ORIGINAL ARTICLE

Auramine-O Staining vs Ziehl Neelsen Staining: Advantages and Disadvantages

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Abstract

Background: Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*Mtb*) is still a major public health concern around the world. Prompt detection of active tuberculosis cases helps in timely therapeutic intervention and reduces community transmission. Despite limited sensitivity, conventional microscopy is still used to diagnose pulmonary tuberculosis in high-burden nations such as India. This study, therefore, was aimed at assessing the diagnostic performance of microscopy by Ziehl Neelsen (ZN) and auramine (AO) staining in the diagnosis of pulmonary tuberculosis.

Materials and methods: A prospective comparative study was done on the sputum samples of 2,395 adult patients from November 2018 to May 2020 suspected of having pulmonary tuberculosis visiting the Designated Microscopic Centre of SGT Medical College, Budhera, Gurugram. Each sample was subjected to ZN staining, and AO staining as per NTEP guidelines.

Results: Out of the 2,395 samples studied, 161 (6.76%) and 224 (9.35%) were positive by ZN and AO staining methods respectively. Pauci-bacillary cases detected by AO were more than ZN staining. There were 63 more sputum samples detected by AO staining which were missed by ZN microscopy.

Conclusion: When compared to conventional ZN staining, the auramine staining technique is more sensitive and takes less time to diagnose pulmonary tuberculosis.

Keywords: Acid-fast bacilli smear microscopy, Cartridge-based nucleic acid amplification test, Pulmonary.

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Abbreviations Used in this Article

AFB = Acid fast bacilli; AO = Auramine-O; AR = Auramine Rhodamine; ERC = Ethical Research Committee; LED-FM = light-emitting diode fluorescent microscopy; MGIT = mycobacterial growth indicator tube; *Mtb* = *Mycobacterium tuberculosis*; TB = Tuberculosis; ZN = Ziehl-Neelsen.

Introduction

Tuberculosis (TB) is an infectious disease, caused by *Mycobacterium tuberculosis* (*Mtb*).[1] It is the second most frequent cause of death worldwide, with an incidence of 10 million new cases per year.[1,2]

One-fourth of the world's population is infected with latent tuberculosis.[3] Out of these cases 10% develop active infection, particularly in diabetic or HIV-positive patients or those undergoing immunotherapy.[4,5]

Chronic cough, blood in the sputum, fever, weight loss, and night sweats which may be moderate for many months are all signs of active TB. Delays in treatment may spread the disease to other people. When an infected individual coughs or sneezes, respiratory secretions in the form of droplets transmit the TB *bacilli.*

Mycobacterium tuberculosis can penetrate and proliferate within the endosomes of pulmonary alveolar macrophages resulting in clinically active disease in about 10% of cases whereas the remaining cases can be arrested by a competent immune response.[6,7] The *bacilli* are, however, totally eliminated from those with arrested cases in around 10% of the people, with the remaining 90% going into a dormant or latent stage where the infection is contained. The pathogens escape the microbicidal action of host immune cells latent TB and the dormant *bacilli* are reactivated whenever the host immune system is seriously compromised (by HIV infection, diabetes mellitus, renal failure, chemotherapy, immunosuppressive therapy, malnutrition, etc.).[7] Over the course of a year, people with active tuberculosis can infect 5–15 other people through close contact. Other organs, in addition to the lungs, can get infected and cause a wide range of symptoms.[8]

The current trend of globalization makes the world as a whole more vulnerable to TB infection than just the high-incidence nations. Therefore, it is urgent to develop rapid and effective diagnostic technologies for TB. Culture is still the gold standard, although results might take weeks, whereas smear microscopy enables quick diagnosis but with poor sensitivity.[9] Smear microscopy is a simpler, less expensive, and more accurate method of detecting and diagnosing pulmonary tuberculosis. It is essential to effectively treat

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those who have the disease and stop it from spreading across the community.[10] The diagnosis of tuberculosis can also be assisted by a number of tests which includes chest radiographs, mycobacteria cultures, and nucleic acid amplification assays.[11]

Mycobacteria can be identified in less than an hour by microscopic examination of Ziehl-Neelsen (ZN) or auramine-O (AO) stained samples. Ziehl-Neelsen staining is the most frequently used method to demonstrate *Mtb* in a smear. Fluorescent staining is the other staining technique where sputum samples are stained by Auramine. The fluorescent staining technique is simpler, and the smear can be examined under higher magnification than ZN. Fluorescent microscopy (FM) is 75% faster than bright field microscopy.[12]

This usefulness is of enormous benefit in many low-resource settings where laboratories are overburdened. Therefore, the present prospective study was undertaken to analyze the sensitivity of fluorescent staining and ZN staining in the diagnosis of pulmonary tuberculosis.

