International Journal of Research in Health Sciences
ISSN: 2321-7251
Available online at: http://www.ijrhs.org/
Original Article

Comparison of Ziehl Neelsen (ZN) and Auramine Phenol (AP) staining method to detect acid-fast bacilli in sputum smear

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Received: 10-07-2017 / Revised Accepted: 09-10-2017 / Published: 03-01-2018

ABSTRACT

Microscopic examination of sputum samples for the presence of Acid fast bacilli is still the mainstay for the rapid presumptive diagnosis of pulmonary tuberculosis though it lacks sensitivity. The objective of this study was to compare Ziehl Neelsen (ZN) staining with Auramine fluorochrome staining in the diagnosis of tuberculosis (TB), to improve upon the sensitivity of sputum smear microscopy. All the positive smears by auramine staining were stained with ZN stain, for confirmation. A total 408 out of 2030 samples were smear positive. In the present study the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the auramine staining Vs ZN staining (under RNTCP) was found to be 100 vs. 85.8%, 99.8 vs 100%, 99.5 vs 100% and 100 vs 96.3% respectively. The present study clearly demonstrates the high sensitivity of auramine staining in comparison to ZN staining, though ZN staining is slightly more specific than the auramine staining for demonstration of acid fast bacilli in the direct sputum smear.

Key Words: AFB; ZN staining; Auramine; sputum; smear

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How to Cite this Article: Deepinder Chhina, Rama Gupta and Ashish Chawla. Comparison of Ziehl Neelsen (ZN) and Auramine Phenol (AP) staining method to detect acid-fast bacilli in sputum smear. Int J Res Health Sci 2018; 6(1): 1-5.

INTRODUCTION

In India, Revised National Tuberculosis Control Programme (RNTCP) was initiated in 1997. The whole country was covered under RNTCP in March 2006. The two objectives of the program are: (a) a cure rate of at least 85% in new sputum smear positive cases of tuberculosis (TB) and (b) to detect at least 70% of such cases in the community. The state of Punjab has been achieving the objective of cure rate but is lagging behind as far as case detection is concerned. [1]

Sputum smear microscopy has a fundamental role in the diagnosis of pulmonary tuberculosis (PTB) under RNTCP program. It is easy to perform at peripheral laboratories and is not expensive. If good diagnostic practices are followed, it is expected that at least 50% of the new patients diagnosed will be smear positive. Further, it is very important to identify TB suspects and diagnose them early in order to effectively treat them and make them non-infectious, because an untreated sputum smear positive pulmonary tuberculosis patient can infect 10-15 persons annually. [2]

Direct microscopy using ZN Staining for AFB is currently the most commonly used microbiological method for the diagnosis and confirmation of PTB and when positive, identifies the contagious cases for the community. [3-4] This method is highly specific and till date, the most rapid and economical method used for detection of AFB in sputum. The only disadvantage of this method is low sensitivity (varying from 50%-80%) relative to culture. [4-8]

Recently, there have been studies indicating that fluorochrome staining (either auramine phenol or auramine rhodamine) of smear for AFB, significantly increases the sensitivity of direct microscopy. [5, 9-10] Although several research groups have investigated the clinical validity and differences in sensitivity between various staining methods, the technical and procedural factors can influence the sensitivity of each staining method. [5, 11] However, there is paucity of data from our country on this aspect of diagnosis of tuberculosis. Therefore, the study was planned to assess the efficacy of fluorescent microscopy in comparison to ZN staining, to increase the case detection rate in the community under RNTCP program.

METHODOLOGY

Study Design: A longitudinal study incorporating five Urban Designated Microscopic Centers (DMCs) working under Distt Ludhiana with a slide positivity rate (SPR) of 9-10% and annual negative slide volume (ANSV) of approximately 500 slides, was carried out at Department of Microbiology, Dayanand Medical College & hospital, Ludhiana.

Designated Microscopy Centre	SPR	ANSV
Civil Hospital Ludhiana (LMCH)	15	9674
Chest clinic – Midhachowk (MC)	12	1777
Salem Tabri, Ludhiana (ST)	11	1913
ESI Model hospital, Ludhiana (ESIC)	9	5063
DMC&H, Ludhiana	16	3168

Sampling: Every 5th tuberculosis suspect reporting to any of the five microscopy centers was included in the study. After receiving the sputum samples (a, b), two sputum smears were made from each sample. One of the smear was stained with ZN stain for routine reporting under RNTCP at respective microscopy center. The second smear after fixation was transported to Department of Microbiology, DMCH Ludhiana for auramine phenol staining. The slides received from various microscopy centers were stained using auramine phenol staining methods. The smears found positive with the auramine staining were destained and then stained with ZN stain for confirmation. Both ZN and auramine phenol staining were done by methods as recommended by WHO. RNTCP guidelines were used for grading the smears. [2] Positive and negative control slides were included with each staining batch for internal quality control of the staining methods.

