of members of the *M. tuberculosis* complex were identified by DNA hybridization technology with nitrocellulose strips (GenoType MTBC; Hain Diagnostika, Nehren, Germany) according to the manufacturer's instructions. The procedure involves the isolation of DNA from cultured material, multiplex amplification with biotinylated primers, and reverse hybridization of the single-stranded, biotin-labeled amplicons onto membrane-bound probes. The resulting banding pattern indicates the species of the *M. tuberculosis* complex. *M. tuberculosis* was differentiated from *Mycobacterium canettii* based on the colony morphology of the isolates on solid media.

Statistical analysis. All laboratory data were entered, cleared, and analyzed by using SPSS, version 13, statistical package software (SPSS Inc., Chicago, IL). The effects of sputum storage time, smear positivity, and sputum volume on the rate of recovery and time to detection of *M. tuberculosis* growth were analyzed in this study. The measurement of time to detection was of a nonnormal distribution, and we have therefore reported summary statistics of medians and ranges. We used a linear regression model to analyze predictors of a successful culture. The differences in the rates of recovery of *M. tuberculosis* among different culture systems were determined by the McNemar modification of the chi-square test. The comparison of the median times for a positive culture among fluid and solid culture systems was made by the Wilcoxon matched-pairs test. One-way analysis of variance (ANOVA) was used for trend analyses across ordered groups. *P* values of less than 0.05 were considered statistically significant.

RESULTS

A total of 319 smear-positive pulmonary tuberculosis patients were enrolled in this study. The median specimen storage time from collection until processing for culture was 132 days (range, 16 to 180 days). The specimen transportation time from the hospitals in Ethiopia to the mycobacteriology laboratory in Germany was 6 days. All samples did arrive thawed at the mycobacteriology laboratory in Germany. Of all specimens, 97/319 (30.4%) had a small sputum volume (<2 ml); some specimens had as little as 500 μl. The majority, 222/319 (69.6%) specimens, had a large volume (2 to 5 ml). Additionally, 29/319 (9.1%) specimens had low-level smear positivity (scores of doubtful and 1+), and 290/319 (90.9%) had a high level of smear positivity, with scores of 2+ and above.

Of all sputum specimens, 283/319 (88.7%) were culture positive by any of the three culture methods, 4/319 (1.3%) were contaminated in all cultures, 26/319 (8.1%) failed to grow in all systems, and 6/319 (1.9%) were found to be contaminated in the BacT/Alert 3D system and failed to grow in both L-J and Gottsacker media (Table 1). *M. tuberculosis* isolates from four contaminated specimens in all cultures were successfully isolated on Middlebrook 7H10 agar medium, thereby increasing the recovery rate to 287/319 specimens (90.0%). PCR was performed for 26 specimens that were negative by all culture methods and 6 specimens that were contaminated in fluid cultures and failed to grow in solid media. The PCR results showed that 27/32 (84.4%) specimens were positive for organisms of the *M. tuberculosis* complex, 3/32 (9.4%) were inhibited, and 2/32 (6.2%) were negative. All positive cultures were identified as *Mycobacterium tuberculosis*. No nontuberculosis mycobacterium (NTM) was isolated from any study subject.

The differences in the rates of recovery of *M. tuberculosis* among the three culture methods were not statistically significant. The contamination rate was found to be high, 42/319 specimens (13.2%), in the BacT/Alert 3D system, compared to

TABLE 1. Rates of recovery of *M. tuberculosis* by the BacT/Alert 3D system, L-J medium, and Gottsacker medium during primary culture[d]

Culture result	No. (%) of specimens with result determined by:				
	BacT/Alert 3D system ($n = 319$)	L-J medium ($n = 319$)	Gottsacker medium ($n = 319$)	L-J and Gottsacker media ($n = 319$)	BacT/Alert 3D system and L-J and Gottsacker media ($n = 319$)
Positive (*M. tuberculosis*)	251 (78.7)	249 (78.0)	236 (74.0)	265 (83.1)[a]	283 (88.7)[a]
Contamination	42 (13.2)	21 (6.6)	16 (5.0)	10 (3.1)[b]	4 (1.3)[b]
Negative	26 (8.1)	49 (15.4)	67 (21.0)	44 (13.8)[c]	26 (8.1)[c]
Contamination in BacT/Alert 3D and negative in solid media					6 (1.9)

[a] At least one positive.
[b] All media were contaminated.
[c] All media were negative.
[d] $P = 0.892$ for the BacT/Alert 3D system versus L-J medium, $P = 0.082$ for the BacT/Alert 3D system versus Gottsacker medium, and $P = 0.072$ for L-J medium versus Gottsacker medium (determined by the McNemar test for differences).

