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MEDICINE

Introducing a Cold Staining technique for demonstrating Acid-Fast Bacilli under field conditions

P. A-K. ADDY, Ph.D., M.Sc. (Med. Microbiology), D.T.M. and P., Dip. Biol., Cert MDS., M.G.I.M.

P. AIKINS-BEKOE, A.I.M.L.T

DEPARTMENT OF CLINICAL MICRO-BIOLOGY,
SCHOOL OF MEDICAL SCIENCES,
UNIVERSITY OF SCIENCE AND TECHNOLOGY,
KUMASI, GHANA.

ABSTRACT

Tuberculosis which is a public health menace in many a developing country has been diagnosed over the years by the Conventional Ziehl-Neelsen's acid-fast staining technique. This technique, however, is tedious, cumbersome and time-consuming. It moreover, does not lend itself to application in the field and, therefore, unsuitable for tuberculosis surveillance work. (eg. Primary Health Care Programme).

The Cold-Acid-fast (C-ZN) staining method reported here has succeeded in correcting some of the short-comings of the Conventional Ziehl-Neelsen (Conv-Zn) technique for demonstrating acid fast bacilli. A field kit has been designed and it is here introduced. The concordance (correlation) between the Cold-Acid fast and the Conventional Ziehl-Neelsen's staining techniques was 93.2%.

Keywords: Tuberculosis, Diagnosis, Field, Technique, Introduction.

INTRODUCTION

Tuberculosis is still a public health problem in many a developing country, despite efforts being made to reduce the level of morbidity and mortality due to this condition by mass vaccination of neonates, small children and susceptible adults with B.C.G. [1]. Effective treatment of tuberculosis is now a problem on account of the development of drug resistance by the causal organism, M. tuberculosis (2, 3), making it therefore, imperative that an early and unequivocal diagnosis of the condition be made to ensure prompt and effective treatment and prevention of spread of the disease to susceptible individuals and the community.

Unfortunately, in developing countries, particularly in the rural areas, where morbidity and mortality figures for tuberculosis are high, quick and reliable supportive laboratory diagnostic facilities are, in most cases, not available. Direct microscopy of sputum smears continues to be the basic microbiological technique in many a developing country with occasional use of culture methods. The conventional Ziehl-Neelsen's (Conv-ZN) method has been the mainstay for demonstrating the tubercle bacillus in sputum smears. However, it is time-consuming and cumbersome [4] and does not lend itself readily to employment under field conditions. It is, therefore, not relied upon for data collection in the field [3].

It is to correct some of the "defects" of the Conv-ZN technique that the authors of this report have developed this new variant which does not make use of heat. A field kit that may be employed in epidemiological studies of tuberculosis has also been designed. The new technique is simple, easy to perform, and is time-saving in terms of time saved on transportation to the laboratory for staining. The new technique described here is very reliable. The avoidance of the use of heat, an essential ingredient in the Conv-ZN method, does not sacrifice the safety of the operator.

This new method is also less expensive in terms of reagents which once prepared, are stable and can be used for a long time under field conditions.

MATERIALS AND METHODS

Source and Pathological state of study specimens

Sputum samples collected over a four month period from 117 (40 in-patients and 77 out-patients) tuberculosis-suspected patients were prepared in duplicate on clean microscope slides. One batch of smears was stained by the new staining method being introduce, ie. the cold acid-fast staining(C-ZN) method, and other by the Conv-ZN method as described by Baker [5].

