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Reduced detection by Ziehl-Neelsen method of acid-fast bacilli in sputum samples preserved in cetylpyridinium chloride solution

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SUMMARY

SETTING: Twelve health facilities implementing the DOTS strategy, and the Tuberculosis Research Centre (TRC), Chennai, India.

OBJECTIVE: To determine the detection rates using Ziehl-Neelsen (ZN) and auramine-phenol to stain acid-fast bacilli (AFB) in sputum samples stored in cetylpyridinium chloride (CPC) solution.

METHODS: Two smears were prepared from each of 988 sputum samples collected in CPC and randomly allocated, one to ZN and the other to auramine-phenol staining. All samples were processed for culture of *Mycobacterium tuberculosis*.

RESULTS: A significantly higher proportion of samples were negative using the ZN method compared to the auramine-phenol method (74.5% vs. 61.8%, McNamara's paired χ^2 test; $P < 0.001$). Among 377 samples that were positive using auramine-phenol, 44% were negative using ZN. There were more culture-positive, smear-negative samples in ZN (52.7%) than in auramine-phenol (30%); the difference attained statistical significance (McNemar's paired χ^2 test; $P < 0.00004$). Using ZN, of the 104 smears made immediately after collection, 52 were positive for AFB, of which only 35 (67.3%) were positive after storage in CPC; the reduction in the number of positive smears attained statistical significance (McNemar's paired χ^2 test; $P = 0.004$).

CONCLUSION: Detection of AFB in sputum samples preserved in CPC is significantly reduced using ZN staining.

KEY WORDS: *Mycobacterium tuberculosis*; AFB; Ziehl-Neelsen; cetylpyridinium chloride

TUBERCULOSIS is a major public health problem in many developing countries, and the World Health Organization (WHO) declared it a global emergency.[1] The DOTS strategy is being implemented throughout the world as an effective strategy to control tuberculosis. To understand the impact of the DOTS strategy in the community in terms of the epidemiology and emergence of drug resistance, isolation of *Mycobacterium tuberculosis* by culture is necessary. In many settings, sputum samples cannot be transported to central laboratories within 3 days for culture, and delays beyond 3 days may result in reduced isolation rates.[2] Therefore, sputum samples need to be collected in a preservative and stored in ambient conditions until transport to central laboratories for culture. As storage in preservative solution results in liquefaction of sputum samples, the rate of detection of acid-fast bacilli (AFB) in smears from such samples could be reduced. There is no documented evidence on the rates of detection of AFB using either the Ziehl-Neelsen (ZN) or the auramine-phenol method of staining in sputum samples preserved in cetylpyridinium chloride (CPC) solution. This study determines the rates of detection of AFB using the ZN and auramine-phenol methods in sputum samples preserved in CPC.

MATERIAL AND METHODS

Sputum samples

A total of 988 sputum samples were collected from pulmonary tuberculosis patients assessed and admitted to receive India's Revised National Tuberculosis Control Programme (RNTCP) regimens in 12 health facilities implementing the DOTS strategy. The patients were instructed to collect sputum in McCartney bottles containing 5 ml of CPC solution.

1% CPC and 2% sodium chloride solution

The stock solution was prepared by dissolving 1 g CPC (Suvidhinath Laboratories, Baroda, India) and

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2 g sodium chloride (Qualigens, Mumbai, India) in 100 ml of distilled water and autoclaved at 121°C for 15 min. The solution was distributed in 5 ml quantities in sterile McCartney bottles. The bottles were put in cardboard containers and distributed to the health units, where they were kept in ambient conditions for 2–7 days before the samples were collected. The sputum samples were stored in the same conditions for 3–7 days after collection and before transportation to TRC.

Direct smears and staining

Two direct smears were prepared from each of the samples and randomly allocated, one to ZN[3] and the other to auramine-phenol[4,5] for staining, and were examined using an oil immersion lens in an Olympus binocular microscope (Model CH 30, Olympus, Tokyo, Japan) and a 40× objective lens in fluorescence microscope (Olympus BX 40, Japan), respectively.

