Research Article

Comparative Study of AFB Detection in Concentrated and Unconcentrated Sputum Sample by Ziehl-Neelsen Staining and Auramine-O Staining of Patients Attending the Microscopic Centre of RNTCP at Darbhanga Medical College & Hospital, Laheriasarai

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Abstract

Ziehl-Neelsen is a common bacteriological staining method used from a long time to stain acid-fast bacilli, especially *Mycobacterium tuberculosis* which causes mainly pulmonary tuberculosis. In recent technologies, fluorescent-staining is considered to be a more reliable method due to more intensive binding of mycolic acids of the bacilli to phenol auramine-O, so the tubercle bacilli is seen more clearly against black background.

Objective: This study was done to compare the efficacy of conventional Ziehl-Neelsen (ZN) and Auramine-O (AO) fluorescent microscopy in detecting acid-fast bacilli in direct and concentrated sputum samples of patients attending the microscopic centre of RNTCP at Darbhanga Medical College & Hospital, Laheriasarai.

Method: One thousand and fifty patients suspected of having pulmonary tuberculosis referred to the RNTCP centre of Darbhanga Medical College and Hospital was included in this study. Spot sputum sample was collected as the clinical sample. Direct smears were prepared from the mucopurulent part of the sputum with a sterile loop. Samples were then concentrated using modified Petroff's method and smear prepared from the concentrated sediment. Both smears were then stained by ZN and AO staining method respectively.

Result: Out of 1050 samples, 165 samples were positive by AO method in direct method and 166 samples were positive by AO method in concentrated method, 147 were positive by ZN staining in direct method and 156 samples were positive by ZN in concentrated method.

Conclusion: FM definitely improves the diagnostic value of the sputum smear especially in patients with low density of bacilli that are likely to be missed on ZN-stained smears, concentrated method on both AO and ZN stain were more sensitive than direct method.

Keywords: Pulmonary tuberculosis, ZN staining, Auramine-O (AO) staining, Modified Petroff's method.

Introduction

Pulmonary tuberculosis is a worldwide public health problem-a disease of lungs, caused mainly by *Mycobacterium tuberculosis.*[1] From villages of India, to the prisons of Russia, new and old civilization is all falling prey to this one organism.

According to World Health Organization (WHO), tubercular infections are currently spreading at the rate of one person per second per million people with three million dying from it.[2] In the words of Charles Dickens *"It is the disease medicine never cured, wealth warded off, or poverty could boast exemption from….which sometimes moves in giant strides and sometimes at tardy sluggish pace, but slow or quick…is never sure and certain."* In developing countries like India, where there is limited resources and infrastructure, sputum smear microscopy remains the most preferred and rapid test for easy and early diagnosis of pulmonary tuberculosis either by ZN or fluorescent-staining technique.[2] Recent studies have shown that auramine staining is a better method for the demonstration of acid-fast bacilli in sputum specimens as compared to ZN method. By fluorescence microscopy, a large number of sputum samples can be examined in comparatively lesser time but it requires greater expertise in reading. It also requires stable power supply and regular supply of costly and short-lived bulbs.[2]

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 labeled to be suffering from pulmonary tuberculosis.[3] In 2014, there was an estimated 9.6 million new TB cases: 5.4 million among men, 3.2 million among women and 1.0 million among children. There were also 1.5 million TB deaths.[4] If properly treated, TB caused by drug-susceptible strains is curable in almost all cases. If untreated, the disease may be fatal within 5 years in 50-65% of cases. Transmission usually takes place through the airborne spread of droplet nuclei produced by patients with infectious pulmonary tuberculosis.

Therefore, the present study was done to compare conventional ZN-staining method with the recent fluorescent-based AO staining method for the detection of mycobacterium bacilli in sputum sample. The second part of the present study compares the sensitivity of AFB detection in direct and concentrated smear.

Materials and Methods

This comparative study was carried out at the Department of Microbiology, Darbhanga Medical College and Hospital, Laheriasarai. Ethical committee clearance was taken from the institution and samples were collected at the microscopic center of RNTCP.

