JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2002, p. 3041–3043 Vol. 40, No. 8
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.8.3041–3043.2002

Inefficiency of 0.3% Carbol Fuchsin in Ziehl-Neelsen Staining for Detecting Acid-Fast Bacilli

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Received 4 February 2002/Returned for modification 25 March 2002/Accepted 17 May 2002

We compared the sensitivity and specificity of a modified Ziehl-Neelsen (modified-ZN) staining method for acid-fast bacilli (AFB) with that of the standard Ziehl-Neelsen (standard-ZN) staining method, using culture results with *Mycobacterium tuberculosis* as the "gold standard." The sensitivity (72%; 101 of 140) of the modified-ZN staining method, which uses 0.3% carbol fuchsin, was significantly lower than that of the standard-ZN staining method (84%; 117 of 140); the modified-ZN method missed 21% of cases detected by the standard-ZN method and 11% more of culture-positive samples than the standard-ZN method. The World Health Organization recommendation of 0.3% carbol fuchsin in the ZN method for staining AFB needs to be reconsidered.

In developing countries, sputum acid-fast bacilli (AFB) smear microscopy is the primary tool for detecting pulmonary tuberculosis (5). The Ziehl-Neelsen (ZN) method is commonly used for staining sputum smears because of its simplicity and low cost. Revised National Tuberculosis Control Programme (RNTCP) guidelines recommend the use of 1% carbol fuchsin in the ZN method (2). However, recent World Health Organization (WHO) guidelines recommend using carbol fuchsin at a concentration of 0.3% (5). However, the reasons for reducing the concentration of carbol fuchsin from 1 to 0.3% are not documented. The efficacy of 0.3% carbol fuchsin over 1% carbol fuchsin in the ZN staining method has not been studied previously.

A total of 586 sputum samples were collected from the same number of patients with symptoms of pulmonary tuberculosis attending two tuberculosis detection centers during the second and third quarters of 2001 in Chennai, India: the Tuberculosis Research Centre, Chetput, and the Government Hospital for Thoracic Medicine, Tambaram Sanatorium. Many of these patients were receiving rifampin-containing short-course regimens for pulmonary tuberculosis and were followed up in controlled clinical trials.

Preparation of reagents. Carbol fuchsin (1%) was prepared from 10 g of basic fuchsin (Hi-Media) dissolved in 100 ml of methanol (Qualigens) and 50 ml of melted phenol (Qualigens) in a flask maintained at 60°C in a water bath. This solution was made up to 1,000 ml with distilled water. Carbol fuchsin (0.3%) was prepared from 33 ml of the above solution diluted to 100 ml with distilled water before use. Sulfuric acid (25%) was prepared from 250 ml of concentrated sulfuric acid (Qualigens) slowly added to 750 ml of distilled water. Methylene blue (0.1%) was prepared from 1 g of methylene blue (Qualigens) dissolved in 1,000 ml of distilled water.

Two direct smears were prepared from each of the 586 sputum samples and coded. One set was stained by the standard-ZN method, in which 1% carbol fuchsin was used, and the other set was stained using the modified-ZN method, in which 0.3% carbol fuchsin was used for staining. The RNTCP guidelines were followed to stain the smears. The air-dried smear slides were fixed over a flame three to five times for 3 to 4 s. The slides were then placed on a staining rack, and filtered carbol fuchsin was poured to cover the entire slide. The slides were heated from underneath for 5 min until vapors started rising. After 5 min, slides were gently rinsed with tap water to remove the excess carbol fuchsin stain. The smears were decolorized with 25% sulfuric acid for 2 to 4 min and again rinsed with water. The slides were counterstained for 30 to 60 s using 0.1% methylene blue solution. The slides were rinsed with tap water, allowed to dry, and examined under a binocular microscope. Technicians who were blinded to the methods of staining read the slides. A senior technician cross-checked all positives and 20% of negative slides. The smear results were decoded and matched for comparison using McNemar's test.

All the sputum samples were processed by the modified Petroff's method for culture of *Mycobacterium tuberculosis* (10). In brief, 3 to 5 ml of sputum was homogenized for 15 min in a shaker using an equal volume of 4% sodium hydroxide. After centrifugation (Heraceus model Megafuge 1.0) at 3,000 rpm for 15 min, the deposit was neutralized with 20 ml of sterile distilled water. The samples were again centrifuged and the deposit was inoculated onto Lowenstein-Jensen medium and incubated for 8 weeks at 37°C. The isolated cultures were confirmed for *M. tuberculosis* by a niacin test, a 68°C catalase test, and growth on *p*-nitrobenzoic acid.

The smear results obtained from the modified-ZN method and the standard-ZN method are compared in Table 1. The agreement in grading (one grade above and one below) between the two methods was 93.2%. The proportion of positive smears detected was 25.4 and 21.7% for the standard-ZN and modified-ZN methods, respectively: the modified-ZN method missed 31 of the 149 (20.8%) slides found positive by the

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TABLE 1. Comparison of the smear results with the modified-ZN and standard-ZN methods[a]

Results with modified-ZN method	No. with result in standard-ZN method						
	3+	2+	1+	Scanty	Any positive	Negative	Total
3+	40	5	3	0	48	0	48
2+	9	17	6	0	32	1	33
1+	2	8	22	1	33	1	34
Scanty	0	0	2	3	5	7	12
Any positive	51	30	33	4	118	9	127
Negative	1	2	11	17	31	428	459
Total	52	32	44	21	149	437	586

[a] 3+, more than 10 AFB per oil immersion field in at least 20 fields; 2+, 2 to 10 AFB per oil immersion field in at least 50 fields; 1+, 1 to 99 AFB in 100 oil immersion fields; Scanty, 1 to 9 AFB in 100 oil immersion fields.

standard-ZN method. The observed difference between the two methods was statistically significant (McNemar's test; $\chi^2 = 11.0$, $P = 0.01$).