Cultures for mycobacteria were made from sediments of each sputum sample which had previously been decontaminated with 4% sodium

Table 1: A COMPARISON OF POSITIVE SPECIMENS BY THE COLD ZN TEST AND THE CONV.ZN. TEST

TYPE OF SPUTUM SPECIMEN TESTED	NUMBER OF SPUTUM SAMPLES STUDIED	COLD-ZIEHL NEELSEN'S METHOD				CONVENTIONAL-ZIEHL NEELSEN'S METHOD			
		Pos.	Neg.	% Pos.	% Pos. of total Tested	Pos.	Neg.	% Pos.	% Pos. of Total Tested
Mucoid	75	75	0	100	64.0	70	5	93.3	59.8
Bloody	12	12	0	100	10.3	11	1	91.7	9.4
Salivary	7	7	0	100	6.0	6	1	85.7	5.1
Purulent	23	23	0	100	19.7	22	1	95.7	18.8
Total	117	117	0	100	100.0	109	8	93.2	93.2

hydroxide (digestant) for 15 minutes at 37°C as described by Canetti and Grosset [6]. Each sediment was inoculated into two tubes of Lowenstein Jensen medium and incubated at 37°C for 8 weeks after which a sputum is declared negative when no specific colonies are observed.

Sputum samples were categorized (according to their pathological states) as purulent, salivary, bloody or mucoid. Their respective proportions are shown in Table 1.

For controls, 10 sputum samples were collected from healthy individuals and treated in the same manner as for those from suspected tuberculosis patients.

Stains

1. Preparation of Stain I:

Strong Alcoholized Carbol Fuchsin with Teepol:

a) A mixture of 25.0 grams basic carbol fuchsin powder and 50 gms. phenol crystals is heated in a conical pyrex flask to completely dissolve the mixture.

b) To the dissolved mixture is added 25 ml. absolute ethyl alcohol followed by the addition of 500ml distilled water. Finally, 0.5 ml. of Teepol (Shell Chemical Ltd.) is added.

c) The whole is mixed thoroughly, filtered through Whatman Filter Paper No.1, bottled in a plastic washing bottle (Fig.1) and labelled.

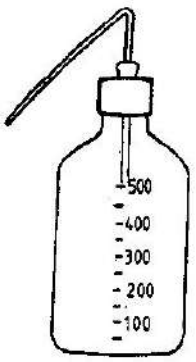

FIG. 1: WASHING BOTTLE (500ml)

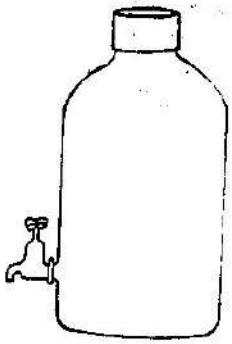

FIG. 2: ASPIRATOR BOTTLE WITH STOPCORK

2. Preparation of Stain II

Loeffler's Methylene Blue in Acid-alcohol

a) Into a one-litre measuring cylinder is delivered 170 ml. absolute ethyl alcohol to which is then added, very slowly, 40 ml. concentrated sulphuric acid. When this solution cools down, 290 ml. distilled water is added.

b) Into a second one-litre measuring cylinder, 3 gms. Loeffler's methylene blue powder is measured.

c) The acid-alcohol solution from the first measuring cylinder (in step 2a) is poured onto the 3 gms. Loeffler's methylene blue powder and mixed thoroughly. This is then filtered through Whatman Filter Paper No.1, bottled in a plastic washing bottle (Fig. 1) and labelled.

Fixative

Formaldehyde - Ether - Absolute Ethyl Alcohol ("FEA") Fixative

To 50 ml. absolute or 95% ethyl alcohol is added 40 ml. ethyl ether or anaesthetic ether (May and Baker Ltd.) and 10 ml. formalin or formaldehyde. This is shaken well to mix and stored in a labelled brown screw-capped bottle.

The Staining Procedure

Sputum smears are made on clean microscope slides, arranged on the grid (Fig.4) which is mounted in the Fluid Collector (Fig.3), and fixed with "FEA" fixative.