Statistical Analysis: The results obtained from ZN staining (under RNTCP), the auramine-phenol staining and ZN staining (under project), were compared. Sensitivity, specificity, positive predictive values and negative predictive values for each method will be calculated.

RESULTS

A total of 2030 slides (2 slides each from 945 patients and one slide each from 202 patients) form 1101 patients were received during the study period. (Table I) A total of 234 patients (21.2%) were found to be positive. Out of these 200 patients were reported positive both with auramine staining (under the project) and ZN staining (performed at

various microscopy centers under RNTCP). Whereas 34 patients were positive only with fluorescent microscopy. Out of these 34 patients, 32 were positive with subsequent ZN staining which was performed after destaining of auramine stained slides at Dayanand Medical College & Hospital, under the project. Two patients were negative with subsequent ZN staining. However, it was found that one of the patients was already on ATT, and was considered false negative by ZN staining performed under the project. The other patient was asked to report after 15 days for fresh sample, but did not reported, and was considered false positive by auramine staining. (Table II)

Hence a positivity of 21.3% and 18.2% was observed with fluorescent microscopy and ZN staining (under RNTCP) respectively. However, with subsequent ZN staining (unde project) of the Auramine stained slides (After destaining) and careful examination of these slides under light microscope confirmed the presence of acid fast organism in all except two of the patients found positive by previous auramine staining, hence giving a positivity of 21.1%. (Table III)

Table IV shows the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the auramine and ZN staining. In the present study the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the auramine staining was found to be 100%, 99.8%, 99.5% and 100% respectively, whereas sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the ZN staining (under RNTCP) was 85.8%, 100%, 100% and 96.3% respectively. However, the results of ZN staining carried out after destaining of the auramine stained slides at DMC & H during the project were comparable with the results of aurmine staining with a sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of 99.5%, 100%, 100% and 99.8% respectively.

Discussion:

World Health Organization (WHO), has estimated immense global burden of disability and death due to tuberculosis (TB) *w*ith an estimate of three million deaths, annually. The HIV-TB co-infection has further increased the morbidity and mortality. [9] India accounts for one- fifth of the world's new TB cases with an estimated 1.9 million cases occurring annually and around 0.9 million have sputum positive pulmonary TB. [12] Conventional Smear microscopy for AFB using ZN staining still remains the mainstay for diagnosis and monitoring treatment of TB as it is simple, inexpensive, widely applicable and highly specific for TB, specifically in developing countries, where the disease is highly endemic, but as the sensitivity of direct AFB smear (ZN stain) is low (50-80%), there is an urgent need to improve the sensitivity of AFB smear microscopy. [5-7] Recently, there have been studies indicating that fluorochrome staining of smear for AFB significantly increases the sensitivity of direct microscopy. The higher sensitivity of this method is attributed to the ease of the detection of a fluorescent rod against a dark background using fluorescent microscope. Therefore, the microbiologist can scan the slide at a lower magnification and observe a larger area than with ZN smears. [9-10]

Habtamu *et al* (2012) observed that, of the 267 sputum samples examined, 74 (28%) and 48 (18%) were acid-fast bacilli (AFB) positive by the, direct auramine and ZN methods, respectively. Direct auramine method ($P = 0.46$), was superior to the ZN microscopy ($P < 0.001$). and yielded 26 (10%) more positives. Also Fluorescent microscopy required a shorter smear reading time (1.5 min on average), while the light microscope took 4 min ($P < 0.001$). They concluded that Fluorescent microscopy with direct smear preparation is rapid and effective. [13]