Preparation of reagents for auramine-phenol staining

Auramine-phenol solution (0.3%) was prepared by dissolving 3 g auramine (New Tech Ltd, Brixworth, Northampton, UK) and 350 ml of ethanol. The contents were made up to 1000 ml by adding 30 g phenol and 650 ml of distilled water; 1% acid-alcohol was prepared by adding 20 ml of concentrated hydrochloric acid to 500 ml of distilled water containing 20 g sodium chloride. The volume was made up to 2000 ml by the addition of ethanol; 0.1% potassium permanganate was prepared by dissolving 1 g potassium permanganate (Rainbow Lab, Chennai, India) in 1000 ml of distilled water.

Blinding of smears for reading

The same technician examined the coded auramine-phenol and ZN smears. A senior technician cross checked all the positive and 20% of the negative smears in both of the methods to ensure correct reading. Discrepant smear results were resolved by a referee; the referee's reading was taken as final. The results were decoded and matched for comparison.

Isolation of M. tuberculosis

The samples were processed for culture using the modified procedure described earlier.[6]

Comparison of sputum smears prepared immediately after collection and 7 days after storage with CPC

In another, separate experiment, 104 sputum samples were collected from patients with symptoms suggestive of pulmonary tuberculosis. Direct smears were prepared immediately and stained by ZN. CPC was added to these samples and stored in ambient conditions for 7 days, after which smears were made and stained by ZN. The smears were coded and examined. The results of smears obtained from CPC preserved samples were compared with those of the direct smears.

Table 1 Comparison of the results of Ziehl-Neelsen and auramine-phenol stained smears of sputum samples collected in CPC solution

Auramine-phenol method†	Ziehl-Neelsen method*						
	1+	2+	3+	SC	Any positive	Negative	Total
1+	58	19	7	57	141	154	295
2+	20	23	10	5	58	11	69
3+	5	1	5	1	12	1	13
Any positive	83	43	22	63	211	166	377
Negative	15	3		21	39	572	611
Total	98	46	22	84	250	738	988

* Negative = no AFB in 100 fields; scanty = 1–9 AFB in 100 fields; 1+ = 10–99 AFB in 100 fields; 2+ = 1 to 9 AFB per field in at least 50 fields; 3+ = more than 10 AFB per field in at least 20 fields

† Negative = no AFB in 100 fields; 1+ = less than 5 AFB per field (a minimum of 4 typical AFB should be seen to grade a smear as 1+); 2+ = 5 and above but less than 100 AFB per field in at least 50 fields; 3+ = more than 100 AFB per field in the majority of the fields.

CPC = cetylpyridinium chloride; SC = scanty; AFB = acid-fast bacilli.

RESULTS

A comparison of the results using the ZN and auramine-phenol methods is shown in Table 1. It is evident that a significantly higher proportion of sputum samples were negative by ZN method compared to auramine-phenol method (74.5%; 738/988 vs. 61.8%; 611/988, McNemar's paired χ^2 test; $P < 0.001$). Among 377 samples that were positive using auramine-phenol, 166 (44%) were negative using ZN.

A comparison of the results of ZN and auramine-phenol stained smears with culture results is given in Table 2. Of 471 specimens that were positive on culture, respectively 248 (52.7%) and 142 (30%) were negative by ZN and auramine-phenol. The difference observed between the two staining methods in culture-positive, smear-negative samples attained statistical significance (McNemar's paired χ^2 test; $P < 0.00004$).

A comparison of the results of 104 ZN smears prepared immediately and after 7 days of storage with CPC is shown in Table 3. Of the 52 specimens that were positive for AFB using ZN staining immediately after collection, only 35 (67.3%) were positive after storage with CPC using the same method; the reduction in the number of positive smears attained statistical significance (McNemar's paired χ^2 test; $P = 0.004$).