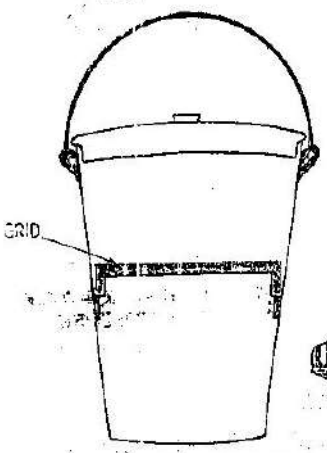

FIG. 3: FUID COLLECTOR WITH GRID

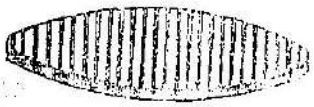

FIG. 4: GRID

Slides which are allowed to dry in air in the Fluid Collector are then flooded with Stain I for 8 minutes at the prevailing field temperature. Slides are washed with water from a plastic Washing Bottle (Fig.1).

Slides are now flooded with Stain II and the stain is left to act for 2-3 minutes and then washed off with water from the Washing Bottle. The slides are dried in air and are examined with the oil-immersion objective of a field light microscope.

The Washing Bottle may be refilled with water from an Aspirator Bottle (Fig.2).

RESULTS:

Acid-fast bacilli appeared bright red against a blue background. All other micro-organisms and tissue cells stained light blue to deep blue.

Sixty-four per cent (64.0%) of the 117 specimens examined were mucoid, while 10.0%, 6.0% and 20.0%, respectively, were bloody, salivary and purulent. The results obtained using the two test methods (Conv.-ZN and C-ZN) are shown in Table 1.

It can be seen from this table that whilst the Conv-ZN method detected 109 of 117 (92.3%) tuberculosis cases, the C-ZN method was able to detect all 177 (100%) tuberculosis cases, including 8 samples which were negative by the Conv-ZN method. These 8 specimens were also culture positive, thus endorsing the positive C-ZN staining results.

Table 2 expresses the results as the number of specimens positive with the Conv-ZN and C-ZN methods, negative with both methods, positive with Conv-ZN but negative with C-ZN and, negative with Conv-ZN and positive with C-ZN.

The Concordance between the Conv-ZN and the C-ZN methods was calculated using the ratio:

$$\frac{\text{Number of sample positive by the two test methods} + \text{Number of negative samples by the two test methods}}{\text{the total number of tested samples}} \times 100$$

Thus the calculated concordance is 93.2%. Concordance levels in relation to the pathological states of the specimens tested are also shown in Table 2.

All ten (10) control specimens collected from healthy individuals and tested by both the Conv-ZN and the C-ZN methods were not only AFB negative but also culture negative.

TABLE 1: CORRELATION BETWEEN CONVENTIONAL ZIEHL-NEELSEN AND COLD ACID-FAST STAINING TECHNIQUES FOR DEMONSTRATING ACID-FAST BACILLI

SPUTUM SAMPLES		RESULTS				
TYPES COLLECTED	TOTAL TESTED	+Conv-ZN +C-ZN	-Conv-ZN -C-ZN	+Conv-ZN -C-ZN	-Conv-ZN +C-ZN	Concordance %
MUCOID	75	70	0	0	5	93.3
BLOODY	12	11	0	0	1	91.7
SALIVARY	7	6	0	0	1	85.7
PURULENT	23	22	0	0	1	95.7
TOTAL	117	109	0	0	8	93.2

From the results it could be deduced that the level of AFB positivity, irrespective of which of the two test methods was used, was related to the pathological state of the specimen. Thus, mucoid specimens yielded the highest (64.1%) AFB and culture positive samples while the rates of AFB and culture positivity for purulent, bloody and salivary specimens were found to be 20.1%, 10.2% and 5.7%, respectively.

DISCUSSION

The Ziehl-Neelsen's technique for staining acid-fast becilli is the most universally and often the only commonly employed staining method for the diagnosis of tuberculosis in most developing countries. This useful staining method, however, has a number of drawbacks, the chief being the application of heat, a procedure which requires precise temperature regulation for success to be achieved. Because of these drawbacks various attempts have been made at various times and by various medical scientists, eg. Gabbet [7], Hok [8], Kinyoun [9], Rao, et al. [10] and recently by Wasanthakumari et al. [11], to develop a method that to some reasonable extent minimizes the said drawbacks.

