Policy Forum

The Feasibility of Sputum Transportation System in China: Effect of Sputum Storage on the Mycobacterial Detection*

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Sputum transportation from county-level to prefecture-level is an ideal strategy to cover the shortage of the laboratory capability in the resource-poor setting. Here, we firstly evaluated the feasibility of sputum transportation system in China by analyzing the culture and molecular diagnosis results from 1982 smear-positive patients with different delay in processing for culture. In this study, the total contamination rate was 2.32% and the total smear positive/culture negative (S+/C-) rate was 7.57%. We found that sputum specimens refrigerated for no more than 7 d before mycobacterial detection did not affect culture significantly. In addition, the invalid result rates among 0-3 d, 3-7 d, and 7+ d group were 3.63%, 3.14%, and 12.48%, respectively. Statistic analysis revealed that molecular diagnostic results while the invalid result rate of genechip for the specimen with more than 7 d delay was significantly higher (*P*<0.001). The refrigerators equipped in county laboratories, transport at low temperature and frequent transport services once a week will ensure the feasibility of sputum transportation system in China.

Tuberculosis (TB) is caused by various strains of mycobacteria, most often *Mycobacterium tuberculosis* (*M. tuberculosis*). The disease remains a major threat to public health worldwide[1-3] due to the emergence of drug-resistant TB, especially multi-drug resistant (MDR) TB[4-5]. Microscopic examination of sputum acid-fast bacilli (AFB) smear is still the most widely available diagnostic tool for tuberculosis, especially in the resource-limited setting[6]. In China, more than 2900 county-level laboratories perform microscopic examination of AFB[7]. The obvious advantages of sputum smear include low price, short turn-around time, and no need for complicated equipment[7-8]. However, the poor sensitivity of sputum smear miscroscopy has been reported to vary from 20% to 80%, which depends on the good specimen quality and well-trained laboratories[8]. Moreover, smear miscroscopy cannot provide valuable information on drug susceptibility of *M. tuberculosis* in the specimen[8-9].

Early diagnosis of TB and its drug susceptibility provides an important approach to preventing transmission of the disease, especially for drug resistant TB[8]. Unfortunately, more than half of the county-level TB laboratories in China do not have the capability to culture mycobacteria, which serves as an essential preparation for conventional drug susceptibility testing (DST)[8]. The appliciation of molecular amplification assays for TB and drug-resistant TB diagnosis in county level laboratories is even worse. Storing and then transporting sputum samples from the county-level to the prefecture-level seems an ideal strategy to solve this dilemma and has been reported in studies from other countries[10-12]. Here we report the first evaluation of the feasibility of a sputum storage and transport system from the county to the prefecture in China.

Three prefectural cities in China-Kaifeng City of Henan Province, Lianyungang City of Jiangsu Province, and Yongchuan District of Chongqing Municipality-representing different administrative divisions in China, were selected for a pilot study. Fourteen counties, including four counties from Lianyungang, five from Kaifeng and five from Yongchuan, were included in this study. From January 2011 to February 2012, all new smear-positive TB cases within the selected counties of the three prefectures were enrolled in the study,

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irrespective of co-morbidities or HIV-status. All patients included in this study provided written informed consent. The study was approved by the Program for Appropriate Technology in Health (PATH) and the Ethics Committee of the Chinese Center for Disease Control and Prevention.

Three sputum specimens, including one spot, one night, and one morning, were collected in the sterile containers. All the sputum samples were examined by smear microscopy in the county-level laboratories. The smears were stained by the Ziehl-Neelsen staining method and screened for acid-fast bacilli (AFB). We stored smear-positive specimens in the 4 °C refrigerator before transporting and monitored the temperature by thermometer daily. The samples were packed with frozen ice bags in insulated cases and were transported in designated vehicles. The transportation time from the county-level to prefectural-level laboratories varied from 2 h to 3 h, depending on the distance. The study required counties to transport the sputum specimens twice per week. The staff of the prefectural laboratories performed the culturing of transferred sputa within 4 h of the time they received the samples, and the molecular diagnosis was completed within 3 d.

Two smear-positive specimens from each TB patient were examined by Löwenstein-Jensen (L-J) culture in the prefectural-level laboratories. On arrival at the prefectural hospitals, sputum specimens were decontaminated with one volume of 4% sodium hydroxide per volume of sputum for 15 min, and a 0.1 mL aliquot of the resulting suspension was inoculated directly onto each of two slopes of the acidified L-J solid medium[13]. The medium bottles were incubated at 37 °C and examined once a week for 8 weeks.

In addition, one smear-positive specimen from each TB patient was examined by Genechip according to the manufacturer's instruction in the prefectural-level laboratories[14].

The condition of the specimens after storage and transport was measured by examining the cultures and the genechip results. For culture, we evaluated the quality of specimen in two ways: by the contamination rate and by the smear positive/culture negative (S+/C-) rate. For genechip, the invalid rate including 'failure to interpretation' and 'no mycobacterium' was calculated to access the quality of specimen. If either rate is high, it indicates deterioration of the specimen.

