Evaluation of a Cold Staining Method for Acid-Fast Bacilli in Sputum

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Comparison between the Ziehl-Neelscn staining method for acid-fast bacilli, applied with and without heating, was carried out in a controlled investigation using smears prepared from 306 sputum samples collected prior to treatment from suspected cases of pulmonary tuberculosis. Smear and culture positively were graded and the colour intensity of bacilli recorded. Results showed that the chance corrected agreement (Kappa) between Z-N and cold methods was only 78%. The sensitivity of the Z-N and cold methods were 84% and 77% respectively when compared with culture results. Assuming 10% smear positivity among symptomatics reporting to Peripheral Health Institutions (PHIs), the positive predictive value of the cold method was very low(53%). When compared to culture, the positive predictive value is 71% for the Z-N method and 57% for the cold method for a symptomatic population with 15% culture positivity.

In the absence of heating. penetration of the stain was significantly reduced and consequently the number of bacilli detected was less. The inability to take the stain without heating was seen in smears from all grades of culture positive samples: thus even heavy positives were missed by the cold method. The evaluation of the cold method against the standard Z-N method highlights its limitations and demonstrates that it is not as reliable as the standard Z-N method.

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Case finding in Tuberculosis Control Programmes of developing countries including India, is done by sputum microscopy using Ziehl-Neelsen (Z-N) staining method. This method involves heating as one of the steps which is considered crucial for the quality of the stained smear and in turn. for the detection of acid-fast bacilli (AFB). The heating procedure. however. is reported to be impractical in certain Peripheral Health institutions (PHIs) because of difficulties in providing alcohol for spirit lamps[1]. Several cold staining variations of the Z-N method, based on modifications of the Fuchsin stain have been reported[2,3].

In 1986, Vasantha Kumari *et al*[1] reported that the carbol fuchsin stain without any modification could be used as a cold stain for sputum smears by merely extending the staining time to 10 minutes to obtain results comparable with that of the standard Z-N method and observed that there was no difference in the colour intensity of the bacilli stained by these two methods. However, the sensitivity of this cold staining method could not be assessed, as the positives were neither graded nor compared with culture results. nor does it appear that the smears were randomised. A similar comparison of 2100 sputum smears conducted under field conditions without randomisation was reported by Gokhale *et al;* in this study, the smear

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postivity was graded but culture examination was not done[4]. In the present study, therefore, the cold staining method[1] was evaluated in a controlled comparison with the standard Z-N method as well as with the culture results.

Material and Methods

A total of 306 sputum specimens collected prior to the start of treatment from patients referred to this Centre with suspected pulmonary tuberculosis were studied. Two smears were prepared from each specimen and were randomly allocated to the two methods of staining. After staining, the slides were coded so that the reader had no knowledge of either the identity of the specimen or the method of staining used.

The stains used and the procedures employed were identical for the cold and Z-N staining methods except for the heating[1]. In brief, the smears were covered with carbol fuchsin stain and heated (Z-N; method) or left unheated (cold method) for 10 minutes. After washing. decolourisation and counter staining was done in a single step using Gabbets methylcne blue which contained 20% H_2SO_4, 30% absolute alcohol and 1% methylene blue.

The smears were examined under oil immersion (7 x 100) taking up to 15 minutes per smear as was done in the original comparison. The smears were graded as follows : < 3 bacilli in the entire smear : negative: 3-99 bacilli in the entire smear : actual count; ≥ 100 bacilli in the entire smear and < 5 per field : 1 +; ≥ 5 per field; 2 + and numerous bacilli or large clumps in several fields : 3 +. In addition to the above grading, the colour intensity of the stained bacilli was also recorded as ‘deep pink’ or ‘faint pink’ as a measure of stain penetration.

Culture was set up from the sputum on L-J medium by the modified Petroff's method[5] and the growth was graded as follows : 1-19 colonies : actual count; 20-99 colonies : 1 +; ≥ 100 discrete colonies : 2 +; and confluent growth : 3 +.

Table 1. *Comparison of Z-N and cold staining methods according to smear grading*

Smear grade by cold staining method	Smear grade by Z-N method				Total	
	0-2	3-99	1+	≥ 2+		
0-2	141	19	7	7		164
3-99	10	18	13	6	47	
1+	1	5	18	20	44	142
≥ 2+	0	3	10	38	51	
		45	43	66		
Total	152		154			306

Results

Five smears stained by the Z-N method and 10 smears stained by the cold method which had only 1 or 2 bacilli were considered as negative.