The cold Ziehl-Neelsen's staining technique developed by us and described in this paper undoubtedly eliminates not only the said drawbacks, but also adds to the safety of acid-fast staining in the field by untrained personnel.

Further advantages to be derived from this new method are as follows:

a) The application of regulated heat to enhance penetration of the dye into the substance of the tubercle bacillus is unnecessary in our staining method. Heat application requires fairly precise temperature control, the operation of which must be performed by an experienced laboratory technician/technologist. Furthermore, more often than not, the heat application method cannot be performed because of lack of rectified spirit [11].

b) The method is suitable for application in the field and therefore, for public health work. To facilitate this, therefore, a kit (depicted in Figs. 1 - 4) for field work has been designed and tested under field conditions. One major advantage of the kit is that the field "Fluid Collector" (Fig.3) ensures that no microorganisms are left behind in gutters or drains or on the ground in a field TB-study. At the end of the day's work in the field, the "Field Collector" is covered with its lid and conveyed safely back to the laboratory for autoclaving. It is then ready for use the next day.
The Aspirator Bottle (Fig.4) is an added advantage for it ensures that clean water is available for use in the field.

c) Unlike the conventional method this new method which can be employed in the field can be relied upon for field data collection purposes. The results obtained by application of the new staining (C-ZN) method correlate well with bacterial isolation frequencies.

d) This Acid-fast bacilli staining method is less expensive, easier to perform, is time-saving in terms of time saved on transportation of specimens to the laboratory for staining, and clinically reliable results are obtainable.

e) this method also has the advantage of minimizing the dangers attendant upon transportation of sputum specimens from the collection point to a central laboratory for processing.

f) furthermore, the addition of cetylpyridium bromide or chloride (for transit digestion and decontamination) to sputum samples which take a long time to get to the processing station or laboratory is eliminated by the use of our staining method.

g) Rural laboratories in developing countries which find it difficult obtaining spirit or gas for the Conventional ZN test, can make use of the new method for laboratory diagnosis of tuberculosis.

h) And finally, this staining method can also be used for demonstrating oocysts of Cryptosporidium in faecal materials.

REFERENCES

1. *Bjarkerdel, T. Host-agent interaction in experimental tuberculosis in guinea pigs, with special reference to the effects of BCG. Vaccination. American Journal of Hygiene, 79: 86 - 106, 1964*

2. *Mitchison, D.A. Drug Resistance in Mycobacteria. British Med. Bull. 40/1: 741 - 743, 1986.*

3. East African and British Councils. Tuberculosis in Kenya: A National sampling survey of drug resistance and other factors. Tubercle, 49: 136 - 169, 1968.

4. Kani, K. The staining properties of isolated cellular component as revealed by Ziehl-Neelsen procedure. American Review of Respiratory Diseases, 85: 442 -443, 1962.

5. Baker, F.G. Handbook of Bacteriological Techniques, 2nd ed. London, Butterworths, 1967.

6. Canett G. and Grosset, J. Techniques et indications des examens Bacteriologique in tuberculose. Ed. de la Tourelle, Paris, 1968.

7. Gabbet, H.S. Lancet, 1: 757, 1987.

8. Hok, T.T. Simple and rapid cold staining method for acid-fast bacteria. American Review of Respiratory Diseases, 85: 753 - 754, 1962.

9. Kinyoun, J.J. American J. Pub. Health, 5: 867, 1915

10. Rao, K.P. et al. A cold staining method for tubercle bacilli using chloroform. Indian Journal of Tuberculosis, 14: 3-7, 1966.

11. Vasanthakumari, R.; Jagannath, K. and Rajasekaran, S. A cold staining method for acid-fast bacilli. Bulletin of the World Health Organization, 64/5: 741 - 743, 1986.