Hooja *et al* (2011) observed a sensitivity of 55.55% for ZN and 71.85% for auramine for the detection of acid fast bacilli, using direct microscopy. Direct fluorescent microscopy detected 9.29% paucibacillary sputum samples that were missed on ZN staining. On concentration, the sensitivity further increased by 6.67% for ZN and 11.11% for auramine. The sensitivity of AFB smear microscopy increased by 27.41% and was statistically significant ($p = < .001$) when both methods were combined. The specificity was 99.19% for both ZN and auramine. They concluded that Fluorescent microscopy has higher sensitivity and comparable specificity which is further enhanced by concentration.(12) In the present study the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the auramine staining was found to be 100%, 99.8%, 99.5% and 100% respectively, whereas sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the ZN staining (Performed at various DMC's under RNTCP) was 85.8%, 100%, 100% and 96.3% respectively; wheras, the results of ZN staining carried out after destaining of the auramine stained slides at DMC & H during the project were comparable with the results of aurmine staining with a sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of 99.7%, 100%, 100% and 99.9% respectively.

However, these obsevations on ZN staining were biased.

Oromocon and Ekuka (2010), in a cross sectional study comparing the performance of Ziehl Neelsen staining and auramine fluorescent microscopy staining techniques in detecting the presence of *Mycobacterium tuberculosis* in sputum, showed that both Ziehl Neelsen and auramine preparations showed 5.6% of positive cases, however, 20% of cases showed false positive with Ziehl Neelsen concentrate technique and 30% showed false positive with auramine concentrate preparation. There was a significant relationships in the performance of ZN and auramine techniques in the detection of AFB, although auramine showed a greater false positive than Ziehl Neelsen method in the detection of AFB . [13] Similarily Musthafa and Syed (2010), have compared the results of the fluorescence microscopy using auramine stain with that of light microscopy using Ziehl- Neelsen (ZN) stain. No difference in the case detection has been reported by both the methods in their study. However it has been analysed that the Fluorescence microscopy offers well described benefits, compared to light microscopy for the evaluation sputum smear samples for tuberculosis, as the mean time to read a negative smear was 2 minutes with fluorescence microscopy and 5 minutes with light microscopy. This reflects a time saving of 60% in fluorescence microscopy. [14]

The present study concludes higher sensitivity of auramine staining for demonstration of acid fast bacilli in the direct sputum smear in comparison to the ZN staining carried out at RNTCP microscopy centers in routine. In addition fluorescent microscopy decreases the time of observation. However the quality of stain/ staining, quality of the examination (Strict adherence to the guidelines), microscope operator expertise remains the factors to be standarised, as careful examination of ZN stained smear previously reported as negative revealed the presence of AFB, though that will further increase the screening time.

Acknowledgement: The study was carried out under operation research project, funded by RNTCP.
Conflict of interest: None

Table I: Distribution of patients/ sputum samples received from various microscopy centers.

MICROSCOPY CENTER	PATIENTS ENROLLED (2 Slides/ 1 Slide)	SLIDE RECEIVED
DMC&H	308 (303/5)	611
LMCH	473 (344/129)	817
ESI	218 (202/16)	404
MIDHA CHOWK	48 (48)	96
SALEM TABRI	54 (48/6)	102
Total	1101 (945/ 202)	2030

Table II: Comparison of ZN staining (Performed at various microscopy centres under RNTCP) and Auramine phenol staining (Performed at DMC &H, Ludhiana under Project).

Microscopy Center	Number of Patients	ZN Positive	Auramine Positive	AURAMINE & ZN +VE	AURAMINE +VE & ZN -VE	Positive Patients (By any Method)
DMC&H	308	68	79	68	11	79
LMCH	473	80	99	80	19	99
ESI	218	40	42	40	2	42
MIDHA CHOWK	48	5	5	5	0	5
SALEM TABRI	54	7	9	7	2	9
Total	1101	200	234	200	34	234

Table III: Comparison of ZN staining and Auramine phenol staining (Performed at DMC &H, Ludhiana under Project).

Microscopy Centre	Number of Patients	ZN Positive	Auramine Positive	Positive Patients (By any Method)
DMC&H	308	79	79	79
LMCH	473	97	99	99
ESI	218	42	42	42
MIDHA CHOWK	48	5	5	5
SALEM TABRI	54	9	9	9
Total	1101	232	234	234

Table IV: Statistical analysis (N=1101)

	Auramine Staining	ZN staining (RNTCP)	ZN Staining (Project)
True Positive	233	200	232
False Positive	1	0	0
True Negative	867	868	868
False Negative	0	33	1
Sensitivity (%)	100	85.8	99.5
Specificity (%)	99.8	100	100
PPV	99.5	100	100
NPV	100	96.3	99.8

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