Patient Inclusion Criteria

Adults and children of both genders suspected of having pulmonary tuberculosis were included in the study. Symptoms of pulmonary tuberculosis included patients having fever, cough for more than three weeks, loss of weight, loss of appetite and with and without hemoptysis. Spot sample was collected. Only mucopurulent samples were accepted for the study.

Patient Exclusion Criteria

Those cases which appeared to be allergy and did not take a course of antibiotics or known cases of carcinoma of lung were excluded. Sputum samples macroscopically resembling saliva were excluded. Samples containing food particles or any other remnants were not included in the study. Patients were advised to give at least 5 ml of the sample.

Sample Size

1050 patients were screened for pulmonary tuberculosis.

Sample Collection Method

On the spot sputum sample was collected from each patient on day 1. Samples were collected in a sterile, leak-proof, wide-mouthed plastic containers properly labeled with name, age and sex of the patient with date and time of collection. Patients were advised to collect sample after rinsing their mouth with water to remove any food remnants, since food particles in smears make acid-fast bacilli difficult to examine. Patients were also advised to avoid betel leaves, tobacco, saliva and nasal secretions in the sample.

Method

After collection of samples at RNTCP centre, they were transported to the Department of Microbiology, Darbhanga Medical College. Specimen containers were tightly closed checked for patient details and for any leakage before their transportation. Samples were placed in the transportation box, tightly closed and then transported to the Microbiology department.

All sputum samples were processed in the Bio safety cabinet, class 11, type A. New, fresh slides were used for smear preparation. From each sample four smears were prepared. Two smears were prepared from the sample before concentration. They were the direct smears which were first allowed air-dry for at least 15 min and then heat-fixed. One of the two direct smears was stained by Auramine-O and the second one by Ziehl-Neelsen staining method. Another set of two smears were prepared from the deposit obtained after concentrating the sample by modified Petroff's method.

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They were the concentrated smears which were air-dried and heat-fixed. Similarly like above one of two concentrated smear was stained by Auramine-O and another by Ziehl Neelsen staining methods. Smears were examined for the presence or absence of acid-fast bacilli using fluorescent and oil immersion microscope (under 100X). Observations were recorded.

Modified Petroff's Method: Kent and Kubica described a recommended standard Petroff's procedure which involved mixing 2 mL of sputum in a test tube with 2 mL of 4% NaOH solution and incubation for 15 min at room temperature. It was homogenized for 15 min in a shaker at 3000 rpm. The deposit was neutralized with 20 mL of sterile distilled water. The sample was again centrifuged at 3000 rpm for 15 mins. From the sediment, smear was made. This method was selected because it required lesser time, had lower contamination rate and resulted in minimal destruction of the bacilli when compared to the NaOH method (Petroff's method).[6] It was also selected because the reagents to be used can be sourced locally.

Zn Staining: A small portion from thick mucopurulent part of the sputum specimen was spread on the slide in a circular movement to prepare a smear of even thickness. The smear was air-dried and heat-fixed and covered with strong carbol fuchsin stain and gently heated to steaming for 5 mins, without letting the stain boil and become dry. The slide was then washed with distilled water and decolorized with 20% sulfuric acid for 1 min and washed with distilled water. The step was repeated until the smear became light pink and color stopped coming out from the smear. In the final step, smear was counterstained with Loeffler's methylene blue for 30 sec and washed with distilled water. The smear was air-dried and examined under oil immersion as bright red rods against the blue background.[7] Image of the bacilli was taken by digital camera.

Auramine-O Staining: The use of Auramine-O (a fluorescent dye) as a staining reagent was first proposed in 1930.[8] Auramine fluoresces when illuminated by blue violet or ultra-violet (UV) light. It is used to demonstrate AFB because it binds to the mycolic acid in the mycobacterium cell wall. No heating of the stain is required. The smear was covered with AO solution and left for 20 min. The slide was then washed with distilled water. In the second step, smear was covered completely with the decolorizer, acid-alcohol, for 2 min. Smear was washed well with distilled water. In the final step, smear was counterstained with 0.1% potassium permanganate for 30 sec.[9] Tubercle bacilli with AO staining appeared as bright yellow against the dark background. Refer to the image that was taken by a digital camera.

