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Original Research Article

Comparative study of different methods of microscopy followed by a culture method in the detection of mycobacterium tuberculosis in clinically and radiologically suspected cases of pulmonary tuberculosis

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ABSTRACT

Aims: To compare the sensitivity of 2 microscopic methods for the diagnosis of Mycobacterium tuberculosis (M.tb) along with culture and drug susceptibility testing to first line drugs.
Settings and design: The cross-sectional study comprises 200 suspected cases of pulmonary tuberculosis both clinically and radiologically in KIMS, Bangalore over a period of 2 years.
Materials and Methods: Samples (sputum/BAL fluid) were collected, processed and stained by Ziehl Neelson (ZN) and Fluorescent methods. Culture and drug susceptibilty testing was done for Streptomycin, Isoniazid, Rifampicin and Ethambutol by Mycobacterium growth indicator tube (MGIT) method after decontamination.
Statistical analysis: Fischer's test
Results: 1. Out of 200 samples: 1.120 were male and 80 were female; 2. 18 were positive by Ziehl Neelson, 21 by Fluorescent and 28 by culture; 3. Majority of the patients belonged to age group 41-50 years (23%); 4. InMGIT, 26 were M.tb and 2 were Non-tubercular mycobacteria; 5. Out of 26 M.tb isolates, 4 were resistant to streptomycin, 6 to isoniazid, 2 to rifampicin and 9 to ethambutol.
Conclusion: 1. The sensitivity of Fluorescent staining (64.28%) is higher than that of Ziehl-Neelson (51.7%); 2. In MGIT, 26 were M.tb and 2 were Non tubercular mycobacteria; 3. 2 were Multi-drug resistant- tuberculosis (MDR-TB)
Key Messages: This study made us aware of the need for prompt detection, identification and appropriate treatment of Tuberculosis due to the rising incidence of MDR-TB.

1. Introduction

Tuberculosis is a chronic granulomatous disease caused by Mycobacterium tuberculosis (M.tuberculosis) and is spread mainly by inhalational route. It has been known to mankind since ancient times and is the oldest malady of human poverty and sufferings and is a significant infectious disease in many parts of the world. Even today after the development of advanced screening, diagnostic and treatment methods for the disease, a third of the world's population has been exposed and infected with the organism. With the advent of HIV infection, there is a resurgence of tuberculosis (TB) with more than 8 million new cases worldwide and more than 2 million dying from it.[1] There is a strong relation between tuberculosis and HIV infection. HIV reactivates a latent TB infection and also makes the disease more serious and renders the treatment ineffective. TB on the other hand, quickens the development of HIV into active disease.[2] Prompt detection, isolation, identification and susceptibility testing of Mycobacterium tuberculosis from a clinical specimen is essential for the appropriate management of patients with TB.

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According to National Tuberculosis Elimination Program (NTEP/RNTCP) annual report, 2019, India accounts for nearly 2.7 million cases of TB out of a global incidence of 10 million[3] and it is estimated that about 40% of the Indian population is infected with TB.

Detection of acid fast bacillus (AFB) in sputum samples constitutes the mainstay of diagnosis of this disease. However, this method has little value in those who don't expectorate significant amount of sputum spontaneously (may be smear negative TB). This problem, the low bacillary load, can be overcome by using culture methods, which are the gold standards for TB diagnosis. Various other samples can be used: Bronchoalveolar lavage (BAL) or gastric lavage (in case of children).

There is a need to check the drug susceptibility of the culture isolates as there is an estimated proportion of MDR-TB in India of 3.4% in new cases and 18% in previously treated.[4] Thus prompt assessment of susceptibility to drugs, helps to decrease the morbidity and mortality and prevent the spread of TB in the population.

2. Objectives

1. To compare the sensitivity of 2 microscopic methods for diagnosis of M.tuberculosis.
2. To detect the growth of M.tuberculosis in culture using Mycobacterium growth indicator tube (MGIT) method and to confirm its growth among clinically and radiologically suspected cases of pulmonary tuberculosis.
3. To detect the drug susceptibility pattern to first line drugs among Mycobacterium isolates.