21/319 (6.6%) in L-J medium and 16/319 (5.0%) in Gottsacker medium. However, the higher contamination rate was compensated for by a higher rate of recovery of *M. tuberculosis* in fluid culture.

The length of time of sputum storage had no significant effect on the proportion of positive culture results for all culture methods. Higher-level smear positivity was more predictive of a positive culture result ($P < 0.001$) for all culture methods. The rate of recovery of *M. tuberculosis* from samples with low-level smear positivity was 4/5 specimens (80%) for doubtful smear-positive samples and 12/24 (50%) for smear-positive samples with a score of 1+. The rates of recovery of *M. tuberculosis* from samples with high-level smear positivity were 37/44 specimens (84.1%) with a score of 2+, 81/89 (91.0%) with a score of 3+, and 149/157 (94.0%) with a score of 4+ for smear-positive samples by all culture methods. The larger volume of specimen was more predictive of a positive culture in L-J medium ($P = 0.023$) and Gottsacker medium ($P < 0.001$) (Table 2).

The median time required for the detection of *M. tuberculosis* growth by each culturing method is summarized in Table 3. The BacT/Alert 3D system detected the growth of *M. tuberculosis* earlier (median, 14 days; range, 6 to 48 days) than did L-J and Gottsacker media (median, 19 days; range, 10 to 60 days) (both $P < 0.001$). The sputum storage time had no significant effect on the time to positive culture by L-J medium and Gottsacker medium, but the longer sputum storage time was significantly associated ($P < 0.001$) with a longer time to positivity by the BacT/Alert 3D system. Sputum smear positivity was inversely related to the time to positivity of the BacT/Alert 3D system ($P < 0.001$); however, it was not statistically significant for L-J medium and Gottsacker medium. The sputum volume was inversely related to the time to positive culture by the BacT/Alert system ($P < 0.001$) and L-J medium ($P = 0.008$).

DISCUSSION

In the present study, the rate of recovery of *M. tuberculosis* from single morning sputum specimens subjected to long-term storage (median, 132 days; range, 16 to 180 days) at −20°C without the addition of chemical preservatives was very high,

TABLE 2. Effects of smear positivity after decontamination, storage time of sputum, and sputum volume on the rate of recovery of *M. tuberculosis*[a]

Predictor	Total no. of specimens	No. (%) of specimens positive by BacT/Alert 3D system	*P* value for BacT/Alert 3D system	No. (%) of specimens positive by L-J medium	*P* value for L-J medium	No. (%) of specimens positive by Gottsacker medium	*P* value for Gottsacker medium
Smear positivity score							
Doubtful	5	3 (60.0)	<0.001	4 (80.0)	<0.001	1 (20.0)	<0.001
1+	24	11 (45.8)		8 (33.3)		7 (29.2)	
2+	44	34 (77.3)		31 (70.5)		23 (52.3)	
3+	89	73 (82.0)		74 (83.1)		68 (76.4)	
4+	157	130 (82.8)		132 (84.1)		137 (87.3)	
Storage time (days)							
16–30	8	6 (75.0)	0.605	7 (87.5)	0.07	7 (87.5)	0.111
31–60	19	12 (63.2)		15 (78.9)		15 (78.9)	
61–90	47	41 (87.2)		41 (87.2)		40 (85.1)	
91–120	69	53 (76.8)		58 (84.1)		59 (85.5)	
121–150	65	49 (75.4)		51 (78.5)		43 (66.2)	
151–180	111	90 (81.1)		77 (69.4)		72 (64.9)	
Sputum vol (ml)							
<2	97	78 (80.4)	0.619	68 (70.1)	0.023	55 (56.7)	<0.001
2–5	222	173 (77.9)		181 (81.5)		181 (81.5)	

[a] $n = 319$.