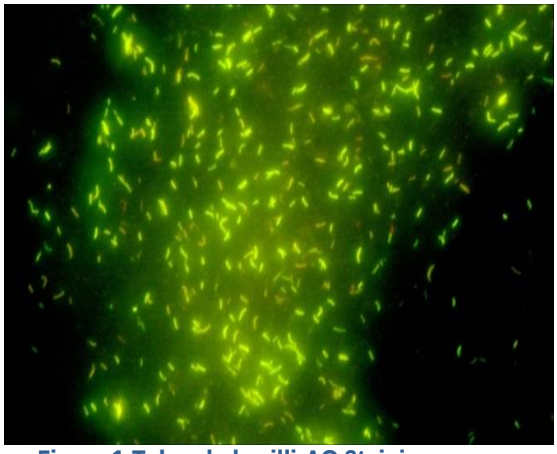


Figure 1.Tubercle bacilli-AO Staining

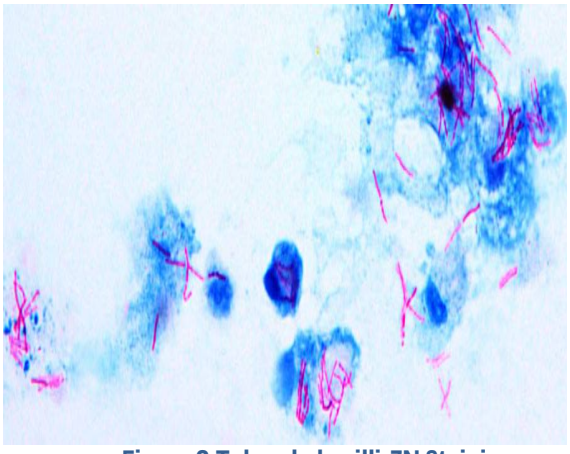


Figure 2.Tubercle bacilli-ZN Staining

Statistical Analysis

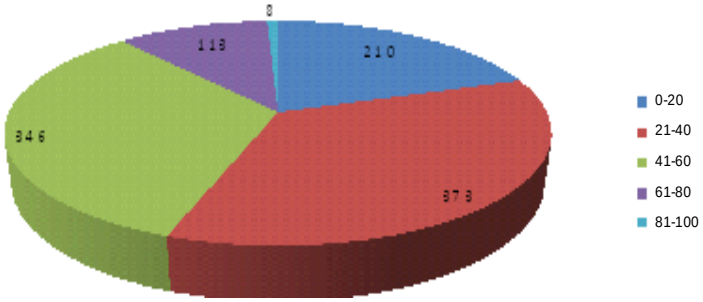
Data collected was analyzed using SPSS (Statistical Package for Social Science) version 21.0.

Results

Comparison of the qualitative result of the above two microscopic methods is depicted in the following tables.

Table 1.Gender-Wise Distribution

Gender	Frequency	Percent
Male	689	65.6
Female	361	34.4
Total	1050	100.0



Graph 1.Agewise distribution of TB Positive people

Table 2.Gender wise distribution of Positive & Negative result in concentrated smear "on spot sample" by AO

AO	Gender		Total
	Male	Female	
Positive	131	35	166
Negative	558	326	884
	689	361	1050

Table 3.Gender wise distribution of Positive & Negative result in concentrated smear "on spot sample" by ZN

ZN	Gender		Total
	Male	Female	
Positive	122	34	156
Negative	567	327	894
	689	361	1050

Table 4.Age-Wise Distribution

Age (years)	Frequency	Percent
0-20	210	20.0
21-40	373	35.5
41-60	346	33.0
61-80	113	10.8
81-100	8	0.8
Total	1050	100.0

Table 5.Number of Samples Positive and Negative by AO Stain in Direct Smear

Samples	Frequency	Percent
Positive	165	15.7
Negative	885	84.3
Total	1050	100.0

Table 6.Number of Samples Positive and Negative by AO Stain in Smear after Concentration

