International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2019): 7.583

A Study on Comparison between Ziehl Neelsen Staining with Auramine Rhodamine Staining in Diagnosis of Paucibacillary Leprosy

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Abstract: ***Diagnosis of paucibacillary leprosy is challenging. There are not many studies corroborating the sensitivity of Auramine Rhodamine staining. Hence this study was done to assess the efficacy of Auramine Rhodamine staining in detecting lepra bacilli in comparison to Ziehl Neelsen staining in paucibacillary leprosy cases. Slit skin smears was taken from 4 sites such as ear lobe, eyebrows, from the lesion, buttocks, and a total of 4 smears from the above sites were prepared on two different slides. One slide was stained with Ziehl Neelsen stain and another was stained with Auramine Rhodamine stain. A total of 60 cases of paucibacillary leprosy were included in this study with maximum number of patients in the age group of 21-40years (46.66%) with male preponderance. Auramine Rhodamine staining was able to detect 42 cases (70%) out of 60 cases and Ziehl Neelsen staining was able to detect 22 cases (36.66%) out of 60 paucibacillary leprosy cases. Auramine Rhodamine staining using fluorescent microscopy is more sensitive than Ziehl Neelsen staining using light microscopy. Hence, it is recommended to be used as supplementary tool to confirm clinically diagnosed cases of paucibacillary leprosy in hospitals where facility for fluorescent microscopy is available.***

Keywords: Paucibacillary leprosy, Ziehl Neelsen staining, Auramine Rhodamine staining, Slit skin smear

1. Introduction

Leprosy is a chronic granulomatous disease caused by infection with Mycobacterium leprae (M.leprae) **.** It is characterized by infection of skin, nerves and associated with immunological damage [1]. The causative agent of leprosy, Mycobacterium leprae was discovered in 1873 by Sir Gerhard Armauer Hansen. Leprosy is an important public health problem in most of the developing countries and it causes physical disabilities affecting people in their most productive stage of life. So, control of communicable disease is based on identifying and destroying or attacking the causative organism [1] [2]. Ridley and Jopling in 1966, defined leprosy into five groups on the basis of clinical, bacteriological, histological and immunological features as tuberculoid leprosy (TT), borderline tuberculoid (BT), borderline borderline (BB), borderline lepromatous (BL) and lepromatous leprosy (LL). In general, Paucibacillary leprosy (PB) disease is equivalent to indeterminate, tuberculoid, pure neuritic and borderline tuberculoid whereas multibacillary leprosy disease is equated with borderline, borderline lepromatous and lepromatous leprosy cases. Using Ridley's bacteriological index (BI) as the criterion, patients (BB, BL, LL) with a BI more than or equal to 2 at any site were classified as multibacillary whereas those patients (TT and BT) with a BI less than 2 at all sites were classified as paucibacillary [3]. The only classification which the WHO advocates for leprosy from the year 1982 is paucibacillary and multibacillary for therapeutic purposes, based on skin smear status and number of skin lesions [4].

The laboratory has an important role in the early diagnosis and documentation of leprosy, as well as in monitoring the response to treatment. Expertise in the laboratory diagnosis of leprosy is now restricted to only few large centres. In expert hands, the specificity and sensitivity of diagnostic tests has greatly increased. There is scope for these centres to offer short training modules to both pathologists and technicians about techniques and reporting of skin smears & biopsies [5]. Ziehl Neelsen (ZN) staining is uncomplicated, cost effective and the most frequently used method for the detection of acid fast bacilli (AFB) especially in resource limited settings. The sensitivity of ZN is inconsistent ranging from 18% to 56%, depending on the study with a low negative predictive value demonstrating the probable high rate of false negative. An acceptable alternative would be Auramine Rhodamine (AR) staining on slit skin smear with a slightly higher percentage of positivity (64%). AR staining is simpler due to the ease of detection of fluorescent stained bacilli and ability to screen the entire field within a short period [6] [7]. In Paucibacillary leprosy cases (indeterminate, pure neuritic, tuberculoid, borderline tuberculoid) where the Slit skin smear has bacteriological index < 2 +, it is difficult to get the bacteriological sensitivity report, which is an important diagnostic criterion. In a comparative study done by Girma S et al, the sensitivity of ZN staining is 59.3% and sensitivity of AR staining is 65.5% in slit skin smears [8]. There are not many studies in literature, corroborating the sensitivity of AR staining. Hence this study was conducted to assess the efficacy of AR staining in detecting lepra bacilli in comparison to ZN staining in paucibacillary leprosy.

