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A novel method of staining acid-fast bacilli in sputum containers

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***Background & objectives*: Making centrifuged deposit smears from sputum to detect acid-fast bacilli (AFB) is considered hazardous. We carried out this study to stain the centrifuged deposits with carbol-fuchsin in sputum containers and to decolourize and counterstain their smears made on glass slides.**

***Methods*: The centrifuged deposits of 180 sputum samples from pulmonary tuberculosis patients were used for making smears (initial deposit smears) and staining by Ziehl-Neelsen (ZN) method for the detection of AFB. Each of the sputum deposit was then treated with one ml of 1 per cent carbol-fuchsin and a smear made between 2 to 3 h was then decolourized and counterstained by the same procedures followed in ZN method (2 h stained deposit smear). The coded initial deposit smears and the corresponding 2 h stained deposit smears were read by the same readers and the results compared.**

***Results*: One hundred and fifty (70 positive and 80 negative) 2 h stained deposit smears were compared with initial deposit smears and the difference was not statistically significant.**

***Interpretation & conclusion*: Centrifuged deposits of sputum in sputum containers can be stained by carbol-fuchsin within 2-3 h and their smears made subsequently on glass slides can then be decolourized and counterstained by the procedures followed in ZN method for detection of AFB by light microscopy.**

Key words AFB - pulmonary tuberculosis - smears - sputum containers - ZN staining

For over a century the diagnosis of pulmonary tuberculosis has been confirmed by staining the acid-fast bacilli (AFB) in direct smears of sputum made on glass slides[1]. Good laboratory practices have to be followed while making direct smears of sputum on glass slides to avoid laboratory acquired tuberculosis infection[1]. Generally, making direct smears from sputum is considered hazardous for laboratory technicians working in developing countries with limited facilities[2]. In some high level laboratories, smears are made from the concentrated deposits after processing sputum samples for

culture. These deposit smears, in addition to generating aerosols, often get peeled off from the slides, resulting in false negative results[3]. In Ziehl-Neelsen (ZN) method, the carbol-fuchsin solution on the slide is to be heated until vapour rises and is allowed to stain the smear for 5 min[4]. In order to overcome these risks, a new technique both simple and non hazardous is needed to stain the AFB. The present study was to stain the AFB by carbol-fuchsin in centrifuged deposits of sputum in sputum containers, with subsequent decolourization and counterstaining of smears made on glass slides for examination under light microscope.

Material & Methods

Chemicals: Basic-fuchsin, methanol, phenol, concentrated hydrochloric acid, ethanol and methylene blue were obtained from Qualigens, Mumbai, India.

Preparation of staining reagents: Carbol-fuchsin (1%) was prepared by dissolving 5 g of basic-fuchsin in 50 ml methanol and 25 ml molten phenol and the solution was made up to 500 ml with distilled water. Acid-alcohol (1%) was prepared by adding 20 ml of concentrated hydrochloric acid to 500 ml of distilled water containing 20 g of sodium chloride. The volume was made up to 2000 ml by the addition of ethanol. Methylene blue (0.1%) was made by dissolving 0.5 g in 500 ml distilled water[1].

Sputum samples and their deposits: The sputum samples (n=180) were collected from pulmonary tuberculosis patients enrolled in the ongoing controlled clinical trials in Tuberculosis Research Centre (TRC), Chennai. These included samples for diagnosis and for follow up examination. The sputum samples were decontaminated and homogenized by the modified Petroff's method for culture of *M. tuberculosis*[5]. The sputum deposits obtained in 0.2 to 0.5 ml volume were used for making smears.

Initial deposit smears: One loop full (5 mm diameter wire loop) of the sputum deposit was used for making uniform smears (initial deposit smears). The smears were air-dried for 15 to 20 min in a Class 1 biological safety cabinets (MAT, Manchester) and fixed over a hot plate maintained at 80°C for 15 min.

Staining of initial deposit smears by ZN method: The initial deposit smears were stained by the standard ZN method[1]. In brief, sputum smears were flooded with filtered 1 per cent carbol-fuchsin and heated until it was steaming and left for five minutes. After rinsing the slides with a gentle stream of water, 1 per cent acid-alcohol was used to decolourize the smears for one minute. The slides were rinsed and counterstained with 0.1 per cent methylene blue for 30 sec. The slides were washed, air-dried and examined using oil immersion objective (100x).

