JCM Accepts, published online ahead of print on 30 September 2009
J. Clin. Microbiol. doi:10.1128/JCM.01295-09

JCM01295-09 Version 2

Recovery of *M. tuberculosis* from sputum treated with Cetyl Pyridinium Chloride

Transportation of sputum samples for culture of Mycobacteria from remote areas to laboratories may take several days which can lead to overgrowth of commensal flora and possible loss of viable mycobacteria (1, 2, 5,8). To overcome this problem, Cetyl-Pyridinum chloride (CPC) is still the most commonly used preservative (5,8). The effect of CPC on mycobacterial culture isolation rate in BACETC MGIT-960® system has scarcely been reported from TB endemic tropical countries (6) where rate of environmental contamination of culture media is high.

We carried out a prospective study to evaluate the effect of CPC on the recovery of mycobacteria in BACTEC-MGIT-960 (Becton-Dickinson™, USA). Sixty patients whose sputa were positive for acid fast bacilli (AFB) were enrolled in the study. All the 60 patients were asked to bring fresh sputum samples on next morning and these freshly collected sputum samples were aliquoted into three equal parts (from all 60 patients), two aliquots were added with equal volume of 1% CPC and stored at $+4^0$C. The third aliquot was processed as such on the same day following standard protocol of smear microscopy and MGIT-960® culture with minor modifications (7). In brief, about 5-6 ml of sputum was mixed with an equal volume of 0.5% NALC – 4% NaOH mixture in 50 ml ridge capped round bottom processing tube, the mixture was vortexed and incubated at 37^0C for 10 min. After incubation, mixture was neutralized with phosphate buffer (pH 6.8) up to a total volume of 50 ml followed by centrifugation at 8000 X g for 10 min. The supernatant was decanted and the pellet was re-suspended in 2 ml of the phosphate buffer. From the decontaminated sample, 0.5 ml of the suspension was inoculated into the BACTEC™ MGIT tubes and 0.1 ml in L-J slants under sterile conditions in the biosafety cabinet type 2 (Kartos, India) and from rest of the sample, smears were prepared for the ZN staining. On the next day of storage, the contents of one aliquot having CPC were washed with phosphate buffer (PB, pH 6.8); centrifuged at 8000×g for 10 min, and the pellet was re-suspended in 1 ml PB for smear and culture. The third aliquot was also taken out from refrigerator and processed as above but without washing with buffer. Both pellets were dissolved in 2 ml of phosphate buffer (pH 6.8) and used for smear and culture as described above. Smears were interpreted using standard criteria for AFB grading. The days taken to give positive or negative signal in MGIT-960® system were recorded. Irrespective of positive or negative signal contents of

all MGI tubes were checked for acid fast bacilli by Z-N staining, after the BACTEC-MGIT-960 system gave positive signal or 42 days of incubation. To check the viability of mycobacterial growth 100 µl culture medium was aspirated out from the MGI tubes and 50 µl yellow MTT {(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide,(Sigma, St Lucia, USA)} was added to it. If the mycobacteria are alive the MTT is reduced to formazan changing the colour of culture medium to purple. For comparison findings of CPC un-added aliquot, which was processed on the same day, were taken as standard. Growths of mycobacteria were identified by using standard protocol in our laboratory, published elsewhere (4).

Results of AFB microscopy are provided in Table 1. Out of 60 samples collected from 60 known pulmonary tuberculosis treatment naïve patients and divided into 180 aliquots (3 each), 59/60 (98.3%) CPC un-added samples, 53/60 (88.8%) CPC washed and 50/60 (83.3%) CPC unwashed samples gave positive signal in MGIT-960® within 42 days of incubation (3). The difference in the recovery rate between samples with CPC un-added and added (irrespective of washing) was statistically significant (Table 1). Surprisingly, on Z-N staining of culture tubes with negative signal, 70% (7/10) CPC unwashed and 71.4% (5/7) CPC washed samples were positive for acid fast bacilli (table 1). On addition of MTT in the MGI Tubes, contents of all the above 17 tubes changed to purple colour indicating presence of viable mycobacteria. Our results show that addition of CPC significantly delayed the mycobacterial detection time in BACTEC-MGIT-960® system ranging from 6-11 days as shown in figure (1). This delay could significantly be reduced by washing the CPC added sample with phosphate buffer.