Materials and Methods

This prospective comparative study was done in the Department of Microbiology and Department of Pulmonary Medicine, Faculty of Medicine and Health Sciences, SGT University, Budhera, Gurugram, Haryana, India. The period of the study was from November 2018 to May 2020 and was approved by the Ethical Research Committee (ERC). Written informed consent was obtained from all participants for the use of their sputum for TB diagnostics and research. Samples from both outpatients and inpatients were included in the study.

Inclusion Criteria

Study subjects included patients with clinical suspicion of pulmonary tuberculosis including symptoms of cough with or without expectoration for >2 weeks with evening rise in temperature and/or/weight loss, fatigue, hemoptysis, and loss of appetite. Patients with or without previous history of tuberculosis and patients who were referred from ICTC (Integrated Counselling and Testing Centre) were included in the study.

Exclusion Criteria

- Patients ≤14 years of age
- Patients already on Antitubercular Treatment (ATT)

Sample Collection

Early morning sputum sample (spot sample if morning sample was unavailable) was collected from 2,395 consecutive patients during the period of study, in clean, sterile, leakproof, wide mouth containers. Two sputum smears were made on glass slides, one for ZN stain and another for Auramine stain. A proforma/questionnaire was used to collect data from the patients at the time of sample collection.

Results

During the study period of 19 months from November 2018 to May 2020 a total of 2,395 adult patients were suspected to have pulmonary tuberculosis based on history, clinical examination, and chest X-ray which were included in the study. Out of the 2,395 patients enrolled, there were 1,581 males (66%) and 814 females (34%). Among these sputum samples, 161 (6.76%) and 224 (9.35%) were found to be positive for acid-fast bacilli by ZN and Auramine O staining respectively. There was no such sample positive by ZN staining which was negative by AO staining. The ZN and Auramine staining positivity rate in this study was 6.76% and 9.35% respectively (Table 1). Out of 161 acid fast bacilli (AFB) positive patients diagnosed by ZN staining, 121 (74.69 %) were males, and 40 (24.8%) patients were females however AO staining detected 172 (76.8%) males and 52 (23.2%) females as AFB-positive. The mean (±SD) age of patients was 45.05 (±18.51) years and ranged from 14 to 89 years (Table 2).

Discussion

The processing of samples was carried out in a biosafety cabinet. Staining was done as per the World Health Organization (WHO) guidelines. Stained smears were examined by a light microscope (Olympus CX21) under a 100X oil immersion lens. The AFB was seen as red-beaded rods against the blue background.

Another method of staining used was fluorescent staining by Auramine O dye. The stained smears were visualized under Fluorescent Microscope (Labomed Lx 400 eFL). The tubercle bacilli appeared as yellow luminous slightly curved rods on a green background. The main advantage of fluorescence microscopy is that it uses a high-power (40X) objective. The field seen is thus many times larger than that seen in conventional bright-field microscopy through an oil-immersion objective. In fluorescence microscopy the field is about 0.34 mm, whereas that seen with an oil-immersion objective is only about 0.02 mm.[2] Fluorescence microscopy allows the same area of a smear to be scanned in a much shorter time than

Table 1: Comparison of ZN staining and Auramine staining (n = 2,395)

Methods used	*Number of positive smears*	*Number of negative smears*
ZN staining	161 (6.76%)	2,234 (93.23%)
Auramine staining	224 (9.35%)	2,171 (90.64%)

Table 2: Demographic features of patients enrolled and AFB-positive patients

	Total patients enrolled		*AFB positive by ZN staining*		*AFB positive by AO staining*	
Age-group	*Male (%)*	*Female (%)*	*Male/total male enrolled in this group (%)*	*Female/total female enrolled in this group (%)*	*Male/total male enrolled in this group (%)*	*Female/total female enrolled in this group (%)*
14–20 years	169 (10.7)	112 (13.7)	14/169 (8.3)	4/112 (3.6)	18/169 (10.7)	7/112 (6.2)
21–40 years	562 (35.5)	310 (38.1)	54/562 (9.6)	18/310 (5.8)	70/562 (12.5)	22/310 (7.1)
41–60 years	490 (31)	221 (27.1)	40/490 (8.2)	10/221 (4.5)	52/490 (10.6)	13/221 (5.9)
61–80 years	334 (21.1)	159 (19.5)	13/334 (3.9)	6/159 (3.8)	32/334 (9.6)	8/159 (5.03)
Above 80 years	26 (1.6)	12 (1.5)	0/26 (0)	2/12 (16.7)	0/26 (0)	2/12 (16.7)
Total	1,581 (66)	814 (34)	121 (7.6)	40 (4.9)	172 (10.9)	52 (6.4)