2. Materials and Methods

Ethical committee approval was obtained before the start of study. Sixty patients attending the outpatient department of

dermatology with clinical diagnosis of paucibacillary leprosy (Indeterminate, pure neuritic, tuberculoid, borderline and tuberculoid leprosy) with BI< 2, from July 2018 to December 2020 were included in the study. Patients who were treated for leprosy and patients with BI>2 were excluded from the study. Patients were counselled regarding the study and written informed consent was taken before enrolling them in the study. A detailed clinical history was elicited from all patients, then meticulous general and cutaneous examination were carried out, following which findings were recorded in a proforma. Slit skin smears were taken from specific sites such as ear lobe, eyebrow, buttocks and hypopigmented patch except in pure neuritic cases where smears were taken from the area of hypoaesthesia along with above mentioned sites. Slit skin smear was obtained by pinching the fold of skin tightly using the thumb and index finger till it is blanched. Using a surgical scalpel, 5 mm long and 2 mm deep cut is made on the skin fold exposing the subepithelial tissue. Then blade was turned to 90°, tissue fragments and fluid were scraped out from the bottom and side of the cut. Material obtained was placed on the clean slide and spread evenly to make a smear of about 8-10 mm in diameter. Then blade was kept on flame and placed it on its stand. Using a second blade, smear was made from the next site. The cooled first blade was used to make the smear from the third site. Procedure was repeated until all the smears were taken. About four smears were made on a single slide and smears were allowed to dry at room temperature.

A total of 4 smears from the above sites was prepared on two different slides. One slide was subjected to ZN staining, which is viewed under light microscopy and another was stained by AR staining, which is viewed under Fluorescent microscopy (Figure 1). ZN-stained skin smears were examined using a light microscope under an oil immersion objective. The number of AFB in each field was counted and graded. Acid fast bacilli appeared bright red to intensive purple, straight or slightly curved rods occurring singly or in groups. The morphology of the bacilli was carefully examined to find out whether the AFB present were live or killed. Solid staining organisms probably were live and viable whereas, the granular, broken and fragmented ones are dead and non-viable. The smears were microscopically examined to measure the bacterial load in the form of Bacteriological Index (BI) as shown in Table 1 and morphological index was also assessed. AR stained slides were examined, where positive interpretation was done when live Acid-fast organisms appeared fluorescent yellow or bright orange and dead organism as red against a dark background. Negative interpretation was done when non-acid fast organisms did not fluoresce. Then smears were graded from 0 to +4 depending on number of acid-fast bacilli seen as shown in Table 2. Results were analysed using appropriate statistical test and expressed in percentage.

3. Results

A total of sixty patients were included, out of which 38 were male and 22 females with maximum number of patients were in the age group of 21-40 years (46%). 32 patients (54%) were in the low socio-economic group and 30 patients (50%) had disease duration less than one year. 38 patients had hypopigmented patch with hypoesthesia whereas 12 patients had only hypopigmented patch and 10 patients had only symptoms like tingling & numbness. Maximum number of patients had hypopigmented patch over the upper extremity (43.33%), followed by back (23.34%), trunk (13.34%), face (10%), head and neck (6.66%) and lower extremity (3.33%). Majority of patients had patches in the range of two to four i.e., 36 (60%), 14 patients (23.33%) had patches in the range of zero to two, 10 patients (16.67%) had no hypopigmented patches. 53.33% of patients had one nerve involvement, 36.67% of patients had more than one nerve involvement, 10% of patients had no nerve involvement. Many patients belonged to BT spectrum (70%) followed by PN (16.67 %), followed by TT (6.67%) and least number belonged to Indeterminate type (6.66 %).

