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Comparative evaluation of Ziehl- Neelsen staining and kinyoun's staining in the diagnosis of clinically suspected cases of tuberculosis

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ABSTRACT

Background: Tuberculosis commonly affects lungs but can also be extra-pulmonary. The increasing prevalence of extra-pulmonary manifestation of tuberculosis is a cause for concern. Microbiological diagnosis is essence for the effective treatment of mycobacterium tuberculosis. The smear detection tests differentiate infectious from non-infectious cases. Both Ziehl-Neelsen (Z-N) and kinyoun stain (KC) methods are over 100 years old; these are most economical in terms of processing time, needed equipment and personnel. The objective of research was to evaluate the two smear microscopy methods for the detection of tubercle bacilli in the sputum and Fine needle aspiration cytology (FNAC) material by comparing the sensitivity, specificity and validities of kinyoun's method against Z-N staining.
Materials and Method: A prospective and comparative study was carried using 500 sputum samples and 100 FNAC material received in the department. All the 600 samples were subjected to two methods:Z-N stain and Kinyoun's cold stain. They were compared for sensitivity and specificity in terms of qualitative results and grades of smear as recommended by RNTCP.
Results: The smear positivity rate was 18% and 20.16% in Ziehl-Neelsen stain and Kinyoun's cold stain respectively. The direct smear microscopy for Z-N staining showed sensitivity, 89.25%, specificity and positive predictive value (PPV) 100% and negative predictive value (NPV) 97.35% whereas KC method showed sensitivity 98.37%, specificity and PPV 100% and NPV 99.58% respectively.
Conclusion: Kinyon's method described is slightly longer to perform than the Ziehl-Neelsen technique but its ease of use and equal efficacy with Z-N method make it universally applicable, especially in those laboratories where facilities are poor.

1. Introduction

Mycobacterium tuberculosis (TB) ranks as the second leading cause of death from an infectious disease worldwide, after the Human Immunodeficiency Virus (HIV). The latest reports shows that one-fourth of the global incident TB cases occurred in India annually making India, the world's highest TB burden country.[1] According to World Health Organization (WHO), tubercular infections are currently spreading at the rate of one person per second per million population.[2]

Tuberculosis commonly affects lungs but can also be extra-pulmonary. The increasing prevalence of extra-pulmonary manifestation of tuberculosis with the HIV scourge is a cause for concern.[3] Lymph nodes tuberculosis is considered the most common form of extra-pulmonary tuberculosis and most common cause of lymphadenopathy in India.[4] Tuberculous lymphadenitis can be considered

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a local manifestation of a systemic disease.[5] Hence microscopic examination of sputum for detection of Acid Fast Bacilli (AFB) is of utmost importance. Early detection can prevent further complication. As per WHO or Revised National Tuberculosis Control Programme (RNTCP), an individual with at least one sputum smear positive for AFB or culture positive for tubercle bacilli is labelled to be suffering from pulmonary tuberculosis. In India with deficit of resources compared to high TB burden, culture facility is not adequately available. Hence most of the TB cases are diagnosed based on Sputum Smear Microscopy.[6] Fine needle aspiration cytology (FNAC) is a precise, cost-effective, and minimal invasive technique for diagnosis of extra-pulmonary tuberculosis.[7] Of the available staining techniques for direct microscopy, the Ziehl-Neelsen (Z-N) and the auramine O fluorescence acid-fast stains are the most widely used. Z-N Sputum smear microscopy is usually low cost procedure but poses problem in the field. It is cumbersome because it requires the heat application during the carbol-fuchsin staining resulting necessity for a flame source;[8] to eliminating heating step of this technique there have been develop a cold method for demonstration of AFB, for example Kinyoun used higher concentration of basic fuchsin and phenol.[9]

2. Aims and Objective

To compare Kinyoun's Cold (KC) staining with the Z-N method, to assess its performance for sensitivity, simplicity and rapidity over the traditional Z-N method, so that it may prove as a good alternative for diagnosis of TB.