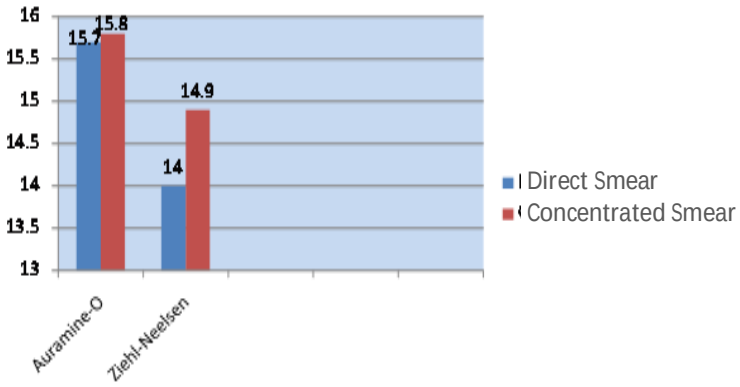
Samples	Frequency	Percent
Positive	166	15.8
Negative	884	84.2
Total	1050	100.0

Table 7.Number of Samples Positive and Negative by ZN Stain in Direct Smear

Samples	Frequency	Percent
Positive	147	14.0
Negative	903	86.0
Total	1050	100.0

Table 8.Number of Samples Positive and Negative by ZN Stain in Smear after Concentration

Samples	Frequency	Percent
Positive	156	14.9
Negative	894	85.1
Total	1050	100.0



Graph 2.Number of Case Positive by AO and ZN in Direct and Concentrated Smear

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Total study population consisted of 1050 OPD patients who attended the RNTCP centre of Darbhanga Medical College and Hospital suspected of having pulmonary tuberculosis. Only those patients were included in the study, who represented with one or two symptoms like fever, loss of weight and most important producing purulent cough with or without hemoptysis. Table 1 shows gender wise distribution. As per the data, 689 (65.6%) were males and 361 (34.4%) were females of the total study samples who reported in the OPD. Table 2 shows that out of total 1050 sputum samples collected, 131 males and 35 females concentrated smear showed the presence of tubercle bacilli by AO staining method. Similarly, Table 3 shows that 122 males and 34 females concentrated smear showed the presence of tubercle bacilli by ZN method of staining. Table 4 shows age-wise distribution of the study population. Middle-age group between 21 and 40 years comprised of 35.5% of the study population and were maximum in number to submit their sample for TB screening. It was followed by age group 41 to 60 years which comprised 33.0% of the study population. Minimum numbers of sputum samples submitted during the study were from the age group 81-100 years and comprised only 0.8% of the study population. 20% of the study population comprised of children and youth who were unfortunately at the risk of developing the disease. Table 5 shows that out of 1050 sputum samples collected on spot, 165 (15.7%) were positive by AO-staining method in direct smears. Table 6 shows that out of 1050 sputum samples collected on spot, 166 (15.8%) were positive by AO-staining method in concentrated smears. On the similar note, Table 7 shows that out of 1050 sputum samples collected on spot, 147 (14.0%) were positive by ZN-staining method in direct smears. Table 8 shows that out of 1050 sputum samples collected on spot, 156 (14.9%) were positive by conventional ZN-staining method in concentrated smears.