All the data were dual-entered and analyzed using Software SPSS 15.0. A chi-square test was used for statistical analysis. If the *P* value was less than 0.05, the difference was judged as significant.

A total of 1982 patients were enrolled in the study and provided 3670 sputum samples. As shown in Table 1, the specimens from 79% (1571/1982) of the patients were transported to the prefectural-level laboratory within 3 d after collection for L-J culture. The specimens from 20% of the patients (397/1982) were refrigerated for 3 to 7 d prior to transport and culture, and less than 1% of specimens took more than 1 week (7 to 14 d) to reach the laboratory. The total contamination rate was 2.32% and the total smear positive/culture negative (S+/C-) rate 7.57%. The total time delay between sputum specimen collection and inoculation onto the medium had no significant effect on the contamination rate or the S+/C- rate (Table 1). In addition, we found that the S+/C- rate of Yongchuan was significantly lower than that of Kaifeng and Lianyungang ($P<0.01$), while there was no statistical difference between Kaifeng and Lianyungang ($P=0.63$).

Among 1982 patients examined by genechip at the prefectural laboratory, 124 (6.26%) showed the invalid results, of which 38/124 (30.6%) and 86/124 (69.4%) belonged to 'failure to interpretation' and 'no mycobacterium', respectively. The invalid result rates among 0-3 d, 3-7 d, and 7+ d group were 3.63%, 3.14%, and 12.48%, respectively. When compared with 0-3 days group, the invalid rate of 7+ d group was significantly higher ($P<0.001$), while that of 3-7 d group showed no difference ($P=0.670$). Similarly, the difference between 3-7 group and 7+ group was statistically significant ($P<0.001$) (Table 2).

Examination of bacteria from sputum samples has played an important role in the diagnosis of pulmonary TB[8]. It has been demonstrated that culture is more sensitive than AFB smear microscopy for detecting *M. tuberculosis*, but establishment of qualified laboratories for direct culture is still a major challenge for resource-poor settings in China. In some pilots, with the support from project, sputum samples were transported from county-level to prefectural-level laboratories to carry out conventional culture, DST, and other molecular diagnoses in China[14]. In accordance with reports from other countries[10-11], our results demonstrated that storage for up to 7 days at 4 °C did not affect culture positivity[10,15]. In contrast, a study by Paramasivan et al. indicated that reduced recovery of *M. tuberculosis*

and rising contamination rates were related to increasing storage time at room temperature[16]. Hence, the temperature at which sputum specimens are stored prior to mycobacterium culture may play an important role in the culture results. Considering the resource-poor settings in some counties of China, purchasing a refrigerator for sample storage seems more accessible.

Table 1. Distribution of Culture Results According to Different Transportation Intervals

Study Area	Interval (days)	Culture Results (No. of culture tubes)					Culture Results (No. of TB patients)				
		Contaminated	Positive	Negative	Total	Contamination rate (%)	Contaminated	Positive	Negative	Total	S+/C- rate (%)*
Lianyungang	0-3	59	1545	268	1872	3.2	4	441	40	485	8.25
	3-7	33	676	165	874	3.8	1	201	22	224	9.82
	7+	0	4	2	6	0.0	0	2	1	3	33.33
	Total	92	2225	435	2752	3.3	5	644	63	712	8.85
Kaifeng	0-3	26	1813	180	2019	1.3	3	602	59	664	8.89
	3-7	8	446	89	543	1.5	0	44	10	54	18.52
	7+	0	0	0	0	0.0	0	0	0	0	0.00
	Total	34	2259	269	2562	1.3	3	646	69	718	9.61
Yongchuan	0-3	27	953	36	1016	2.7	2	405	15	422	3.55
	3-7	17	898	43	958	1.8	0	116	3	119	2.52
	7+	0	51	1	52	0.0	0	11	0	11	0.00
	Total	44	1902	80	2026	2.2	2	532	18	552	3.26
Total	0-3	112	4311	484	4907	2.28	9	1448	114	1571	7.26
	3-7	58	2020	297	2375	2.44	1	361	35	397	8.82
	7+	0	55	3	58	0.00	0	13	1	14	7.14
	Total	170	6386	784	7340	2.32#	10	1822	150	1982	7.57#

Note. *Smear positive/Culture negative. #Differences were not statistically significant at the P=0.05 level.