TABLE 3. Effects of smear positivity after decontamination, storage time of sputum, and sputum volume on time to detection of *M. tuberculosis* growth[b]

Predictor	BacT/Alert 3D system			L-J medium			Gottsacker medium		
	No. (%) of positive samples	Median TTD (days) (range)	*P* (trend)	No. (%) of positive samples	Median TTD (days) (range)	*P* (trend)	No. (%) of positive samples	Median TTD (days) (range)	*P* (trend)
Smear positivity score									
Doubtful	3 (1.2)	26 (21–40)	<0.001	4 (1.6)	45 (28–60)	0.065	1 (0.4)	60 (60–60)	0.845
1+	11 (4.4)	23 (15–48)		8 (3.2)	31 (20–60)		7 (3.0)	60 (20–60)	
2+	34 (13.5)	18 (6–39)		31 (12.4)	22 (15–60)		23 (9.7)	22 (15–30)	
3+	73 (29.1)	16 (6–46)		74 (29.7)	22 (11–60)		68 (28.8)	22 (11–60)	
4+	138 (51.8)	10 (6–30)		132 (53.0)	18 (10–49)		137 (58.1)	18 (10–60)	
Total[a]	251 (100.0)	14 (6–48)		249 (100.0)	19 (10–60)		236 (100.0)	19 (10–60)	
Storage time (days)									
16–30	6 (2.4)	11 (6–15)	<0.001	7 (2.8)	19 (17–22)	0.208	7 (3.0)	19 (17–22)	0.177
31–60	12 (4.8)	8 (6–15)		15 (6.0)	17 (14–60)		15 (6.4)	17 (14–28)	
61–90	41 (16.3)	10 (6–48)		41 (16.5)	18 (10–30)		40 (16.9)	18 (10–29)	
91–120	53 (21.1)	11 (6–23)		58 (23.3)	19 (11–49)		59 (25.0)	19 (11–60)	
121–150	49 (19.5)	15 (6–42)		51 (20.5)	22 (12–60)		43 (18.2)	22 (12–60)	
151–180	90 (35.9)	18 (8–46)		77 (30.9)	23 (15–60)		72 (30.5)	23 (15–60)	
Total	251 (100.0)	14 (6–48)		249 (100.0)	19 (10–60)		236 (100.0)	19 (10–60)	
Sputum vol (ml)									
0.5	8 (3.2)	26 (17–46)	<0.001	7 (2.8)	25 (24–60)	0.008	4 (1.7)	43 (24–60)	0.674
1	38 (15.1)	22 (6–36)		34 (13.7)	24 (10–60)		28 (11.9)	24 (10–60)	
1.5	32 (12.8)	21 (13–40)		27 (10.8)	27 (17–60)		23 (9.7)	27 (17–60)	
2	35 (13.9)	12 (7–48)		34 (13.7)	18 (11–49)		33 (14.0)	18 (11–60)	
3	25 (10.0)	13 (7–30)		26 (10.4)	19 (10–38)		26 (11.0)	21 (10–60)	
4	14 (5.6)	13 (6–42)		14 (5.6)	21 (12–36)		12 (5.1)	20 (11–33)	
5	99 (39.4)	10 (6–35)		107 (43.0)	18 (10–60)		110 (46.6)	18 (10–60)	
Total	251 (100.0)	14 (6–48)		249 (100.0)	19 (10–60)		236 (100.0)	19 (10–60)	

[a] $P < 0.001$ for the BacT/Alert system versus L-J medium and $P < 0.001$ for the BacT/Alert system versus Gottsacker medium (by Wilcoxon matched-pairs test).
[b] $n = 319$. TTD, time to detection of *M. tuberculosis* growth in days.

287/319 specimens (90.0%). The World Health Organization recommends that when specimens are likely to be exposed to room temperature for more than 48 h, an equal volume of either 0.6% CPB or 1% CPC should be added to homogenize and decontaminate the sample (17, 18). However, once CPB or CPC has been added to a specimen, refrigeration as a storage method is no longer possible, as these preservatives are likely to crystallize and become inactive (9). Once preserved specimens have reached the culture laboratory, a centrifugation step without refrigeration is necessary to remove the preservative prior to culture (12).