3. Materials and Methods

The present prospective and comparative study was conducted after obtaining clearance from institutional ethical committee, in the department of Microbiology and Pathology, over a period of one year (October 2021-September 2022). Total 600 samples (500 sputum samples and 100 FNAC samples) of suspected tuberculosis were screened for AFB using the traditional Z-N hot method and the KC method.

3.1. Inclusion criteria

All sputum samples received in the Department of Microbiology for Acid Fast Staining by Z-N Staining method and the samples received in the Department of Pathology, from indoor as well as outdoor patients from all age groups, for FNAC of lymph nodes with clinical suspicion of tuberculosis were included in the study.

3.2. Exclusion criteria

Salivary samples and the cases diagnosed as malignancy on routine cytology were excluded from the study.

3.3. Ethical consideration

Informed consent was taken and FNAC procedure was explained to the patients.

FNAC was done by using 22-gauge needle and 20 ml syringe. All the aspirates which yielded whitish pus or pus mixed material were expressed on to the glass slide. Two smears were made from each aspirate sample and sputum sample. The slides were coded, air dried and heat fixed. One smear each from sputum and aspirate was stained with Z-N method and another smear were stained with KC method.

3.4. Examination of slides

The coded slides were examined using an oil immersion objective (1000x) independently by 2 groups of experienced doctors. Both group had no information on the staining method used for each slide and the result of the others, so as to reduce the bias. AFB was seen as red, beaded and slightly curved rod against a bluish background. The sputum smears were then graded depending on the number of bacilli observed. The data was processed and the sensitivity, specificity and positive and negative predictive values were calculated.

4. Results

A total of 600 samples were subjected to Z-N stain and KC stain. The smear positivity for AFB on Z-N staining method was 18% (108/600), while the positivity increased to 20.16% (121/600) when the KC method was used. Thus, the KC method detected AFB in additional 6 cases, which was statistically significant. In our study out of 108 positive smears, 2 smears were positive by Z-N method only and same were negative by Kinyoun's stain. Comparison of the qualitative result of the two microscopic methods is depicted in Table 1. Out of 500 sputum samples smear positivity for AFB by Z-N method was 16.8% (84/500) and by KC method was 18.4% (92/500) while in 100 FNAC material smear positivity was 24% and 28% respectively. (Table 2) Comparison of Ziehl- Neelsen staining with Kinyoun's cold staining by grading of the AFB-smear positivity in sputum samples is depicted in Table 3.

The direct smear microscopy for Z-N staining showed sensitivity, 89.25%, specificity and positive predictive value (PPV) 100% and negative predictive value (NPV) 97.35% whereas KC method showed sensitivity 98.37%, specificity and PPV 100% and NPV 99.58% respectively.(Table 4)

5. Discussion

The definitive diagnosis of tuberculosis is by the isolation and identification of M. tuberculosis. Culture remains the gold standard diagnostic method for tuberculosis, it requires weeks before a positive culture can be identified; moreover culture requires at least a moderately well equipped

Table 1: Comparison of the qualitative result of the two microscopic methods in total 600 samples

Staining method	Ziehl-Neelsen staining 600	Kinyoun's cold staining 600
Positive	108(18%)	121 (20.16%)
Negative	492 (82%)	479 (79.84%)

Table 2: Comparison of the qualitative results of Ziehl- Neelsen method and Kinyoun's cold staining method in sputum and FNAC material

Staining method	Ziehl-Neelsen method 600		Kinyoun's cold staining 600	
	Sputum (500)	FNAC material(100)	Sputum (500)	FNAC material(100)
Positive	84(16.8%)	24(24%)	92(18.4%)	28 (28%)
Negative	416(83.2%)	76 (76%)	408(81.6%)	72 (72%)

Table 3: Comparison of Ziehl- Neelsen staining with Kinyoun's cold staining by grading of the AFB-smear positivity in sputum samples

Result by grading	Sputum staining method	
	Ziehl-Neelsen method %	Kinyoun's cold method %
Negative	416(83.2%)	408(81.6%)
1+	30	37
2+	26	25
3+	28	30
Total	500	500

Table 4: Comparison of Sensitivity, Specificity, Positive and Negative Predictive Values in Z.N. Method and Kinyoun's Method