DISCUSSION

Sputum AFB smear microscopy, a very simple, inexpensive and rapid method, is the key tool in the diagnosis of pulmonary tuberculosis in many disease burdened countries. Under ideal conditions, as many as 80–85% of culture-proven pulmonary tuberculosis patients can be diagnosed by the examination of two

Table 2 Comparison of the results of Ziehl-Neelsen and auramine-phenol methods with culture results of sputum samples collected in CPC solution

		Culture results*							
	Colonies	1+	2+	3+	Any positive	Contaminated culture	UMB	Negative	Total
Ziehl-Neelsen method†									
1+	12	17	36	25	90	2		6	98
2+	4	9	20	12	45			1	46
3+		1	8	10	19	3			22
Scanty	12	20	30	7	69		2	13	84
Any positive	28	47	94	54	223	5	2	20	250
Negative	107	71	58	12	248	7	7	476	738
Auramine-phenol method†									
1+	48	72	104	27	251	6	4	34	295
2+	2	9	26	30	67	1		1	69
3+			3	8	11	2			13
Any positive	50	81	133	65	329	9	4	35	377
Negative	85	37	19	1	142	3	5	461	611
Total	135	118	152	66	471	12	9	496	988

* Negative = no growth; Colonies = 1–19 colonies; 1+ = 20–100 colonies; 2+ = innumerable colonies; 3+ = confluent growth.
† As given in Table 1.
CPC = cetylpyridinium chloride; UMB = unclassified mycobacteria.

sputum smears. However, the sensitivity of smear microscopy, which is extensively used in many developing countries with satisfactory results, varies from 30% to 80%, depending on various factors.[7] In the DOTS strategy, diagnosis, smear conversion rates at the end of the intensive phase of treatment and cure rates at the end of chemotherapy are determined based on sputum examinations. Therefore, any procedure or modification that would alter the sensitivity of the method needs to be carefully analysed before meaningful conclusions can be drawn.

It has been very well documented that sputum samples can be collected in CPC solution and transported to the central laboratories for culture of *M. tuberculosis*.[5,6,8–11] However, the rates of detection of AFB in sputum samples preserved in CPC solution by smear microscopy, using either the ZN or the auramine-phenol method, is not known. It is therefore imperative to know the sensitivity of these two methods for detecting AFB in sputum samples preserved in CPC. This baseline information will be useful for interpreting the value of ZN smear results obtained in studies involving transportation of sputum samples in CPC solution to the central laboratories.

Table 3 Comparison of Ziehl-Neelsen stained smears prepared immediately and after 7 days storage with CPC solution

7 day CPC-ZN smear	Direct Ziehl-Neelsen (ZN) smear						
	3+*	2+	1+	Scanty	Any positive	Negative	Total
3+	2	0	1	0	3	0	3
2+	3	3	0	0	6	0	6
1+	4	10	4	0	18	2	20
Scanty	3	2	1	2	8	1	9
Any positive	12	15	6	2	35	3	38
Negative	2	6	8	1	17	49	66
Total	14	21	14	3	52	52	104

* As shown in Table 1.
CPC = cetylpyridinium chloride.

The results of the present study reveal that the sensitivity of the ZN method in detecting AFB in sputum samples preserved in CPC is significantly lower than that of the auramine-phenol method, particularly in low-grade sputum samples. It was also observed that a significant proportion of smears made from CPC-preserved sputum samples were negative compared to corresponding smears made immediately after collection. These limitations should be taken into consideration in laboratories using ZN staining for samples collected in CPC solution.

The dilution and subsequent liquefaction of sputum in CPC solution could have caused reduced detection rates. The dilution factor could have varied by one to four times, depending on the volume of sputum collected in 5 ml of CPC solution. Another important limitation of the ZN method is the examination of smears under higher magnification. The area of smear examined using the 40× lens in fluorescence microscopy is approximately 6.25 times higher than when the 100× lens in a light microscope is used.[12] This difference in the area of smears covered in the two methods could have contributed to the reduced detection rate in ZN method. In order to compensate this difference, 200 more fields should be examined using light microscopes. Nevertheless, the recommended time and the number of fields to be examined using light microscopes are optimised under programme conditions.