Table 3: Studies similar with the current study

S No.	Year of study	Place of study	Authors	Total samples	Positive by ZN staining	Positive by AO staining
1	2020	Warangal, India	Padmaja et al.[22]	188	63 (33.5%)	77 (40.9%)
2	2020	Ethiopia, Africa	Gizaw et al.[18]	346	50 (14.5%)	72 (20.8%)
3	2019	Aligarh, India	Ahmed et al.[17]	1,503	781 (51.96%)	904 (60.15%)
4	2019	Kade, Ghana	Dzodanu et al.[23]	200	46 (23.0%)	71 (35.5%)
5	2018	Sudan, Africa	Ahmed[16]	241	52 (21.5%)	62 (25.7%)
6	2015	Mumbai, India	Bhadade et al.[21]	800	33 (4.12%)	130 (16.25%)
7	2015	Kathamandu, India	Timalsina et al.[24]	299	57 (19.06%)	87 (29.1%)
8	2014	Chinnakakani, India	Mamilla and Suhasini[20]	500	60 (12%)	99 (19.8%)
9	2013	Muzaffarnagar, India	Goyal and Kumar[25]	388	29 (7.47%)	57 (14.69%)
10	2012	Chennai, India	Kumar et al.[26]	225	28 (12.4%)	43 (19.1%)
11	2009	Manipur, India	Laifangbam et al.[27]	306	45 (44.1%)	73 (71.6%)
12	2022	Gurugram, India	Current study	2,395	161 (6.72%)	224 (9.35%)

can be achieved by conventional microscopy after staining with the Ziehl-Neelsen technique.[13] Grading of slides was done as per the International Union Against Tuberculosis and Lung Disease (IUATLD)

The sensitivity of smear microscopy is highly variable, and sometimes almost half of the positive smears are reported as negative due to low sensitivity.[14] As a consequence, many TB patients remained undetected by smear microscopy and no anti-TB treatment can be initiated. Not only the patient is deprived of the anti-TB treatment but it also results in the spread of infection to others, whether in a hospital or in the community. In most cases, low sensitivity is due to failure of detecting "scanty" positive smears with very few *bacilli* present, and only 10% of the smeared area is examined using commonly recommended practice.[15]

In the present study out of 2,395 samples examined, 161 (6.72%) and 224 (9.35%) TB cases were detected by ZN and AO staining methods respectively (Table 1). It was observed that a total of 63 sputum smears that were negative by the ZN staining method were positive by AO staining. There was no such sample positive by ZN stain which was negative by AO. Similar results have been reported by Ahmed.[16] in Sudan (Africa). They examined two hundred forty-one sputum samples by ZN and AO Staining techniques and found that 52 (21.75%) of them showed the characteristics of AFB appearance of serpentine cords under oil immersion field while 62 (25.7%) sputum specimens showed the typical characteristics of AFB *bacteria* using Auramine staining while 67 (27.8%) were shown *Mtb* detected by cartridge-based nucleic acid amplification test (CBNAAT). A study was conducted by Ahmed et al.[17] in Aligarh. They processed and subjected 1,503 samples of sputum to ZN, AO staining, and solid culture for the detection of acid-fast *bacilli*. Out of 1,503 samples, 781 (51.96%) were found to be AFB positive by ZN stain while 904 (60.15%) samples were positive by AO Staining, and 843 (56.09%) showed growth of mycobacteria on LJ Medium. A cross-sectional study done in Ethiopia by Gizaw et al.[18] on 346 pulmonary TB suspected patients. They evaluated the importance of Auramine-O staining in direct and concentrated sputum against conventional ZN and mycobacterial culture on mycobacterial growth indicator tube (MGIT) 960. Out of 346 samples, 50 (14.5%) sputum samples turned out to be positive by direct ZN staining while 72 (20.8%) and 90 (26%) samples were positive by direct and concentrated AO staining respectively. There were 110 patients who were positive for MGIT 960. One more study by Golia et al.[19] in Bengaluru was conducted on 634 sputum samples, collected from suspected cases of pulmonary tuberculosis. The sputum samples were processed and subjected to ZN and Auramine staining for the detection of TB. Out of 634 sputum smears, 67 (10.57%) and 105 (16.56%) were positive by ZN and AO respectively. A study in Chinnakakani (Andhra Pradesh) by Mamilla and Suhasini.[20] compared the sensitivity of FM staining and ZN staining in diagnosing sputum smear-positive pulmonary tuberculosis. The authors collected 500 sputum samples, processed and subjected them to ZN and FM staining for the detection of AFB. They found 60 (12%) and 99 (19.8%) smears positive by ZN and FM staining respectively. Bhadade et al.[21] conducted a study to determine the utility of light-emitting diode fluorescent microscopy for the diagnosis of pulmonary tuberculosis in 400 HIV-infected patients. They collected two sputum specimens from each patient. Two smears were prepared from each sputum specimen, one was stained with the ZN method and another by the Auramine–O method. There were 130 (16.25) such samples that came out to be positive by the light-emitting diode fluorescent microscopy (LED-FM) method while 33 (4.2) were positive by ZN Method and 77 (9.6) specimens showed growth of *Mtb* on the LJ medium.