Of the 306 smears examined, 154 (50%) were positive by the Z-N method compared to 142 (46%) by the cold method (Table 1). Further, 131 were positive and 141 negative, by both the methods showing an overall agreement of 89%. However, the chance corrected agreement (Kappa) was only 78%. The sensitivity and specificity of the cold method as compared against Z-N were 85% and 93%. respectively, and the positive and negative predictive values were 92% and 86%. respectively.

Disagreement between the two methods was observed in 23 positives by the Z-N method that were not detected by the cold method and 11 *vice versa.* This difference bordered on statistical significance (P = 0.06).

Identical smear gradings were obtained by the two methods in 215 (70%) of the 306 samples. Among those which differed, the cold method gave lower grading (P < 0.001) more often than the Z-N method as can be seen from table I, where the numbers above and below the diagonal are 62 and 29 respectively.

The effect of heating was assessed based on colour intensity of bacilli seen. Table 2 shows that the bacilli in 149 (97%) of the 154 Z-N positives stained deep pink, while only 114 (80%) of 142 positives among the cold stained smears had the same depth of colour (P < 0.001). Irrespective of the method of staining employed, a larger number of bacilli were detected when the smears had deeply stained bacilli. Thus, ≥ 100 bacilli were detected in 107 (72%) of 149 Z-N smears and 86 (75%) of 114 cold stained smears with deeply stained bacilli compared to only 10 (36%) of 28 with faintly stained bacilli among the cold stained smears (P < 0.001).

Table 2. *Association of grading of smear positivity with the colour intensity of bacilli*

Colour intensity of bacilli	Method	Total no. of smears	Number of bacilli seen			
			< 100		≥ 100	
			No.	%	No.	%
Deep pink	Z-N	149	42	28	107	72
	Cold	II?	28	25	86	75
Faint pink	Z-N	5	4	(80)*	1	(20)*
	cold	28	18	64	10	36

* Figures in parantheses indicate percentage based on fewer than 25 observations.

Table 3. *Relative agreement in the classification of specimens by culture and the two smear methods*

Agreement* between Cold stain smear method and culture	Agreement* between Z-N smear method and culture		Total
	Present	Absent	
Present	222	14	136
Absent	28	21	49
Total	250	35	285

*Agreement between results: ***Present:*** Positive or negative by both smear and culture methods.
Absent : Positive by one and negative by the other method.

The reliability of the smear results by both methods was ascertained by comparing them with the corresponding culture results (Table 3). For this analysis, detection of even 1 bacillus by smear was considered as positive since that is the criterion for smear positivity in the NTP of India". Of the 306 samples, culture results were available for 285 specimens. The classification of positives and negatives was identical to the culture method in 250 (88%) by the Z-N method as compared to 236 (83%) by the cold method, 222 showing agreement by all the 3 methods (Table 3). Neither of the smear methods (Z-N or cold) agreed with culture method in 21 of the remaining 63 specimens; a further 28 disagreed with the culture results by the cold method only and 14 disagreed with the culture result by the Z-N method only; this difference was statistically significant (P = 0.03).

Table 4. *Association of culture results with smear results*

Culture results	No. of cases	Smear results (No. of bacilli)					
		Z-N method			Cold method		
		0	1-2	>3	0	1-2	>3
Negative	112	105	1	6	102	8	2
< 20 colonies	14	11	0	3	9	0	5
20-99 colonies	31	13	1	17	17	I	13
Numerous colonies	71			65	17		59
Confluent growth	57	0	0	57	2	0	55
Contaminated	11	4	I	6	3	0	8
Non-tuberculous mycobacteria	10	10	0	0	10	0	0
Total	306	147	5	154	154	10	142

The association of culture results with smear results is presented in table 4. There were 128 specimens which were heavy positives by culture (71 with numerous colonies and 57 with confluent growth). The cold method failed to detect 13 of these and the Z-N method only 4 ($P < 0.02$). Analysis (not tabulated here) showed that even among the 115 heavy culture positive specimens detected by the cold method, ; 9 had only faintly stained bacilli whereas of 124 such specimens detected by the Z-N method only 1 had faintly stained bacilli ($P < 0.001$).

Discussion

It has been reported by Vasantha Kumari *et al*[1] that the Z-N staining method for acid-&t bacilli in sputum can be performed as a cold staining method. without any modification of the stains and still obtain comparable degree of smear positivity by merely extending the staining time to IO minutes. The report of Gokhale *et al*[4] based on 21 sputum smears showed that the cold method was less sensitive when compared to the Z-N method though 100% specific. The results of our study showed that there was 89% overall agreement between the two methods in classifying the smears as positives or negatives. The sensitivity and specificity of the cold method as compared with Z-N method were 85% and 93% respectively and the positive and negative predictive values were 92% and 86% respectively.