In addition, previous studies have shown the negative effects of CPC on microscopic examination and culture systems, including (i) a significant reduction in the detection of AFB with Ziehl-Neelsen staining (14, 16); (ii) an inhibition of mycobacterial growth, especially when inoculated into culture media, including Middlebrook 7H9 and 7H10 media, which have an insufficient neutralizing activity for this quaternary ammonium compound (17); and (iii) a negative effect on the Bactec MGIT 960 system, decreasing the mycobacterial detection rate and increasing the time required for mycobacterial growth readings (13). Therefore, these findings suggest a need for a simpler and cost-effective alternative to the current WHO recommendations for conducting epidemiological and drug resistance studies in resource-poor settings.

According to World Health Organization protocols for the surveillance of drug resistance, two sputum specimens should be collected to ensure a high yield of positive cultures (19). Epidemiological and drug resistance studies are expensive and technically demanding, due in part to this requirement. This recommendation has a considerable impact, as it necessitates additional logistics of storage, transportation, and dual-laboratory processing for culturing. Our results demonstrate an acceptably high yield of positive cultures from a single morning sputum specimen, with considerable time and expense savings.

Our study revealed that increasing the storage time at −20°C without the addition of preservatives had no significant effect on the rate of recovery of *M. tuberculosis*. However, in other study, an increase of the storage time of up to 7 days at room temperature resulted in a reduced rate of recovery of *M. tuberculosis* and rising contamination rates (9). In this study, a higher degree of smear positivity was highly predictive of a positive culture result ($P < 0.001$) by all culture methods. This result was in agreement with the findings of another study, where specimen smear positivity was a factor in the recovery of *M. tuberculosis* (10). The volume of the sputum used for decontamination and homogenization was significantly predictive of a positive culture with L-J medium ($P = 0.023$) and Gottsacker medium ($P < 0.001$) but not by the BacT/Alert 3D system. This difference in the volume of the specimen and the predictive value of a positive culture among solid media and the BacT/Alert 3D system might be due to differences in the

amounts of decontaminated specimen inoculated into the media. The amount of the specimen inoculated into the BacT/Alert 3D system is 2.5 times higher than the amount inoculated into solid media. Moreover, in the BacT/Alert 3D system, small-volume specimens had a higher degree of culture positivity than specimens with a larger volume (Table 2). This might be due to the lower contamination rate, 2/97 (2.1%) of samples, with a smaller volume (<2 ml) than the contamination rate, 40/222 (18.0%) of samples, with a larger volume (2 to 5 ml). The higher contamination rate for the larger volume might be due to the higher load of contaminants in the larger sputum volume that could overcome the effect of the decontamination solution and antibiotic supplements in the fluid culture system.

In agreement with data from other studies (5, 6), the BacT/Alert 3D system showed a significant advantage over L-J and Gottsacker media in the early detection of *M. tuberculosis* (both $P < 0.001$) because of its built-in colorimetric sensor of early bacterial growth in the culture broth. However, the BacT/Alert 3D system has shown a higher contamination rate than those of Lowenstein-Jensen and Gottsacker media. This is in agreement with the culture results of the sputa processed for routine patient diagnosis in this mycobacteriology laboratory and the results reported previously in another study (11). The high degree of contamination of the BacT/Alert cultures could have a considerable effect on the time to identification and cost, as it necessitates additional laboratory processing for culturing. However, these effects were minimized by the use of the BacT/Alert 3D system together with Lowenstein-Jensen and Gottsacker solid media.

In conclusion, single morning sputum specimens collected from smear-positive patients at remote settings, frozen at −20°C for long periods of time without the addition of chemical preservatives, can yield a high level of positive culture results. These findings suggest an alternative method of sputum storage with potential logistic simplification and cost savings for epidemiological and drug resistance studies in low-resource countries. Specimens were not kept at −20°C while in transport, which might support the growth of contaminants and decrease the viability of *M. tuberculosis*. Therefore, sample transport would require the addition of dry ice instead of ice packs to keep the specimens at a lower temperature during delivery. The level of smear positivity of patients included in this study was high; therefore, further studies are required to confirm these findings with other patients with low-level smear positivity and smear-negative pulmonary tuberculosis. Additionally, a sufficient volume of sputum specimens should be collected from smear-positive TB patients and stored at −20°C prior to transport to the mycobacteriology laboratory for culturing.

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