Of the 586 samples cultured, 140, 23, and 1 yielded *M. tuberculosis*, contaminants, and nontuberculous mycobacteria (NTM), respectively, and the remaining 422 samples were negative for culture. Of the 23 specimens which yielded contaminants, 16 were negative and 5 were positive for AFB by both staining methods; 2 were positive by modified-ZN and negative by the standard-ZN method. The one specimen with NTM was smear negative by both methods. After excluding the contamination and NTM results, the remaining 562 specimens were used for comparison.

The performance of the modified-ZN method (Table 2) and that of the standard-ZN method were evaluated using culture as the "gold standard." The proportion of smear-negative but culture-positive specimens was higher with the modified-ZN method (27.9%) than with standard-ZN (16.4%): the modified-ZN method missed 16 more of the 140 (11.4%) culture-positive specimens than the standard-ZN method did. The observed difference between the modified-ZN smear results and the culture results was statistically significant (McNemar's test; $\chi^2 = 6.22$, $P = 0.01$), while the difference between the standard-ZN method and the culture results was not statistically significant. Nineteen and 23 of 140 culture-proven cases were found to be smear negative by modified-ZN and standard-ZN methods, respectively. This could be attributable to the collection of sputum samples from tuberculosis patients receiving rifampin-containing short-course chemotherapy regimens in controlled clinical trials.

Directly observed treatment short-course programs throughout the world examine millions of sputum smears each year. The RNTCP in India alone, which now covers approximately half of the population, will examine more than 7 million sputum smears in 2002. Sputum smear microscopy is a simple and cost-effective tool which provides not only a preliminary confirmation of the disease but also a quantitative estimate of the number of bacilli. Smear positivity correlates well with the severity of pulmonary disease, infectiousness, and risk of death if untreated. The ZN method for detection of AFB is easy, rapid, and inexpensive. Cultures are generally not performed in developing countries, and in any case they require several weeks to become positive. The quest for rapidity and efficacy has resulted in several modifications to simplify the ZN method. The approaches have included bulk staining of slides (3), staining of slides by using a microwave oven (4), use of a decalcifying agent (1), chloroform (8), carbol fuchsin-impregnated strips (11), and cold staining (6, 7, 9, 12). However, none of these modified methods have gained wide acceptance and are not used. Recently, the WHO recommended the use of 0.3% carbol fuchsin (5). This study suggests that use of 0.3% carbol fuchsin in ZN staining may result in missing 20% of smear-positive patients and is less sensitive and no more specific in detecting AFB in sputum. The modified-ZN method was found to be significantly less sensitive than the standard-ZN method that uses 1% carbol fuchsin (72.1 versus 83.6%). The modified-ZN method had 11% more smear-negative culture-positive samples than the standard-ZN method.

TABLE 2. Comparison of sputum smear results of the modified-ZN method and standard-ZN method with culture results

Method	Smear result[d]	No. with culture result[a]								
		3+	2+	1+	COL	Any positive	Negative	Cont.	NTM	Total
Standard-ZN[b]	3+	38	6	3	1	48	1	3	0	52
	2+	16	9	2	0	27	4	1	0	32
	1+	23	6	5	2	36	8	0	0	44
	Scanty	3	2	0	1	06	14	1	0	21
	Any positive	80	23	10	4	117	27	5	0	149
	Negative	1	3	9	10	23	395	18	1	437
Modified-ZN[c]	3+	35	5	3	1	44	0	4	0	48
	2+	21	6	0	0	27	6	0	0	33
	1+	14	7	4	2	27	7	0	0	34
	Scanty	0	1	2	0	03	6	3	0	12
	Any positive	70	19	9	3	101	19	7	0	127
	Negative	11	7	10	11	39	403	16	1	459
	Total	81	26	19	14	140	422	23	1	586

[a] 3+, confluent growth; 2+, innumerable number of colonies; 1+, 20 and above but less than 100 colonies; COL, 1 to 19 colonies; Cont, contamination.
[b] Agreement, 91.1%; sensitivity, 83.6%; specificity, 93.6%.
[c] Agreement, 89.7%; sensitivity, 72.1%; specificity, 95.5%.
[d] As shown in Table 1.

Bias in reading slides was eliminated by blinding the technicians to the staining methods and having the same technicians read the corresponding slides. Quality control of reading the smears was established by a second senior technician who cross-checked all of the positive slides and 20% of the negative slides.

The WHO recommendation of 0.3% carbol fuchsin in the ZN method needs to be reconsidered, as 0.3% carbol fuchsin may result in 20% of the smear-positive patients being missed.

We gratefully acknowledge John Arokiadas, R. Shahabuddin, and S. Sudhamathi for their excellent technical support and V. Sundaram and K. Thyagarajan for statistical help.

This research was supported in part by WHO with funds from the U.S. Agency for International Development.

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