Discussion

In reference to gender wise distribution (Table 1) it was found that males were more in number submitting their sample than females. In the present study the difference in sex could be because more males attended the OPD seeking health care facility while females did not. Fear of being left alone from the society and family members, late realization of the disease and the need of medical help, feeling shame in talking about the disease and poverty are few reasons that might have prohibited females from attending the OPD. It was also found from the study that females were busy taking care of the house and children and some of them were working also thus leaving them with no time for themselves and thus late realization of the disease. In reference to Tables 2 and 3: Smear positive males and females by Auramine-O staining method were 19.0% and 9.7% respectively. And by ZN staining method, smear positive males and females were 17.7% and 9.41% respectively. Ramakrisna et al in his study found 76.3% incidence in male and 23.7% in female[10]. K. Prashanthi et al found in their study that 70% of TB patients were males and 30% were females[11]. S.Rao in his study sample of total 446 patients found that males were 308 (69%) and females were 138 (31%) in number. Out of which 248 (68%) smear positive were males and females were 116 (32%); in the ratio of 2:1.[12] Biological factors could be the cause of differences in resistance to infection/disease between men and women. Sex steroid hormones and the anti mycobacterial immune response, the genetic makeup of the sex chromosomes, and sex-specific metabolic features may play some role in gender difference towards TB[13]. All age-groups get infected by TB in the same way ai8nd that is by inhaling TB bacteria which are in the air as a result of being released into the air by someone with active TB. In reference to the age wise distribution the present study showed maximum patients who developed pulmonary TB belonged to the middle-age group between 21 and 40 years followed by age group 41 to 60 years. From the data collected it was found that patients belonging to the age group 21 to 40 years was the most active group occupied mainly as drivers, labourers and rickshaw pullers. They had developed the infection due to their weak immune system which was because they were exposed to drinking and smoking. They belonged to the lower socio-economic level, had poor diet and lived in small rooms with large families. The present study showed that these poor people who were categorized as daily earners of bread and butter were in maximum number affected by TB. This age group also included young students who were found to be at the risk of developing or had already developed pulmonary tuberculosis from either their close friend, family member, neighbor or from their colleague. The present study showed in reference to Tables 5-8 that the use of concentration method increased the sensitivity of identifying positive TB cases when compared to the direct method. Out of 1050 sputum samples 165 (15.7%) were positive by AO and 147 (14%) were positive by ZN-staining method in direct smears. The percentage of positive numbers increased to 166 (15.8%) by AO and 156 (14.9%) by ZN in concentrated smears. It can be seen from Graph 2 that concentration method significantly increased the rate of detection of Acid Fast Bacilli in concentrated sputum smears than in the direct smears. Many parts of our country still depend on ZN method of staining for AFB detection in conventional direct smears. Despite the fact that concentration method can be superior to the direct method, it is not being followed in routine diagnosis of AFB in sputum smears. Concentration method increases

the chance of identifying patients with low burden of TB bacilli which could have been missed in direct method. Finance, lack of training, lack of adequate infrastructure and health risk associated with it may be responsible for not including the concentration method in routine AFB microscopy. Even by using a conventional method of ZN-staining, sensitivity of AFB microscopy of sputum samples can be increased by following concentration smear microscopy in place of direct microscopy. A similar study conducted by Hooja et al. showed that on concentration the sensitivity of ZN staining increased by 6.67% and 11.11% for AO.[14]

The present study showed that since fluorescent microscopy gave a better contrast and a larger field to examine, there was a significant increase in the detection of Acid Fast Bacilli in sputum smears by AO-staining method. Previous studies have also shown similar results. Mamilla and Sanda in their study collected 500 sputum samples and found 12% and 19.8% positive cases by ZN and FM staining respectively.[15] Kumar et al. conducted a study to compare ZN-and FM-staining methods in diagnosing PTB in 2012 in Madras. Out of 400 samples, 11% and 17% are positive by ZN-and AO-staining respectively.[16] Timalsina et al. in their study found that out of 299 sputum samples collected, 19.06% were positive by ZN and 29.1% were positive by AO respectively.[17] Khatun et al. conducted similar studies in Bangladesh in 2011 and out of 300 sputum samples collected from suspected pulmonary tuberculosis patients, 10.4% and 17.8% were positive by ZN-and AO-staining respectively.[18] Fluorescent microscopy increases the diagnostic value of sputum smear especially in patients with low bacterial count which could have been missed by ZN staining.

As it can be seen from the Graph 2 that AO gave better result than conventional ZN stain in direct as well as in the concentrated smear. Under fluorescent light, tubercle bacilli appeared as bright yellow against dark background because of which it was easy to identify them. Since there was a contrast, AFB bacilli were readily seen and large area of the smear was examined in less time. Screening of smears by AO method took lesser time as compared to conventional ZN method in which we had to carefully search for the tubercle bacilli. Thus, AO-staining method is a recent technology strongly recommended for laboratories and RNTCP centers handling large number of sputum samples. The difference in the case detection rate was also found to be statistically significant with better method of microscopy by AO method than ZN method ($p<0.01$).

Conclusion

In spite of sputum culture being the gold standard method of detecting the tubercle bacilli, smear microscopy remains the key diagnostic tool for early and easy detection of tubercle bacilli. Considering sensitivity, advantage of clear and easy visibility of the tubercle bacilli, AO stain is found to be superior to conventional ZN staining. False-negative results reported with AO staining were comparatively lesser compared to ZN staining. AO staining took relatively shorter time for identification of the tubercle bacilli than ZN staining and thus more slides were processed in shorter duration of time. AO staining method was helpful in early and accurate detection of patients with low burden of tubercle bacilli (paucibacillary cases). AO staining technology is the recent and reliable method to be introduced at centers handling high number of specimen. AO staining method is superior to conventional ZN staining method. For the success of TB control program, emphasis should be on early detection and treatment of the pulmonary tuberculosis. And the target can be achieved by the recent AO-staining method over conventional ZN method and by examining concentrated smears than the direct smears.

