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Cetyl-Pyridinium Chloride Is Useful for Isolation of *Mycobacterium tuberculosis* from Sputa Subjected to Long-Term Storage

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Recovery of *Mycobacterium tuberculosis* from sputa treated with cetyl-pyridinium chloride (CPC) and stored for 20 ± 9 days was significantly higher than that from sputa that were untreated and processed by the *N*-acetyl-L-cisteine–NaOH method. Addition of CPC is useful for isolation of *M. tuberculosis* from sputa subjected to long-term storage received from remote areas of the world.

Tuberculosis (TB) remains one of the major public health problems worldwide, with 95% of cases and 98% of deaths occurring in developing countries (9). The transportation of sputa for culture for *Mycobacterium tuberculosis* from these countries to laboratories located abroad usually takes more than one week and results in an increased contamination rate and a loss of positive cultures. This is due to several factors, including inadequacy of refrigeration and a lack of preservative addition.

In previous studies, cetyl-pyridinium chloride (CPC), a substance known to kill pathogenic fungi from sputum specimens (4), was shown to increase the culture positivity and reduce the contamination rate (1, 6–7) in sputa cultured on solid media after 8 days (6–7) or 2 weeks (1, 8) from collection, in comparison with the *N*-acetyl-L-cisteine–NaOH (NALC) decontamination method (3). An advantage of the CPC method is that specimens can be decontaminated during mail shipment when processing is to be delayed. In this study we examined the effect of CPC on the recovery of *M. tuberculosis* from sputa subjected to long-term storage in comparison with CPC-untreated sputa collected from the same patients and processed by the NALC method. Such a long period between sputum collection and culture may occur in places where transportation from the collection site is subject to heavy logistic constraints and samples are gathered to limit the cost of shipment.

A total of 276 sputum samples were collected at Guliripchi Tuberculosis Hospital, Sukhumi, Abkhazia, a region of the former Soviet Union along the Black Sea coast, from March 2003 to November 2003. For each patient, two sputum samples were collected on two consecutive days, but to only one was added an approximately equal volume of a CPC (Sigma, St. Louis, Mo.) solution (final concentration [f.c.]: CPC, 0.5%; NaCl, 1%;) (2). At Guliripchi Hospital, smear microscopy by the Ziehl-Neelsen method (2, 3) was performed on all unconcentrated specimens. CPC was added randomly to either the first or second specimen from the same patient. Sputa were kept refrigerated at +4°C and mailed by a courier in groups of 20 to 30 sputa per parcel to the Istituto Superiore di Sanità, Rome, for culture; overall, the mean time between collection and sample processing was 20 ± 9 days (range, 7 to 36 days). CPC-treated sputa (approximately 3 ml of sputum and 3 ml of CPC) were added with Bacto neutralizing buffer (Difco, Detroit, Mich.) to inactivate residual CPC (2) up to a 30-ml volume in a 50-ml Falcon tube and centrifuged at 3,000 × *g* for 30 min at room temperature, and the sediment was inoculated in Lowenstein-Jensen (LJ) slants (100 μl) (Biomérieux, Marcy l'Etoile, France) and Middlebrook 7H10 (7H10) (Difco) plates (100 μl). The non-CPC-treated sputa were processed by the NALC method, using a commercial kit (MycoPrep; Becton Dickinson, Cockeysville, Md.) (1% NaOH, f.c.), and the sediment was suspended in phosphate-buffered saline and inoculated in LJ and 7H10 media. An aliquot of each sediment was frozen at −80°C; if the first cultures of CPC- and/or NALC-treated sputa were contaminated, the frozen aliquot was thawed and submitted to a retreatment with NaOH alone (2%, f.c.) for 15 min at room temperature, diluted with phosphate-buffered saline, and centrifuged, and the sediments (100 μl) were plated on LJ and 7H10 media. The LJ and 7H10 media were incubated at 37°C in 5% CO_2 and examined weekly for 8 weeks. *M. tuberculosis* was identified in positive cultures by a DNA-RNA hybridization (Accuprobe; Gene Probe, San Diego, Calif.) (3).