Table 2. Distribution of Invalid Genechip Results According to Different Transportation Intervals

Study Area	Interval (days)	Invalid Result			Total Specimens for Genechip	Invalidity Rate of Result (%)
		Failure to interpretation§	no mycobacterium	Total		
Lianyungang	0-3	1	5	6	103	5.83
	3-7	3	9	14	298	4.70
	7+	19	28	47	311	15.11
	Total	23	42	65	712	9.13
Kaifeng	0-3	0	5	5	166	3.01
	3-7	1	9	10	380	2.63
	7+	3	17	20	172	11.63
	Total	4	31	35	718	4.87
Yongchuan	0-3	0	1	1	62	1.61
	3-7	5	2	7	308	2.27
	7+	6	10	16	182	8.79
	Total	11	13	24	552	4.35
Total	0-3	1	11	12	331	3.63
	3-7	9	20	31	986	3.14*
	7+	28	55	83	665	12.48
	Total	38	86	124	1982	6.26

Note. *χ^2=0.182, P=0.670 (0-3 group *vs.* 3-7 group); χ^2=20.087, P<0.001 (0-3 group *vs.* 7+ group); χ^2=53.864, P<0.001 (3-7 group *vs.* 7+ group). §Failure to interpretation represented the genechip result with unsuccessful output at rifampicin or/and isoniazid matrix of genechip. No mycobacterium represented that both the rifampicin and isoniazid matrixes showed that no mycobacterium was detected from the specimen.

Although the quality control for solid culture is acceptable in the three prefectures where this study was conducted, our data also revealed that the S+/C- rate of Yongchuan was significantly lower than the other two pilots. The S+/C- rate of mycobacterial culture is considered an indicator of the proficiency of laboratory staff and the quality of equipment in the laboratory. Among the three pilot study areas, the laboratory of Yongchuan belongs to a comprehensive, class AAA hospital with better laboratory conditions and more highly trained laboratory staff, so it is understandable that the quality control of Yongchuan is the best of the three. In addition, we observed that the S+/C- rate of 7+ group in Lianyuangang seemed higher than those of the other two groups, while the differences were not statistically significant due to the low number of sputum samples in this group.

As described previously, the contamination rate for cultures that had delays of more than 7 days is usually higher than that with 3-7 days' delay[16]. In contrast, we observed there was zero contamination for the 56 cultures that had delays of more than 7 d. One possible explanation is that the sample size of cultures with more than 7 d delays is small (56 cultures) when compared with that of other groups (more than 2000 samples), so the contamination rate for the more than 7 days' group may not be representative for this storage time.

PCR-based amplification assay is often performed with specimens which have been stored for an extended period[17]. Several previous literatures have demonstrated that viral DNA remains quantitatively stable over at least 6 months when it is stored as unextracted DNA in a whole cerebrospinal fluid specimen frozen at -20 °C[17-18]. In contrast, we found that the >7 d storage of sputum at 4 °C would increase the invalid rate of genechip significantly. One possible explanation may be that the higher storage temperature of sputum samples will produce adverse impact on the quality of DNA. In addition, the complex components of sputum, including mucoprotein and epithelial cell, may also reduce DNA stability of *M. tuberculosis* strains and increase the amplification inhibitor of purified DNA in the clinical specimen. In a recent study by Guio and colleagues, *M. tuberculosis* stability spotted on an FTA® cards at room temperature was up to 6 months, which provides a alternative method for the collection, storage and transport of TB specimens for later molecular testing[6].

In addition, the laboratory's capacity to carry out specimen processing is another important issue that may affect the feasibility of sputum transport. In the present study, staffs in the prefectural laboratory are able to perform the solid culture for a maximum of 20 specimens per day, when considering their routine work and the sputum specimens from the counties. The specimens from our study did not exceed this level, so laboratory staffs were not overloaded. Although this analysis of workload is suitable for the most prefectures of China, separate analyses should be considered for prefectures with larger populations.

We also realized that this study had several obvious limitations. First, we did not compare the culture and genechip results on the same specimens. The variability of specimens, including quality, quantity, and bacterial load may play an important role on the detection effect of both culture and genechip. If each specimen or pooled specimens were aliquoted and tested at varying intervals of storage, this limitation would be eliminated. Second, the cost of specimen transportation is another important issue affecting the feasibility of sputum transportation system, which was not analyzed in the present study. Hence, further research will be carried out to evaluate the cost-effectiveness of sputum transportation in the tuberculosis control programmes.

Based on our experience in this study, three points must be followed to ensure the quality of sputum specimens that are stored and transported. First, a refrigerator should be available in the county-level laboratory, and routine temperature recording must be performed to ensure that the refrigerator is working well. Second, an approved insulated transport case must be provided to keep the specimens at low temperatures during transport. Finally, the frequency of transport services must be adequate, at least once a week.

There are several benefits from using a storage and transport approach for sputum culturing. Transporting specimens rather than allowing smear-positive patients to travel from county to prefecture by public transportation will reduce the possibility of TB transmission in the community. Another benefit is that smear-positive patients will obtain more detailed and rapid diagnostic results from prefecture laboratories compared with those from the county, and this helps to generate an effective anti-TB chemotherapy regimen for the patient.

In summary, sputum specimens were stored for up to 7 days at 4 °C before performing mycobact-

erial culture without affecting culture and molecular diagnostic results significantly. In addition, our data demonstrated that the quality of specimens would be diminished for molecular tests after prolonged refrigerated storage. Access to refrigerators in county laboratories, transport at low temperature, and frequent transport services (i.e., once a week) can ensure the feasibility of a sputum storage and transport system in China.

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