Values	Ziehl-Neelsen method %	Kinyoun's method %
Sensitivity	89.25	98.37
Specificity	100	100
Positive predictive value	100	100
Negative predictive value	97.35	99.58

laboratory. For this reason, microscopic examination for AFB has been the mainstay of the diagnosis of pulmonary tuberculosis while the results of sputum cultures were pending.[10] One advantage of the AFB sputum smear is its close correlation with infectiousness: patients who are sputum smear positive/culture positive are far more likely to be infectious than persons who are culture positive but smear negative.[11] To achieve the goal of Tuberculosis Eradication Programme we need to perform sputum smear examination in a large number throughout the country, as Z-N staining method can be practiced even at remote areas.[12]

However, the Z-N method poses problems, such as the need for a regular supply of spirit/liquid petroleum gas, which is used for heating. It is also a cumbersome procedure and can be hazardous. Various attempts have been made to develop a cold staining technique in order to omit the step that involves the heating of carbol-fuchsin.[8,13,14] The Kinyuon's cold stain method is an alternative method to the Z-N stain. The drive for the use of this method lies in the elimination of heating step which prevents production of harmful phenol fumes, fire from flammable phenol, cost of heating gas or spirit and the breakage of slides during heating.[10] The color contrasts of the smears stained by both the methods were visually identical.

In present study, out of 600 samples 108(18 %) samples were positive for AFB by Z-N stain and 121(20.16%) by the KC method, where as other workers reported 16% and 10% positivity respectively.[15] The slide positivity rate of KC method is slightly higher than Z-N stain (2.16%)in our study whereas other workers reported slightly lower than Z-N stain (2.75%).[6]

In our study sputum samples smear positivity for AFB by Z-N stain method was 16.8% and by KC method was 18.4% whereas other workers reported 53.1% positivity by Z-N method and 46.21% by cold staining method.[16] The Z-N staining has been shown to be highly specific in areas with a high prevalence of TB but with varying sensitivity (20– 80%).[17]

In the present study out of 100 FNAC material smear positivity for Z-N method and Kinyoun's method was 24% and 28% respectively. Whereas other workers reported 42.8% positivity by conventional Z-N method and 82.1% positivity by cold method in the suppurative lymphadenitis.[18] This increase in positivity could be due to increased number of the bacilli per field and clean background due to digestion of cellular elements by cold method. The morphology of AFB also appeared to be better preserved, and they were thicker and longer than the routine Z-N smears. This could probably be due to swelling of

bacilli in the solution. The above mentioned observations were also noted by other workers in their studies.[19,20]

The sensitivity of the Z-N and Kinyoun's methods were 89.25% and 98.37% respectively whereas specificities were same for both methods (100%). Although the true negative rate was very high in both staining methods, false-positive results were comparatively rare and therefore a positive smear could be relied upon as a good diagnostic indicator.

In this study the positive predictive values and the negative predictive values were high for both the staining methods. The positive predictive values of the Z-N and KC methods were 100% respectively and the negative predictive values were 97.35% and 99.58% respectively. These data suggested that these methods have sufficient validity to predict the presence or absence of the disease in a featured population. But ZN method has some disadvantages:

1. In this procedure, carbol fuschin stain has to be filtered every day before use.
2. Heating of stain on slides requires spirit/gas connection: supply of a sufficient quantity of spirit poses technical and organizational problems and many centres are not provided with LPG connections.[21]
3. Trained personnel are required.

Whereas a KC staining procedure has few distinct advantages:

1. A high level of technical skill is not required, thus simplifying the training of personnel.
2. The problem of procuring rectified spirit for spirit lamps is removed, making it suitable for use even in remote areas and heat fixation of smears on slides can be carried out on any available source of dry heat.
3. Easy to use.

To conclude the Kinyon's method described here is slightly longer to perform than the conventional Ziehl-Neelsen technique but its ease of use and equal efficacy with ZN method make it universally applicable, especially in those laboratories where facilities are poor.

6. Conflict of Interest

The authors declare no relevant conflicts of interest.

7. Source of Funding

None.

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