Treatment of the sputa with CPC significantly increased the rate of positive *M. tuberculosis* cultures (from 47.1 to 63.8% for NALC and CPC, respectively; $P < 0.05$, Fisher's exact test) and decreased the contamination rate (from 42.0 to 21.0% for NALC and CPC, respectively; $P < 0.01$); the negative cultures were 10.9 and 15.2% for NALC and CPC, respectively. After retreatment of the contaminated sputa with NaOH, overall positivity (positivity after the first culture plus positivity after retreatment) of CPC-treated samples was significantly higher than that for samples processed with the NALC method (78.3 and 65.9%, respectively; $P < 0.05$); the contamination rate was

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TABLE 1. Comparison of smear and overall culture results

Smear score	Culture result[a]							
	No. (%) positive		No. (%) negative		No. (%) contaminated		Total no. of specimens	
	CPC	NALC	CPC	NALC	CPC	NALC	CPC	NALC
− or doubtful	14 (48.3)*	6 (19.3)*	14 (48.3)	22 (71)	1 (3.4)	3 (9.7)	29	31
1+	20 (71.4)	12 (54.5)	7 (25)	9 (40.9)	1 (3.6)	1 (4.5)	28	22
2+	16 (72.7)	22 (68.8)	6 (27.3)	10 (31.3)			22	32
3+	22 (95.7)	16 (94.1)		1 (5.9)	1 (4.3)		23	17
4+	36 (100)	35 (97.2)				1 (2.8)	36	36
Total							138	138

[a] *, $P < 0.05$, Fisher's exact text.

very low (2.2 and 3.6%, respectively), and the rates of negative cultures were 30.4 and 19.5% for CPC and NALC, respectively. These observations indicated that the yield of positive cultures in sputa subjected to long-term storage is higher in samples collected with CPC than in those collected without the addition of this substance; these results are in keeping with those of previous reports (1, 6–8) but extend them to a time period longer than those previously described.

To evaluate the effect of CPC and NALC methods on *M. tuberculosis* viability, smear scores and overall culture results obtained after the second decontamination steps were compared (Table 1). The data showed that while 4+, 3+, 2+, or 1+ smear-positive CPC- or NALC-processed sputa did not significantly differ in culture positivity, for those reported as negative or doubtful, which should contain fewer than 5,000 to 10,000 organisms per ml (3), culture positivity was significantly higher for CPC-treated (48.3%) than for NALC-treated (19.3%) sputa ($P < 0.05$, Fisher's exact text). To verify whether the quality of the specimen could affect the sensitivity of the method, a statistical analysis was performed including only the patients from which sputa with identical smear results were obtained; also, in this case, culture positivity of smear-negative (or doubtful) sputa collected with CPC was statistically higher than that of sputa collected without CPC ($P = 0.03$, McNemar binomial test for paired samples). These observations indicated that in samples containing relatively low numbers of mycobacterial cells, CPC preserves *M. tuberculosis* viability to an extent significantly higher than that with NALC. From the clinical point of view, this is very important for diagnosis and treatment adaptation, because smear-negative patients have a 2.5-times-higher chance of having a positive culture result if sputa are collected with CPC (95% confidence interval, 1.1 to 5.6; Epi Info 6.04 [Centers for Disease Control, Atlanta, Ga.]). Also, in 1+ to 4+ smear-positive, culture-positive samples, CPC-treated sputa had higher positivity rates than those with NALC, even if the differences did not reach a significant level. Among 1+, 2+ and 3+ smear-positive samples, 26% in the CPC group (13 out of 50) and 28.2% in the NALC group (20 out of 71) were culture negative; of these, 77.9% (10 out of 13) and 55% (11 out of 20) in the CPC and NALC groups, respectively, were collected from patients on anti-TB treatment, who are known to harbor nonviable *M. tuberculosis* bacilli**;** these percentages are in agreement with those reported during follow-up of TB treatment (5). A total of eight patients on anti-TB treatment overlapped among the culture-negative samples from the CPC and NALC groups. Nonviable *M. tuberculosis* cells or CPC-susceptible mycobacteria other than *M. tuberculosis* organisms were probably present in the remaining (3 out of 13) smear-positive, culture-negative sputa treated with CPC.

Overall, our observations indicated that CPC is a satisfactory preservative for mailing sputa for TB diagnostics because it decontaminates specimens collected in remote areas of the world and increases the yield of positive cultures after 20 days or longer.

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