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TECHNICAL NOTE

Comparison of variants of carbol-fuchsin solution in Ziehl-Neelsen for detection of acid-fast bacilli

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SUMMARY

To evaluate Ziehl-Neelsen (ZN) staining using variants of carbol-fuchsin solution, duplicate smears from 416 samples were stained with ZN, one set with 1% basic fuchsin and the other 0.3%. Another set of duplicate smears from 398 samples were stained with ZN, one with 1% basic fuchsin and the other 0.1%. The coded smears were read and discrepancies resolved. All samples underwent mycobacterial culture. The sensitivity of ZN using 0.3% (65%) and 1% basic fuchsin (62%) was comparable, while it was reduced using 0.1% (74%) compared to 1% basic fuchsin (83%). Reducing the concentration of basic fuchsin below 0.3% in ZN staining was found to significantly reduce its sensitivity.

KEY WORDS: pulmonary tuberculosis; Ziehl-Neelsen staining; carbol-fuchsin; AFB

PULMONARY TUBERCULOSIS is diagnosed by the detection of acid-fast bacilli (AFB) in sputum smears by the Ziehl-Neelsen (ZN) method, which is commonly used in many high TB burden countries due to its simplicity and low cost.[1] Since Robert Koch first described it in 1882, several modifications[2] have been attempted to improve its sensitivity. The Government of India's Revised National Tuberculosis Control Programme (RNTCP) guidelines[1] recommend 1% basic fuchsin in the ZN method, while the World Health Organization (WHO) and the International Union Against Tuberculosis and Lung Disease (IUATLD) guidelines recommend 0.3%.[3,4] The reasons for reducing the concentration from 1% to 0.3% are not known. The need to document the adequacy of 0.3% over 1% basic fuchsin in ZN staining has been stressed.[5] The present controlled laboratory study was designed to compare the efficiency of 0.3% and 0.1% basic fuchsin in ZN staining.

MATERIALS AND METHODS

Chemicals and reagents

Ethanol, phenol, sulphuric acid, methylene blue (Qualigens, Mumbai, India) and basic fuchsin (Hi-Media, Mumbai, India) were used in the study.

Staining methods

The Indian RNTCP guidelines were followed for the preparation of smears and reagents, and for the staining, examination and grading of direct smears.[1]

Carbol-fuchsin solution (1%)

Basic fuchsin 10 g was dissolved in 100 ml ethanol and 50 ml molten phenol in a flask maintained at 60°C in a water bath. The solution was made up to 1000 ml with distilled water.

Carbol-fuchsin solution (0.3%)

A stock solution of 3% basic fuchsin was prepared by dissolving 3 g basic fuchsin in 100 ml ethanol. A stock solution of 5% phenol was prepared by adding 25 ml molten phenol to 475 ml distilled water. Carbol-fuchsin (0.3%) solution was prepared by adding 10 ml of 3% basic fuchsin solution to 90 ml of 5% phenol solution.

Carbol-fuchsin solution (0.1%)

A stock solution of 1% basic fuchsin was prepared by dissolving 1 g basic fuchsin in 100 ml ethanol. Carbol-fuchsin solution (0.1%) was prepared by mixing 10 ml of 1% basic fuchsin solution with 90 ml of 5% phenol solution.

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Sulphuric acid (25%)

Concentrated sulphuric acid 250 ml was slowly added to 750 ml distilled water.

Methylene blue (0.1%)

Methylene blue 1 g was dissolved in 1000 ml distilled water.

Comparison of ZN methods using 1% and 0.3% basic fuchsin solutions

Four hundred and sixteen sputum samples were obtained from TB patients attending the Tuberculosis Research Centre (TRC) Clinic. They included patients newly registered for treatment, patients assessed for clinical trails and patients being followed up in ongoing clinical trials. Duplicate smears were made from each sample and stained with ZN, one using 1% and the other using 0.3% basic fuchsin.

Comparison of ZN methods using 1% and 0.1% basic fuchsin solutions

Three hundred and ninety eight sputum samples of unknown (diagnostic/follow-up) status were received from different parts of the country. Duplicate smears were prepared from each sample and stained with ZN, one using 1% and the other using 0.1% basic fuchsin.

Reading of smears

The duplicate smears made from each of the samples, and allocated to 1% and 0.3% basic fuchsin or to 1% and 0.1% basic fuchsin were read by the same technicians. The two technicians examined equal proportions of smears made for the different staining methods. All the smears were coded before reading. As part of the supervision, all positives and 20% of negative slides were checked by a senior technician. Referee reading resolved any discrepancies in smear results. The results of the referee reading were taken for analysis.

