

Transportation of sputum samples in cetylpyridinium chloride for drug resistance studies from remote areas of Odisha, India

Dasarathi Das, Bhagirathi Dwibedi, Shantanu Kumar Kar

Regional Medical Research Centre, Bhubaneswar, Odisha, India

Abstract

Introduction: Antimicrobial susceptibility testing of *Mycobacterium tuberculosis* is required for successful treatment of patients, mainly in retreatment cases which necessitate isolation of mycobacteria from sputum samples within 24-48 hours. In situations where transportation of sputum is required, the use of cetylpyridinium chloride (CPC) effectively sustains the viability of mycobacteria up to two weeks.

Methodology: Sputum samples were collected from pulmonary TB patients attending designated microscopy centres (DMC), stored in CPC solution and transported to a culture drug susceptibility testing laboratory using overnight bus transport facilities. For culture, the sputum specimens were processed and inoculated in Lowenstein- Jensen (LJ) medium. Growth on LJ was identified by colony morphology, growth rate and biochemical tests, and transit time was calculated as the time taken from the date of sample collection to the inoculation date.

Results: Out of the 816 sputum samples collected in CPC, 691 (84.7%) yielded *M. tuberculosis*, 97 (11.9%) yielded no growth, 21 (2.6%) grew contaminants and 7 (0.8%) were nontuberculous mycobacteria. CPC containing sputum samples processed within two weeks showed 88.6% culture positivity, while positivity was significantly affected beyond two weeks.

Conclusions: CPC is cheap, easy to use, inhibits the growth of other organisms and can effectively be used to transport sputum specimens within two weeks from hard to reach areas to central locations without compromising culture positivity. Bus transport services can also help in reducing delay and the cost of transportation from remote areas.

Key words: CPC; Sputum transportation; remote areas.

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Introduction

Tuberculosis is one of the major public health problems in many remote areas of low and middle income countries of the world. In such countries this situation is further challenged by the emergence of multi drug resistant (MDR) and extensively drug resistant (XDR) strains. For successful treatment of patients infected with tuberculosis and aiming at generating area-specific drug resistance patterns, drug susceptibility testing is being carried out in many remote parts of the globe, both in rural and urban areas. The processes involve transporting sputum samples for culture to specialized laboratories usually located in major towns. Studies have shown that, sputum specimens kept at room temperature beyond 3 days without preservatives, suffer a significantly low yield of isolation of *Mycobacterium tuberculosis* [1].

Only one study on drug resistance which used cetylpyridinium chloride (CPC) as a transport medium [2] has been previously conducted in the Mayurbhanj

district, state of Odisha (India), that has hilly and forest covered areas similar to the area of Rayagada.

In India, CPC has been found to provide a good transport medium, and sample processing within a week is therefore strongly recommended [3-5]. Logistic problems related to transportation of sputum specimens from remote, hilly, forest areas have often contributed to delay and hence exposed *M. tuberculosis* culture specimens to the risk of being stored for a long period of time in CPC.

Methodology

This study was included into a cross-sectional study undertaken in collaboration with the state and district TB department belonging to the government of Odisha, between June 2011 and May 2013, for assessment of anti-TB drug resistance in Rayagada district. The district is located between 19°0' and 19°58' north latitude and 82°5' and 84°2' east longitude in the southern part of Odisha, with 43% of the region covered by forests. According to the 2001

census, the district has a population of 830 thousand, living in 2,667 rural villages and five urban/towns. Out of the 20 designated microscopy centres (DMC), five are placed in the most important towns. Except few areas, communication by public transport to the district interior is not frequent and in some areas it is scantily available and extremely complicated in rainy seasons.