Even though CPC still remains the first choice to preserve the viability of mycobacteria and simultaneously minimize the overgrowth of bacterial and fungal flora, this study clearly shows a negative effect of CPC in the BACTEC-MGIT-960® system. We found that probably mainly affects the levels of fluorescence thereby decreasing the mycobacterial detection rate and increased duration for mycobacterial growth readings in the system. However, most important observation of the study was false negative signals given by the system despite having mycobacterial growth in the medium. Findings of acid fast bacilli in a high (70%) number of signal negative culture tubes is alarming. Recently introduced TB control programs recommend use of BACTEC-MGIIT-960 for regional laboratories which cater services to various primary health care laboratories. Our results

indicate that in these programs it should clearly be highlight that even if the samples need to transported to regional laboratories, use of CPC should be discouraged .Nevertheless our study also shows that, if unavoidable, the detrimental effect of CPC can be minimized by washing the samples with phosphate buffer. We also recommend that all BACTEC-MGIT-960® tubes with negative signal must be rechecked for the presence of acid false bacilli before releasing culture negative reports.

We acknowledge Mr. Balbir Singh, Mr. Mahesh Paliwal for their technical help and we thank Department of Biotechnology, Govt. of India for funding (BT/ PR7761/ MED/ 14/1105/2006) this work.

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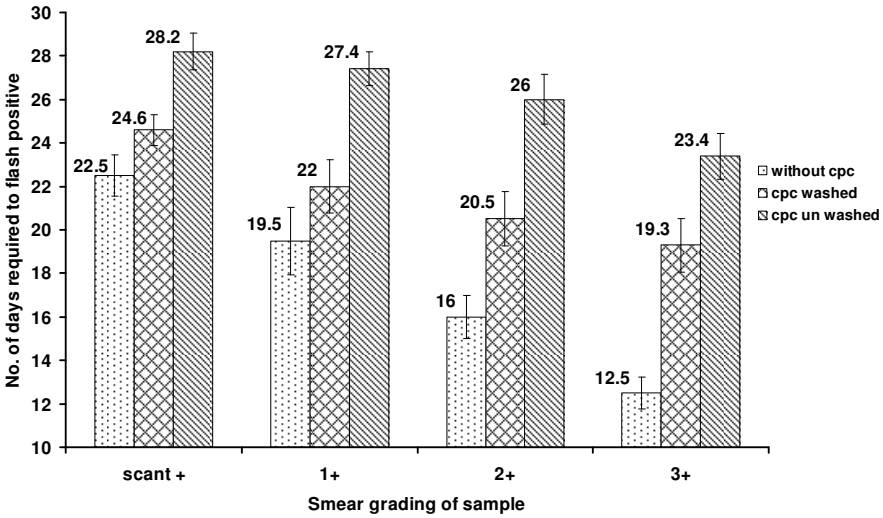
Legends for Figure 1.

Comparison of time taken to flash BACTEC- MGIT-960 ® culture positive and load of acid-fast bacilli in sputum samples.



Smear grade: 3 + (>10 AFB /field in at least 20 fields)

Smear grade: 2 + (1-9 AFB /field in at least 50 fields)

Smear Grade: 1 + 10 – 99 AFB in 100 fields

Smear Grade Scanty: <10 AFB in 100 fields

Smear Negative: no AFB seen in entire smear

Legends for Table 1.

Table showing Mycobacterial detection rate in BACTEC-MGIT-960 ® system and the rate of false negative signals.

Sample Processing protocol (n=60)	Flashed Positive	Flashed Negative	Smear Negative	False Negative BACTEC-MGIT culture
1. Without CPC (n=60)	59	1	1	0/1 (0%)
2. CPC Washed (n=60)	53	7	2	5 /7 (71.4%)
3. CPC Unwashed (n=60)	50	10	3	7 /10 (70.0%)

P value: a & b = <0.0001; a & c = <0.0001; b & c = >0.5

Each of the 60 samples was divided into 3 parts and one aliquot had no CPC, another had CPC but washed while the third aliquot had CPC and was processed without washing it. The Bactec-MGIT-960 tube was labeled as false negative when it gave negative signal but on smear examination it contained mycobacteria.