Two hour stained deposit smears: After making the initial deposit smear, one ml of 1 per cent carbol-fuchsin was added to the remaining sputum deposit in each of the sputum containers (McCartney bottles/Universal containers) and kept at room temperature (22° to 26°C). From each sputum deposit, another smear was subsequently made as described above within 2 to 3 h (2 h stained deposit smear). The smears were then immediately heat fixed by passing over the flame 5 to 6 times.

Decolourization and counterstaining of 2 h stained deposit smears: The 2 h stained deposit smears were decolourized and counterstained by the same procedures followed in ZN staining method using the same reagents.

Reading smears: All the coded initial deposit smears and the corresponding 2 h stained deposit smears were read by the same readers (first readers) and compared.

Resolving discrepant results: If the results of two smears from a sample were same (AFB positive

or AFB negative), they were considered as smears with concordant results. If there was any disagreement in a set of two smears, both were read blindly by second reader and the smears with discrepant results were read blindly by an umpire. The results of umpire were considered as final for discrepant smears.

Statistical analysis: The data were analysed using Microsoft Excel. The statistical significance of the observed difference between the different types of smears was determined using McNemar's chi-square test. Kappa statistic was used to know the extent of agreement in smear grading between the smears. The results of initial deposit smears (after discrepancy resolving) were considered as gold standard to determine the sensitivity and specificity of 2 h stained deposit smears.

Results & Discussion

The results of 2 h stained deposit smears were comparable (kappa = 0.67) to initial deposit smears (Table I) and the difference observed was not statistically significant (P = 0.9). The comparison of smear results after resolving the discrepancies (Table II) revealed few errors (kappa = 0.89).

If the sputum deposits can be stained in the sputum container itself, this technique will have the following advantages to mycobacteriologists and medical technologists working in sputum AFB microscopy centres all over the world: (*i*) The process of making smears is less hazardous as the deposit treated with carbol-fuchsin is completely sterile and technicians can work safely without fear. (*ii*) Staining procedure becomes simple as it does not involve heating of carbol-fuchsin, which is crucial in the ZN method. (*iii*) Problems encountered during heating process, such as disfiguration of AFB in smears due to over-heating, and drying of stain if unattended for a long time due to busy work hours can be overcome/prevented. (*iv*) The sputum containers become less contagious and disposal becomes easy. (*v*) It is cost-effective as the quantity of carbol-fuchsin used for staining is 3 to 4 times less than normally used in the hot ZN method.

It is generally observed that sputum samples are received in microscopy centres until the last

Table I. Comparison of smear results obtained in the first reading

		2 h stained deposit smears						
		SC	1+	2+	3+	Any positive	Negative	Total
Initial deposit smears	SC	1	3	–	–	4	4	8
	1+	–	3	5	3	11	6	17
	2+	1	5	4	6	16	2	18
	3+	1	3	6	29	39	4	43
	Any positive	3	14	15	38	70	16	86
	Negative	10	1	2	1	14	80	94
	Total	13	15	17	39	84	96	180

SC, scanty = 1-9 AFB in 100 fields; 1+ = 10-99 AFB in 100 fields; 2+ = 1 to 9 AFB in at least 50 fields; 3+ = > 10 AFB in at least 20 fields; Negative = no AFB in 100 fields

Table II. Comparison of smear results* after resolving discrepancies

		2 h stained deposit smears						
		Scanty	1+	2+	3+	Any positive	Negative	Total
Initial deposit smears	Scanty*	3	3	–	–	6	1	7
	1+	1	4	6	3	14	3	17
	2+	2	6	3	6	17	2	19
	3+	1	4	7	32	44	–	44
	Any positive	7	17	16	41	81	6	87
	Negative	3	1	–	–	4	89	93
	Total	10	18	16	41	85	95	180

*As shown in the previous Table
Kappa = 0.89; sensitivity = 93%; specificity = 96%

outpatient was seen by the medical officer. The smears are then made for staining and examination which usually takes 2 to 4 h. The present study explored the possibility of staining the AFB within 2 to 4 h as the sputum deposits were found to be adequately stained only after 2 h.

Practical limitation of this method is its applicability only on deposits of sputum samples, processed using a high speed centrifuge available in high level mycobacteriology laboratories. This can be explored further to stain the deposits of sputum samples obtained in phenol ammonium sulphate sediment method[2] or in-household bleach method[6] so as to utilize this methodology in sputum AFB microscopy centres of the national TB control programmes.

In conclusion, deposits of decontaminated and homogenized sputum samples in sputum containers can be stained with carbol-fuchsin between 2 to 3 h, and their smears made subsequently on glass slides can then be decolourized and counterstained for detection of AFB under a light microscope as in the traditional ZN method.

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