Sputum samples used for this study were collected from pulmonary TB patients attending 18 out of 20 DMC's, as the number of patients enrolled in two DMC's was very low (less than 30 per year). The DMCs were supplied with freshly prepared 1% CPC in 2% Sodium chloride solution and replaced fortnightly with fresh provisions. Sputum samples were collected in sterile 50 mL Falcon tubes to which equal volumes of CPC were added, tightly capped, sealed, labeled and kept at room temperature in the DMC by the Revised National Tuberculosis Control Programme (RNTCP) laboratory technician. The sputum samples were usually collected within a week by the project staff posted at the Regional Medical Research Centre Field Unit (RMRC-FU) at the Rayagada district headquarter hospital (DHH), following a phone communication from the respective DMC laboratory technician. Samples were transported by the project staff from the DMC's to RMRC-FU by using the public transport system. At the RMRC-FU the sputum samples collected from various DMC's throughout the week were packed (triple packing) and transported weekly to Bhubaneswar city using an overnight public transport facility, preferably on Monday or Tuesday. Specimens were delivered by a person in charge from the bus service and processed on the same day at the Revised National Tuberculosis Control Programme approved Tuberculosis Culture and Drug Susceptibility Testing Laboratory of RMRC in Bhubaneswar, based on the standard method [3].

For culture, the specimens were centrifuged at

3000 × g for 15 minutes; sediment was re-suspended again in about 40-45 ml sterile distilled water and centrifuged at 3000 × g for 15 minutes. Sediment was resuspended in 1-2 ml of sterile distilled water and a loopful (10ul) was inoculated in Lowenstein- Jensen (LJ) medium. The medium bottles were incubated at 37°C in a walk-in-incubator and were examined for growth of *M. tuberculosis* once per week, up to 8 weeks. The growth of *M. tuberculosis* complex was graded as follows: actual number of colonies if between 1 and 19 colonies; 1+ for growth of > 20 but < 100 colonies; 2+ for growth of > 100 colonies; and 3+ for confluent growth. Growth on LJ was identified by colony morphology, growth rate and biochemical tests, including niacin production, catalase activity at 68°C and susceptibility to p-nitrobenzoic acid. The transit time was calculated as the time taken from the date of sample collection to the inoculation date. Written informed consent was obtained from all patients. The Ethical Committee of RMRC, Bhubaneswar approved the study protocol.

Results

Out of 816 sputum samples collected in CPC, 691 (84.7%) yielded *M. tuberculosis*, 97 (11.9%) yielded no growth, 21 (2.6%) grew contaminants and 7 (0.8%) were nontuberculous mycobacteria. It was observed that 43.0%, 69.1% and 84.3% of sputum samples were processed within the first, second and third week respectively (Table 1). About 30.9% of sputum specimens could not be processed within two weeks, as transport took a longer time. The isolation of positive culture was not statistically significant among sputum samples processed within 2 weeks. It was observed that beyond 2 weeks the culture positivity was significantly affected ($p < 0.05$) in comparison to samples processed up to two weeks, while there was no effect on contamination rate. Specimens processed beyond 3 weeks were considerably affected ($p <$

Table 1. Effect of CPC on isolation of *M. tuberculosis* in LJ medium.

Number of sputum samples(Processed within days)	Culture positive N (%)	Culture negative N (%)	Contamination N (%)	NTM N (%)
351 (1-7)	311 (88.6%)	34 (9.6%)	6 (1.7%)	0 (0%)
213 (8-14)	189 (88.7%)	14 (6.6%)	7 (3.3%)	3 (1.4%)
124 (15-21)	101 (81.4%)	16 (12.9%)	5 (4.0%)	2 (1.6%)
64 (22-28)	45 (70.3%)	16 (25.0%)	2 (3.1%)	1 (1.6%)
34 (29-35)	25 (73.5%)	8 (23.5%)	0 (0%)	1 (2.9%)
20 (36-42)	14 (70.0%)	5 (25%)	1 (5.0%)	0 (0%)
6 (43-49)	4 (66.7%)	2 (33.3%)	0 (0%)	0 (0%)
4 (50-56)	2 (50.0%)	2 (50.0%)	0 (0%)	0 (0%)
Total	691 (84.7%)	97 (11.9%)	21 (2.6%)	7 (0.8%)

CPC-Cetylpyridinium chloride, LJ- Lowenstein-Jensen, NTM- Non tuberculous mycobacteria

0.0001) in comparison to two weeks exposure to CPC. Smear grade was compared with culture result and specimens with negative or scanty smear grade yielded significantly more negative cultures ($p < 0.001$) than smears with 1+, 2+ or 3+ grades (Table 2). The growth of colonies in LJ medium was dependent on smear grade and significantly less confluent growth occurred in sputum samples incubated up to 8 weeks with negative or scanty acid fast bacilli (AFB).