In our study, ZN staining was able to detect 18 cases of BT leprosy (42.85%), two cases of PN (20%) and TT (50%), out of 22 ZN +ve cases (Figure 2). BI for indeterminate leprosy was negative (BI 0) and BI was 1+ for pure neuritic, tuberculoid, borderline tuberculoid [Table 3]. 38 patients had BI 0 and 22 patients had BI 1+. Out of 42 AR Positive cases, AR Staining was able to detect 32 cases of BT (76.19%), 6 cases of PN Leprosy (60%), 4 cases of TT (100 %) and 0 cases in I type (Figure 3). AR grading was done which showed negative for indeterminate leprosy and 1+ for pure neuritic, tuberculoid, borderline tuberculoid spectrum [Table 4]. When comparing ZN stain with AR Staining, AR Staining was able to detect 42 cases (70%) whereas ZN staining was able to detect 22 cases (36.66%) out of 60 Paucibacillary leprosy cases [Table 5]. AR staining showed overall sensitivity of 70% (n=42) out of which 32 (76.19%) were BT, 4 (100%) were TT and 6 (60%) were PN cases whereas ZN staining showed overall sensitivity of 36.66% (n=22) out of which 18 (42.85%) were BT, 2 (50%) were TT and 2 (20%) were PN.

4. Discussion

Worldwide, two to three million people are estimated to be permanently disabled because of leprosy. India has the greatest number of cases, with Brazil second and Indonesia third. India reports over 50% of the world's leprosy cases [9]. Various spectrum of leprosy has been described like Indeterminate, Pure neuritic, Tuberculoid, Borderline leprosy, Borderline Tuberculoid, Borderline leprosy, Borderline lepromatous and Lepromatous leprosy. Laboratory diagnosis of leprosy plays an important role for diagnosis and management of leprosy. Of all the laboratory tests in leprosy service, slit-skin smear examination is the most simple and valuable. Laboratory investigations of leprosy patients for diagnosis, classification and monitoring of chemotherapy have been depending on slit-skin smear examinations. Smear is stained by various staining methods, preferably undertaken at room temperature [10] and different staining techniques used are Ziehl Neelsen stain (ZN), Auramine Rhodamine Stain (AR), Haematoxilin and eosin stain (H & E), Modified Fite Faraco stain (FF).

Acid-fast stained smears of clinical specimens require at least 10^4 acid fast bacilli per millimeter for detection from concentrated specimens. Fluorochrome stain is the screening procedure recommended for those laboratories that possess a fluorescent microscope [11]. This stain is more sensitive than

the conventional carbol fuschin stains because the Fluorescent bacilli stand out brightly against the black background as yellow coloured or bright orange-coloured bacilli which is viewed under fluorescent microscope. Also, smears can be examined at low magnification, more fields can be visualized in a shorter time. In a study by Bramhne et al, smears from 74 known smear negative cases of leprosy were examined after staining with Auramine O and they found that 40.54% were positive for Fluorescent bacilli [15]. Girma S et al done a comparative study, in which performance of the fluorescent Auramine O (AO) staining and polymerase chain reaction (PCR) was assessed. Various staining methods like AO, ZN, FF, H and E were used on slit skin smears and punch biopsies collected from 141 clinically confirmed leprosy cases. They found that sensitivities were 87.6% , 59.3%,77% for H and E, ZN and FF respectively, whereas it reached 65.5% and 77.9% for AO in slit skin smears and tissue sections respectively. Samples with low bacillary index had sensitivity of 61.8% for AO staining which is similar to FF staining (60%). They recommended AO staining for the diagnosis of leprosy in lower health facilities such as health centres and district hospitals [8].