The sputum specimens used in this study were from suspected cases of pulmonary tuberculosis and consisted of about 50% positives by smears. A high level of positivity observed is not expected in the NTP, or field surveys. Assuming 10% smear positivity among symptomatics for the same level of sensitivity and specificity as in the present study, the positive predictive value of the cold method will drop down to 57% indicating a 43% false positivity by this method. This is a setback for the applicability of the method in situations where the expected rate of positivity is low. When compared with the culture results, the Z-N method showed 84% sensitivity and 94% specificity. The corresponding figures for the cold method were 77% and 91% respectively. The positive predictive value of the two smear methods in relation to culture finding were 95 and 93%. Assuming 15% positivity by culture as may be expected in the programme, the positive predictive value will be 71% for the Z-N method and 57% for the cold method. The analysis of disagreements between culture and smear results shows that it occurred significantly more often with the cold method than Z-N method. Further, 13 of 128 specimens with heavy culture positivity were not detected by the cold method as compared to 4 not detected by the Z-N method ($P = 0.02$). Thus, the cold method was less reliable than the Z-N method.

Our results showed that heating enhanced the penetration of‘ stain, as significantly larger proportion of Z-N stained smears had deeply stained bacilli. Similar observations were made by Pathan and Arain[7] and recently by Chandrasekaran and others,[8] who observed that the colour of bacilli was faint with cold staining. However. neither of them reported estimation by grading the colour intensity. The poor penetration of stain adversely affected the detection of bacilli; thus fewer bacilli were detected when the bacilli were faintly stained. In the present investigation, the faintly stained bacilli could be detected primarily because the examination

was carried out by an experienced person in a research laboratory. As the level of accuracy in a research lab may not be expected in routine practice in the field as observed by Toman[9]. the sensitivity of the cold method could be further reduced. Moreover in places, which have a wide range of ambient temperatured, the cold staining method may not yield consistent results.

The practical difficulty in hearing the stain is said to be non availability of alcohol for use in spirit lamps[1,4]. Therefore, alternatively sources of heat may be considered in a laboratory, such as candies[5], rolled up paper fags or swabs dipped in spirit-based antiseptics. The stain can also be heated in a test tube in a water bath or sterilizer. Therefore, non-availability of spirit in a PHI need not be a deterrent for employing the Z-N staining method.

In the National TB Programme, detection of a bacteriologically positive case is the first step in effectively checking the chain of transmission, and in practice case finding is dependent most often on a single sputum smear examination. Hence, all efforts must be aimed at improving the reliabilty, of the existing method of smear examination. This controlled comparison of the cold and heated Z-N methods for the detection of acid-fast bacilli in sputum demonstrates that the cold method is not as reliable as the regular heated Z-N method.

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References

1. Vasantha Kumari R, Jagannathan K, Rajasekharan S. A cold staining method for acid-fast bacilli. *Bull WHO* 1986: **64** : 741.

2. Kinyoun JJ. A note on Uhlenhuth's method for sputum examination for tubercle bacilli. *Am J Pub Hlth* 1915: **5** : 867.

3. Rau KP, Naganathan N, Nair SS. A cold staining method for tubercle bacilli using chloroform Indian J Tub 1966; **14** : 3.

4. Gokhale S, Qadir S, Nagra JS, Chakraborty AK. Efficiency of cold staining method of AFB in sputum: A comparison with Ziehl-Neelsen method under field conditions. *Indian J Tub* 1990; **37** : 135.

5. Tuberculosis Research Centre, Madras. A controlled comparison of a twice weekly and three once weekly regimens in the initial treatment of pulmonary tuberculosis. *Bull WHO* 1970; **43** : 143.

6. District Tuberculosis Programme. NTI. *Laboratory Technician Manual.* National Tuberculosis Institute, 1977..

7. Pathan AJ, Arian AR. Comparative study of cold stain and Ziehl-Neelsen stain. *Tubercle* 1988: **69** 227.

8. Chandrasekaran Sujatha, Sreenivas TR, Chaudhri S. Modified sputum microscooy techniques sparing the use of alcohol for field application. *Indian J Tub* 1991: **38** : 87.

9. Toman K. *Tuberculosis Case Finding and Chemotherapy : Questions and Answers*. WHO Publication 1979; p. 18.