Culture method

All sputum samples were processed by the modified Petroff's method for culture of *Mycobacterium tuberculosis*.[6]

Statistical analysis

The statistical significance of observed differences in smear results between the different staining methods was determined using McNemar's test. A P value < 0.05 was considered significant. The level of agreement in smear results between the ZN methods was determined using kappa statistics.

RESULTS

Comparison of smear results obtained from ZN staining using 0.3% and 1% basic fuchsin revealed close agreement ($\kappa = 0.79$): respectively 138 (33%) and 137 (33%) of the 416 samples were positive (Table 1).

Comparison of smear results obtained from ZN staining using 0.1% and 1% basic fuchsin revealed a significant difference ($P < 0.008$): respectively 161 (40%) and 184 (46%) of the 398 smears were positive (Table 1). ZN using 0.1% basic fuchsin detected only 75% (138/184) of the smears found positive by ZN using 1% basic fuchsin.

The performance of ZN staining using 0.3%, 0.1% and 1% basic fuchsin was evaluated against culture results as the gold standard (Table 2). The sensitivity and specificity of ZN staining using 0.3% and 1% basic fuchsin were respectively 64% (129/200)

Table 1 Smear results obtained from ZN methods using 0.3% and 0.1% compared to 1% basic fuchsin

	1% basic fuchsin						
	Scanty*	1+	2+	3+	Any positive	Negative	Total
0.3% basic fuchsin							
Scanty	4	5	0	0	9	7	16
1+	7	27	13	1	48	9	57
2+	0	11	10	7	28	4	32
3+	0	1	5	27	33	0	33
Any positive	11	44	28	35	118	20	138
Negative	12	4	3	0	19	259	278
Total	23	48	31	35	137	279	416
0.1% basic fuchsin							
Scanty	5	11	2	1	19	14	33
1+	5	18	25	4	52	9	61
2+	0	9	14	14	37	0	37
3+	0	0	3	27	30	0	30
Any positive	10	38	44	46	138	23	161
Negative	25	17	4	0	46	191	237
Total	35	55	48	46	184	214	398

* 3+ = >10 AFB/oil immersion field in at least 20 fields; 2+ = 1–9 AFB/oil immersion field in at least 50 fields; 1+ = 10–99 AFB/100 oil immersion fields; Scanty = 1–9 AFB/100 oil immersion fields.
ZN = Ziehl-Neelsen; AFB = acid-fast bacilli.

Table 2 Comparison of smear results obtained from ZN staining using 0.3%, 0.1% and 1% carbol-fuchsin with culture results

Method and smear results*	Culture results†								
	Col	1+	2+	3+	Any positive	Neg	Cont	NTM	Total
0.3% basic fuchsin									
Scanty	0	5	6	3	14	2	0	0	16
1+	1	8	25	19	53	0	3	1	57
2+	0	2	7	21	30	2	0	0	32
3+	0	2	9	21	32	1	0	0	33
Any positive	1	17	47	64	129	5	3	1	138
Negative	12	35	13	11	71	187	10	10	278
Total	13	52	60	75	200	192	13	11	416
1% basic fuchsin									
Scanty	1	4	8	3	16	5	1	1	23
1+	2	7	18	19	46	1	1	0	48
2+	0	3	9	17	29	1	0	1	31
3+	0	2	7	24	33	2	0	0	35
Any positive	3	16	42	63	124	9	2	2	137
Negative	10	36	18	12	76	183	11	9	279
Total	13	52	60	75	200	192	13	11	416
0.1% basic fuchsin									
Scanty	3	6	9	8	26	7	0	0	33
1+	4	6	15	23	48	12	1	0	61
2+	1	3	13	17	34	3	0	0	37
3+	0	1	7	21	29	1	0	0	30
Any positive	8	16	44	69	137	23	1	0	161
Negative	14	15	10	9	48	171	8	10	237
Total	22	31	54	78	185	194	9	10	398
1% basic fuchsin									
Scanty	3	6	5	9	23	9	1	2	35
1+	2	11	19	16	48	6	0	1	55
2+	2	6	10	22	40	8	0	0	48
3+	3	1	13	26	43	3	0	0	46
Any positive	10	24	47	73	154	26	1	3	184
Negative	12	7	7	5	31	168	8	7	214
Total	22	31	54	78	185	194	9	10	398

* As shown in the previous table.
† 3+ = confluent growth; 2+ = innumerable colonies; 1+ = ⩾20 colonies but <100 colonies; Cols = 1–19 colonies; Any positive = total positives; Neg = no growth of *M. tuberculosis*; Cont = contamination.
NTM = non-tuberculous mycobacteria.

and 96% (187/192), and 62% (124/200) and 95% (183/192). The sensitivity and specificity using 0.1% and 1% basic fuchsin were respectively 74% (137/185) and 88% (171/194), and 83% (154/185) and 87% (168/194).