Discussion

The primary requirement for a solid culture-based drug susceptibility testing is the need of a sputum sample free from contamination by other organisms than mycobacterium and its confluent growth in LJ media. We noted lower contamination rate ($< 5\%$) from samples processed up to 6 weeks (samples processed beyond 6 weeks were few in number) from collection. This may be due to quick addition of CPC to the sputa by the laboratory technician as the patients were already found positive by Ziehl Neelsen microscopy. Previous studies had shown that CPC kills pathogenic fungi from sputum specimens [6] and reduce the contamination rate in sputa cultured in solid media [3]. The earlier study in Odisha reported a contamination rate of 9.9% and attributed it to non-compliance of immediate CPC addition and transport delays [2]. In comparison to earlier studies, these results show an overall culture positivity of 84.7%. However, the samples tested within two weeks of storage in CPC showed 88.6% culture positivity. The culture positivity observed beyond three weeks was comparable and in agreement with earlier reports in which samples were processed after 20 days or longer [4]. While processing the sputum samples it was noted that single centrifugation as recommended [7,8] was not able to remove traces of CPC and it caused a deepening at the site of inoculation on LJ slants. We suggest a further wash with sterile distilled water for good colonies of *M. tuberculosis* on LJ slants, as reported by a previous study [9]. The culture

negativity was significantly higher ($p < 0.001$) in negative and scanty smears in comparison to smears with 1+ or more AFB grades. Out of 691 culture positives, 81.5% specimens showed confluent growth within eight weeks of incubation (Table 2). It was observed that a higher number of sputum specimens with 1+ or higher AFB grades showed confluent growth within eight weeks compared to scanty and negative smear specimens. During our study, sputum specimens were collected both from newly diagnosed and previously treated cases with anti TB treatment and not differentiated, as CPC has no effect on isolation of mycobacteria [10]. We used public bus services to transport pooled sputum samples from Rayagada district to RMRC, the Bhubaneswar laboratory situated at a distance of 500 kms and found that this is effective in reducing transportation costs, human resources requirements and delay in delivery, as previously reported for difficult areas [11].

Conclusion

The study showed that using public bus services to transport sputum from remote, hilly, forest and hard to reach areas located as far as 500 kms away from testing point was effective in reducing transportation costs, human resources requirements and minimized delays pertaining to delivery of specimen. In addition, CPC is inexpensive, easy to use, inhibits the growth of other organisms and can effectively be used to transport sputum specimens within two weeks from hard to reach areas to central locations, without compromising culture positivity. This may therefore be a possible alternative and an appropriate solution in most resource-constrained countries where long-distance transportation of sputum specimen for drug susceptibility testing is required.

Other low income countries, having geographical limitations such as rural location, remoteness, hilly and forest covered areas or hard to reach and/or underserved communities, may use the method described in this study to improve effectiveness of

Table 2. Comparison of smear grade with culture negativity and growth rate up to 8 weeks.

AFB Smear grade (Number of samples)	Culture negative N (%)	Confluent growth observed up to 8 weeks out of culture positives N (%)
Negative (28)	7 (25%)	7 (33.3%)
Scanty (46)	14 (30.4%)	23 (71.9%)
1+ (343)	41 (12%)	230 (79.6%)
2+ (217)	20 (9.2%)	162 (86.6%)
3+ (182)	15 (8.2%)	141 (87.0%)
Total	97 (11.9%)	563 (81.5%)

AFB-Acid fast bacilli, *AFB grade could not be evaluated for 9 samples

delivering sputum and at the same time to reduce risk of damage related to isolation of Mycobacteria. There may be the need to make CPC-sodium chloride solution always available in such areas so that the quality of sputum is ensured and the risk to jeopardize culture positivity reduced.

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Corresponding author

Shantanu Kumar Kar
Regional Medical Research Centre, Bhubaneswar-751023,
Odisha, India
Phone: 91-674-2301322
Fax: 91-674-2301351
Email: skk@icmr.org.in

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