Similarly, comparative study was conducted by Somoskovi et al, where standard smears were examined microscopically following staining by the Ziehl Neelsen staining, Auramine staining and silver methanamine methods. They found sensitivity and specificity of the ZN and fluorochrome methods are comparable and they also concluded, because of the rapidity of the fluorochrome method, laboratories with large specimen numbers should use this technique [Somoskovi]. Adiga et al in their retrospective study has compared three staining methods i.e., ZN Stain, FF stain and fluorescent stain. They concluded Fluorescent method is more sensitive than FF stain and due to its high sensitivity, paucibacillary cases can be upgraded to multibacillary cases, thus changing the treatment of leprosy [12]. Similarly, Nayak et al has compared Fluorescent staining with FF staining in the detection of M. Leprae. They concluded that, fluorescent method was more useful than modified Fite Faraco method, particularly in paucibacillary cases. They also observed that Fluorescent microscopy has the advantage of speed, ease of screening and reduces observer fatigue [13]. Bhatia et al has conducted comparative study of Auramine staining with Ziehl Neelsen staining of M.Leprae in skin smear slide. They found that Auramine method was found to be more sensitive than ZN method and they claimed it to be useful in detecting small number of M.Leprae in skin smears and concluded that inter observer variance was minimal with auramine staining [14]. Similarly, in our study, AR staining was able to detect 42 cases (70%) whereas ZN staining was able to detect 22 cases (36.66%) out of 60 cases.

5. Conclusion

Auramine Rhodamine staining using fluorescent microscopy is more sensitive than ZN staining using light microscopy. Hence it is recommended to be used as supplementary tool to confirm clinically diagnosed cases of paucibacillary leprosy in hospitals, where facility for fluorescent microscopy is available.

No conflicts of interest

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Table 1[10]: Grading of Bacterial load (Bacteriological Index)

6+	Over 1,000 Bacilli and Globi in an average microscopic field
5+	Over 100 bacilli but less than 1,000 in an average microscopic field
4+	Over 10 bacilli but less than 100 in an average microscopic field
3+	1–10 bacilli in an average field
2+	1–10 bacilli in 10 microscopic fields
1+	1–10 bacilli in 100 microscopic fields
0	No bacilli observed after searching at least 100 microscopic fields.

Table 2[16]: Grading of smears based on number of AFB seen (AR Staining).

Number of AFB seen (450x magnification)	*Number of AFB seen (250x magnification)*	*Reported as*
0 AFB per 70 Field	0 AFB per 30 Fields	AFB not seen
1-2 AFB per 70 Fields	1-2 AFB per 30 Fields	Doubtful; Repeat with another specimen
2-18 AFB per 50 Fields	1-9 AFB per 10 Fields	1+
4-36 AFB per 10 Fields	1-9 AFB per Field	2+
4-36 AFB per Field	10-90AFB per Field	3+
>36 AFB per Field	>90 AFB per Field	4+

Table 3: ZN staining in different clinical spectrums of leprosy

Clinical spectrum	*ZN +ve cases (%)*	*BI*	*ZN -ve cases*	*Total*
Indeterminate	0	Negative	4	4
Pure neuritic	2(20)	1+	8	10
Tuberculoid	2(50)	1+	2	4
BT	18(42.85)	1+	24	42
Total	22(36.66)		38	60

Table 4: Auramine Rhodamine staining in different clinical spectrums of leprosy

Clinical spectrum	*Auramine Rhodamine +ve (%)*	*AR Grading*	*Auramine Rhodamine -ve*	*Total*
Indeterminate	0	Negative	4	4
Pure neuritic	6 (60)	1+	4	10
Tuberculoid	4 (100)	1+	0	4
BT	32 (76.19)	1+	10	42
Total	42 (70%)		18 (30%)	60

Table 5: Comparison of diagnostic efficacy between ZN staining and Auramine Rhodamine staining in different spectrums of leprosy

Clinical Types	*Number of cases*	*ZN Positive (%)*	*AR Positive (%)*
Indeterminate	4	0	0
Pure Neuritic	10	2 (20%)	6 (60%)
Tuberculoid	4	2 (50%)	4 (100%)
Borderline Tuberculoid	42	18 (42.85%)	32 (76.19%)
Total	60	22 (36.66%)	42 (70%)