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Conflict of Interest: None

References

1. Ananthanarayan R, Paniker CJ. Textbook of Microbiology. 6th Edn. Hyderabad: *Orient Longman,* 2000: 324-47.
2. Forbes B, Rieder HL. A comparison of florescence microscopy with the Zeil-Neelson technique in the examination of sputum for acid-fast bacilli. *Int J Tubercle Lung Disease* 1999; 3(12): 1101-105.
3. Laifangbam S, Singh HL, Singh NB et al. A comparative study of fluorescent microscopy with Ziehl-Neelsen staining and culture for the diagnosis of pulmonary tuberculosis. *Kathmandu Univ Med J (KUMJ)* Jul-Sep 2009; 7(27): 226-30.
4. WHO. Global Tuberculosis Report. 2015.

5. Parsons LM, Somoskövi Á, Gutierrez C et al. Laboratory diagnosis of tuberculosis in resource-poor countries: Challenges and opportunities. *Clin Microbiol Rev* 2011; 24(2): 314-50.
6. Kent PT, Kubica GPW. Public Health Mycobacteriology. A guide for the Level III Laboratory. Atlanta: *Centers for Disease Control* 1985: 21-44.
7. National Tuberculosis Centre. A Manual for AFB Staining, India.
8. Hagemann PKH. Fluoreszenzfärbungvon Tuberkel bakterienmit Auramin. *Münch Med Wschr* 1999; 85: 1066-68.
9. Manual for Sputum Smear Fluorescence Microscopy. Central TB division, Directorate General of Health Services, Ministry of Health and Family Welfare, Nirman Bhavan, New Delhi-110011.
10. Ramakrishna R, Bharavi C. A comparative study of sputum smears by ziehl neelsen staining versus fluorescent staining in the diagnosis of pulmonary tuberculosis in patients suffering with cough for 2 weeks or more. *Journal of Evidence based Medicine and Healthcare* 2015; 2(11); 1663-74.
11. Prasanthi K, Kumari AR. Efficacy of fluorochrome stain in the diagnosis of pulmonary tuberculosis co-infected with HIV. *Indian J Med Microbiol* 2005; 23: 179-81.
12. Rao S. Tuberculosis and patient gender: An analysis and its implications in tuberculosis control. *Lung India* 2009; 26(2): 46-47.
13. Neyrolles O, Quintana-Murci L. Sexual inequality in tuberculosis. *PLOS Medicine* 2009.
14. Hooja S, Pal N, Malhotra B et al. Comparison of Ziehl Neelsen & Auramine O staining methods on direct and concentrated smears in clinical specimens. *Indian Journal of Tuberculosis* Apr 2011; 58(2): 72-76.
15. Mamilla R, Sanda S. Sensitivity of FM staining versus ZN staining in diagnosing sputum smear positive PTB. *IOSR Journal of Dental and Medical Sciences* 1999; 14(3): 29-33.
16. Kumar SJ, Chandrasekar C, Rajasekaran S. Comparison of conventional and fluorescent staining methods in diagnosis of pulmonary tuberculosis among HIV seropositive individuals. *J Evol Med Dent Sci* 2012; 1: 463-66.
17. Timalsina B, Kutur B, Pradhan R et al. A comparative study on Ziehl-Neelsen staining (light microscopy), Auramine O staining (iLED fluorescent microscopy) and culture on LJ media of sputum samples for the diagnosis of pulmonary tuberculosis. *IJMBS* 2015; 1(1): 12-22.
18. Khatun Z, Kamal M, Roy CK et al. Usefulness of light emitting diode (LED) fluorescent microscopy as a tool for rapid and effective method for the diagnosis of pulmonary tuberculosis. *Bangladesh Med Res Counc Bull* 2011; 37: 7-10.

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