All the above studies have similar findings and support the present study. They concluded that AO staining is better than ZN staining (Table 3).

In contrast to this, an ICMR study was done by Madhusudhan and Amrithalingeswaran[28] to compare the ZN stain with fluorescent microscopy and modified cold stain to detect acid-fast bacilli from sputum samples. They concluded that the results of fluorescent microscopy and the modified cold stain method were inferior to the ZN stain, unlike other studies. They mentioned that the reason could be due to the FM and ZN smear being screened by different persons.

A cross-sectional study was conducted by Gelalcha et al.[29] in west-eastern Ethiopia. They diagnosed the sputum samples using LED-fluorescent microscopy (FM), Gene Xpert, concentrated Ziehl-Neelsen (cZN) staining, and Lowenstein–Jensen culture. Out of 362 sputum samples collected and processed 36 (9.9%) samples were positive by LED-FM, 42 (11.6%) samples were positive by cZN staining, and in 50 (13.8%) samples, *Mtb* was detected by Gene Xpert. There were 45 (13.6) samples that were confirmed as mycobacteria by culture. The higher sensitivity of cZN than that of LED-FM observed in this study was due to the concentration of sputum. As concentration has the potential to increase smear sensitivity.

However, Kulkarni et al.[30] in Davangere, Karnataka compared Ziehl-Neelsen staining with acridine orange and auramine Rhodamine (AR) staining for the diagnosis of tuberculosis. They

Table 4: Distribution of slides by grading and methods used

	Positive by	
Grading	*ZN staining*	*Auramine staining*
Scanty	13 (8.02%)	48 (21.42%)
1+	55 (34.16%)	65 (29.01%)
2+	58 (35.80%)	54 (24.10%)
3+	35 (21.60%)	57 (25.44%)
Total	161	224

Table 5: Distribution of paucibacillary and multibacillary slides detected by ZN staining and auramine staining

Staining methods	*No. of Paucibacillary slides*	*No. of Multibacillary slides*	*AFB positive*
ZN staining	68 (42.59%)	93 (57.40%)	161
Auramine staining	113 (50.44%)	111 (49.55%)	224

processed 2,715 sputum samples by ZN, Acridine Orange, and AR staining procedures. Results of the study revealed that 373 (13.73%) samples were smear positive by ZN Staining while 349 (12.85%) and 249 (10.82%) were detected positive by Acridine Orange and AR Staining methods respectively.

We can conclude that the findings of most of the studies where ZN and AO staining methods were compared, remained in favor of our study. In the present study sputum samples of suspected pulmonary tuberculosis patients were processed and subjected to ZN and AO staining and grading was done according to standard WHO criteria. Out of 2,395 sputum smears 161 (6.72%) and 224 (9.35%) were positive by ZN and AO respectively (Table 1). It was observed that a total of 63 sputum smears that were negative by the ZN staining method were positive by AO staining moreover AO staining method with LED microscopy was found to be more efficient over ZN staining in determining the paucibacillary cases. Auramine staining could detect 113 Paucibacillary (scanty, 1+) cases whereas ZN staining detected only 68 of them (Tables 4 and 5). However, there were 111 multibacillary (2+, 3+) cases detected by AO while only 93 cases were detected by ZN staining which proved that paucibacillary cases showed more gain in the missed positive cases by AO staining. The use of AO staining alone could be a reliable microscopic method as there was no smear-positive case by ZN staining where AO was negative. Hence, we can conclude that LED-FM detects paucibacillary cases better than ZN microscopy.

Conclusion

Based on the findings of the present study, we may conclude that microscopy has significance in the diagnosis of tuberculosis in resource-limited nations such as India. Auramine staining has a higher positivity rate, is more sensitive in detecting paucibacillary instances, and is less time-consuming than the ZN staining. The screening of smears in fluorescent microscopy is done at high magnification (40X) and takes less time than the ZN staining method (100X oil immersion) in the diagnosis of tuberculosis. The FM with LED is easier to use, faster, and less expensive, especially in centers that process a significant number of sputum specimens. The fluorescing *bacilli* are easily identifiable and cause less eye strain. Although the culture of mycobacteria is the most sensitive method than smear microscopy, it is time-consuming and requires proper laboratory set-ups, which is not possible in remote and rural areas with poor resources. Hence, we can conclude that AO staining is quite economical in terms of time and expense and it is recommended for busy laboratories where the burden of samples is relatively high.

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