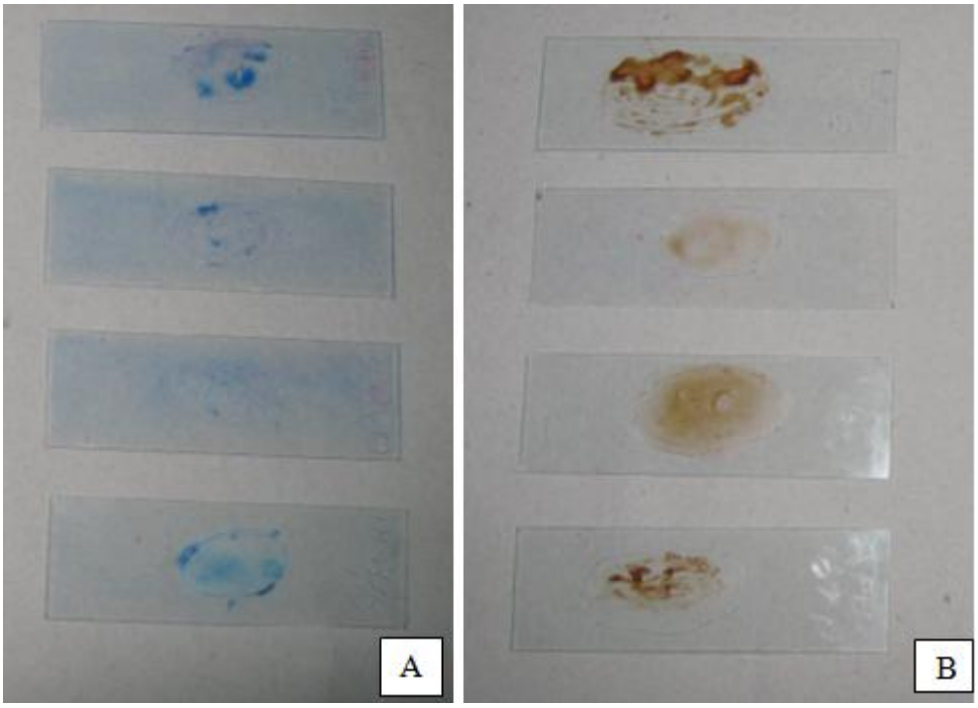

Figure 1: A. Slides stained with ZN Stain and B. Slides stained with AR Stain.