DISCUSSION

The quest for rapidity and efficacy has resulted in several modifications to simplify the ZN staining method.[2] However, none of these has gained wide acceptance, except for the cold staining method. In this study, ZN staining using 0.3% basic fuchsin was found to be as sensitive and specific as that using 1% basic fuchsin. Using this reagent can avoid wastage of basic fuchsin and reduce the cost of staining. Reducing the concentration of basic fuchsin below 0.3%, however, was found to affect the sensitivity of ZN. This shows that the purity of basic fuchsin is crucial in the preparation of carbol-fuchsin solution. Uncertainty about the purity of the basic fuchsin available on the market and ageing of carbol-fuchsin due to prolonged use may be reasons for recommending higher concentrations. In a recently published paper, it was shown that ZN staining using diluted carbol-fuchsin (0.3% basic fuchsin and 1.7% phenol) missed 21% of smears detected as positive by ZN using 1% basic fuchsin.[6] In other words, reducing the concentration of phenol in carbol-fuchsin solution will result in significant reduction in the detection of smear-positive cases.

The reduction in the specificity of ZN staining using 1% basic fuchsin in the experiment using 1% and 0.3% basic fuchsin (12%) compared to that using 1% and 0.1% (5%) can be attributed to the inclusion of follow-up specimens from patients in ongoing controlled clinical trials, as it is well known that 20–25% of samples collected from patients during treatment follow-up yield smear-positive, culture-negative results.[7]

In the present study, the basic fuchsin and phenol solutions were mixed just before staining, as per WHO guidelines.[3] This will pose some operational and technical problems in the field, as many microscopy

centres are not provided with measuring cylinders, and appropriate mixing of stock solutions cannot be ensured in these settings. Preparation of complete 0.3% basic fuchsin reagent, as done in the preparation of 1% basic fuchsin, may overcome those issues. Moreover, like the IUATLD guidelines, the Indian RNTCP guidelines recommend preparation of reagents by the laboratory supervisors in the Tuberculosis Units to be supplied to the peripheral centres on a monthly basis.

CONCLUSION

Reducing the concentration of basic fuchsin below 0.3% was found to significantly reduce the sensitivity of ZN staining.

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RÉSUMÉ

Pour évaluer la méthode de coloration de Ziehl-Neelsen (ZN) en utilisant des variantes de la solution de fuchsine basique, on a coloré une série de paires de frottis provenant de 416 échantillons avec la méthode de ZN, utilisant la fuchsine basique une fois à 1% et l'autre fois à 0,3%. Une autre série de paires de frottis provenant de 398 échantillons ont été colorés avec la méthode ZN, l'un utilisant une solution à 1% de fuchsine basique et l'autre utilisant 0,1%. Les frottis encodés ont été lus et les discordances résolues. Tous les échantillons ont été soumis à culture pour les mycobactéries. La sensibilité des méthodes de ZN utilisant de la fuchsine basique à 0,3% (65%) ou à 1% (62%) est comparable, mais elle diminue lorsqu'on utilise la fuchsine basique à 0,1% (74%) par comparaison à 1% (83%). La sensibilité de la méthode de ZN s'avère significativement diminuée par une diminution de la concentration de la fuchsine basique en dessous de 0,3%.

RESUMEN

Para evaluar la técnica de tinción de Ziehl-Neelsen (ZN) utilizando variantes de la solución de carbolfuscina, se tiñó una serie doble de láminas de 416 muestras de esputo con la técnica de ZN, uno utilizando fuscina básica al 1% y otro al 0,3%. Se tiñó otra serie doble de láminas de 398 muestras con la técnica ZN, una con solución de fuscina básica al 1% y la otra al 0,1%. Se leyeron las láminas codificadas y se resolvieron las discrepancias. Todas las muestras se sometieron a cultivo para micobacterias. La sensibilidad de la técnica de ZN fue equivalente cuando se utilizó fuscina básica al 0,3% (65%) y al 1% (62%), pero fue inferior cuando se utilizó fuscina básica al 0,1% (74%), en comparación con 1% (83%). La disminución de la concentración de fuscina básica por debajo del 0,3% en la técnica de coloración de Ziehl-Neelsen reduce significativamente su sensibilidad.