Physical and chemical injury to the integrity of the cell wall is known to cause tubercle bacilli to lose their acid-fastness.[13] It is not known what the CPC does to affect the cell wall integrity of the AFB that

they become less stainable by the ZN method. Phillips and Kaplan reported that the morphology and the stain ability of pathogenic fungi and *Nocardia asteroides*, another member of *Mycobacteriaceae*, in sputum samples treated with CPC were unaffected by the fluorescent antibody method.[14] However, they also observed that the background material in smears made from CPC-treated samples retained the conjugate and that this made weakly fluorescing organisms more difficult to detect. It is also of interest to note that *N. asteroides* is not affected by treatment with CPC and that the morphology and stainability were not altered in the Kinyoun acid-fast method used to stain mycobacteria.[14]

In conclusion, using the ZN method, the rate of detection of AFB in sputum samples preserved in CPC solution was found to be reduced.

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RÉSUMÉ

CONTEXTE : Douze services de santé mettant en œuvre la stratégie DOTS et le Centre de Recherche de la Tuberculose (TRC) à Chennai, Inde.

OBJECTIF : Déterminer les taux de détection par les méthodes de Ziehl-Neelsen (ZN) et auramine-phénol pour la coloration des bacilles acido-résistants (BAAR) dans les échantillons d'expectoration conservés dans une solution de chlorure de cetylpyridium (CPC).

METHODES : On a préparé deux frottis à partir de chacun des 988 échantillons d'expectoration recueillis dans le CPC et les a attribués au hasard, l'un à la méthode de coloration de ZN et l'autre à l'auramine-phénol. Tous les échantillons ont été mis en culture pour *Mycobacterium tuberculosis*.

RÉSULTATS : Une proportion significativement plus élevée des échantillons a été négative à la méthode de ZN par comparaison avec la méthode à l'auramine-phénol (74,5% vs. 618% ; test χ^2 apparié de McNamara, $P <$ 0,001). Sur 377 échantillons positifs à la méthode auramine-phénol, 44% étaient négatifs au ZN. Les échantillons à bacilloscopie négative et à culture positive ont été plus fréquents après ZN (52,7%) qu'après l'auramine-phénol (30%) ; la différence est statistiquement significative (test χ^2 apparié de McNamara, $P <$ 0,00004). Utilisant ZN, de 104 frottis préparés immédiatement après collection, 52 étaient positifs en directe, dont seulement 35 (67,3%) l'étaient après conservation dans CPC ; la réduction dans le nombre de frottis positifs était statistiquement significative (test χ^2 apparié de McNamara, $P = 0{,}004$).

CONCLUSION : La détection des BAAR dans les échantillons d'expectoration conservés dans une solution de CPC est significativement plus diminuée utilisant la méthode ZN.

RESUMEN

CONTEXTO : Doce establecimientos de salud que aplican la estrategia de DOTS, y el Centro de Investigaciones en Tuberculosis (TRC), Chennai, India.

OBJETIVO : Determinar las tasa de detección por los métodos de tinción de Ziehl-Neelsen (ZN) y de auramina-fenol, para los bacilos ácido-alcohol resistentes (BAAR) en muestras de esputo conservadas en solución de cloruro de cetilpiridinium (CPC).

MÉTODOS : Se prepararon dos frotis a partir de cada una de las 988 muestras de esputo recolectadas en solución CPC y asignadas al azar, una al método de tinción ZN y la otra al método de auramina-fenol. Todas las muestras fueron procesadas para el cultivo de *Mycobacterium tuberculosis.*

RESULTADOS : Una proporción significativa de muestras era negativa con ZN, en comparación con el auramina-fenol (74,5% vs. 61,8% ; test de χ^2 pareado de McNamara, $P < 0{,}001$). De 377 muestras positivas con el método de auramina-fenol, el 44% de ellas eran negativas con el método ZN. El número de muestras con baciloscopia negativa y cultivo positivo era superior con ZN (52,7%) que con el método auramina-fenol (30%) ; la diferencia es estadísticamente significativa (test de χ^2 pareado de McNamara, $P < 0{,}00004$). De 104 frotis efectuados inmediatamente después de la recolección, 52 eran positivos para BAAR ; sólo 35 de ellos (67,3%) eran positivos después de conservación en solución CPC, utilizando el método ZN ; la reducción del número de frotis positivos era estadísticamente significativa (test de χ^2 pareado de McNamara, $P = 0{,}004$).

CONCLUSIÓN : La detección de BAAR en muestras de esputo conservadas en solución CPC se reduce significativamente cuando se utiliza el método ZN.