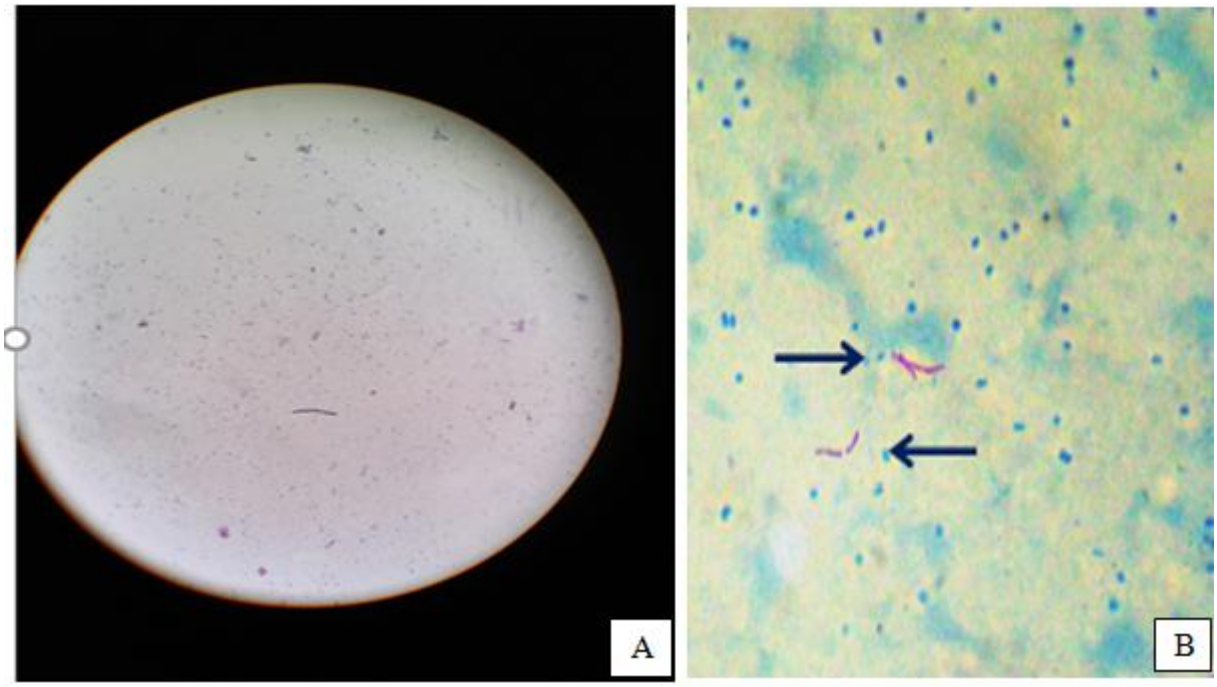

Figure 2: A. ZN negative slide of patient with Indeterminate leprosy and B. ZN positive slide of patient with Borderline tuberculoid leprosy

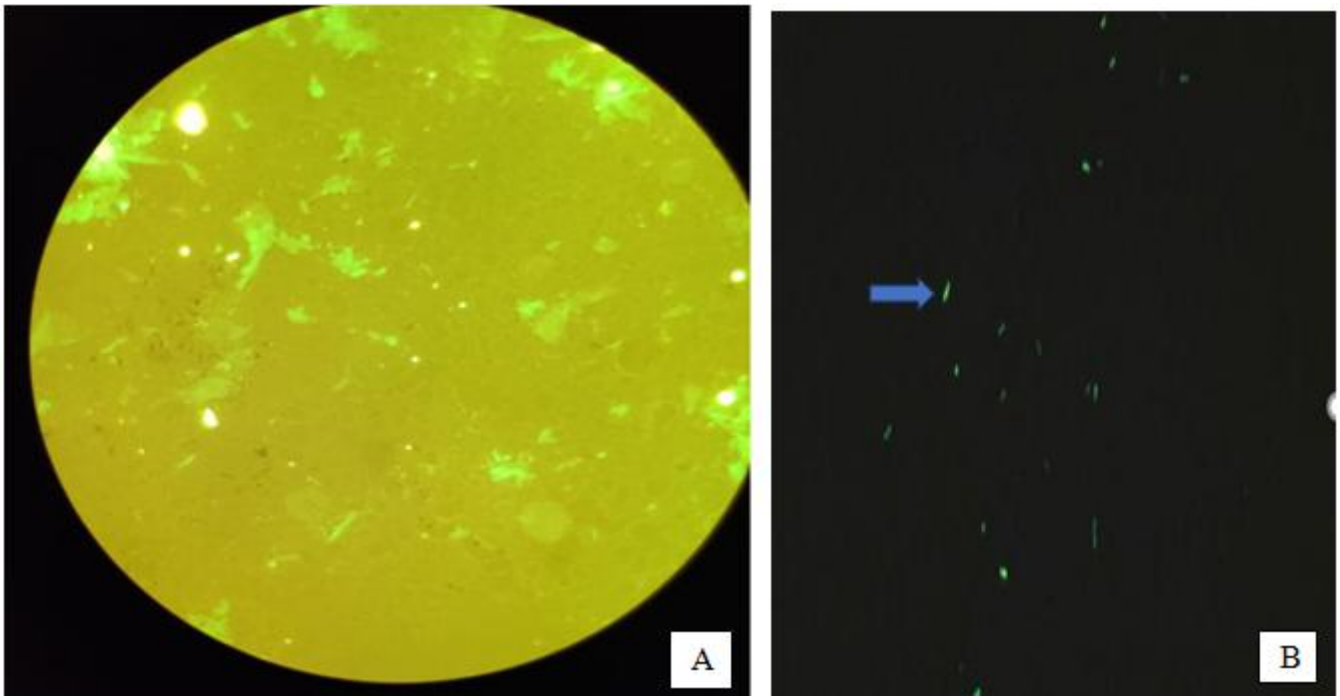

Figure 3: A. AR negative slide of patient with Indeterminate leprosy and B. AR positive slide of patient with Borderline tuberculoid leprosy showing bacilli (Arrow head)