3. Materials and Methods

Source of data: The study comprises suspected cases of pulmonary tuberculosis both clinically and radiologically in Kempegowda Institute of Medical Sciences (KIMS), Bangalore.

3.1. Selection criteria

1. Patients with radiological lesions suggestive of active pulmonary tuberculosis.
2. Those with strong clinical evidence.

3.2. Study type

Cross-sectional study

3.3. Duration

December 2017- December 2020

3.4. Sample size

200

3.5. Data collection

Patients admitted in KIMS hospital, Bangalore fullfilling the selection criteria were examined. Their details, symptoms, lab investigations, radiological investigations were noted and informed consent was taken.

3.6. Methodology

3.6.1. Identification of pulmonary tuberculosis patients

- Clinically, a pulmonary tuberculosis patient, is any person who has a cough for 2 weeks or more. (along with loss of weight, loss of appetite and fever)
- Radiologically, the following signs are suggestive of pulmonary tuberculosis.

1. Fibrosis of upper lobe/ apical segment of lower lobe
2. Hilar lymph node enlargement
3. Consolidation of upper or apical segment of lower lobe.
4. Cavity (single/multiple)
5. Pleural thickening
6. Dense non-homogenous opacity in the upper lobe or apical segment of lower lobe.
7. Any radiological shadow mimicking TB

3.6.2. Specimen collection

Sputum: Patient was instructed to take a deep breath, hold it momentarily and cough deeply and vigorously. Patient was also instructed to cover their mouth carefully and spit it into cup.[5] 2 samples were collected.

Broncho alveolar lavage fluid (BAL): 5ml of BAL was collected in a wide mouthed container and transported to the laboratory and processed within 4 hours of collection.

3.6.3. Transport

All samples were transmitted to the laboratory and processed as early as possible. If there was any delay, they were stored at 4*C.

3.6.4. Sample processing

Smear was prepared from the purulent portion of the specimen on a clean slide on an area of 2x1 cm. They were air dried, heat fixed in biosafety cabinet (2B) and subjected to ZN staining and fluorescent staining.

Ziehl neelson (ZN) staining: The smear was flooded with 1% concentrated carbol fuschin and heated from below till vapours were seen. Heating was done intermittently and slide was washed with tap water after 5 minutes. It was decolourised with 25% sulphuric acid for about 3 minutes. This procedure was repeated until the specimen became colourless. The slide was then counterstained with 0.1% methylene blue for 30 seconds and washed with tap water once again. The slide was dried in air and observed under oil immersion lens.

Fluorescent staining: The smear was stained with freshly filtered auramine-O and was left to stand for 20 minutes. It was washed with running water. Then decolourized by covering completely with acid-alcohol for 3 minutes. Smear was washed and counterstained with 0.1% potassium permanganate for 1 minute, washed with water. Smears were dried and observed under high power magnification (40X).

According to the NTEP (RNTCP) guidelines, The smears were graded as follows:

3.6.5. Decontamination

Modified Petroff's method using N-acetyl cysteine (NALC) was done:

1. Prepared fresh digestant by adding equal amounts of 4% NaOH and 2.9% sodium citrate. Added 0.5g NALC powder per 100 mL of sodium hydroxide-sodium citrate solution. After the NALC was added, the digestant was used within 24 hours.
2. Added the collected specimen to a 50 mL plastic centrifuge tube. The specimen should not exceed 10 mL. Added NALC solution in equal volume to that of the specimen. With the cap tightened, the tube was vortexed (about 5 - 20 seconds /tube) and inverted to ensure that the NALC solution contacted all surfaces of tube and cap.
3. The specimen was allowed to stand 15 - 20 minutes.
4. The tube was filled to 50 mL with a sterile phosphate buffer solution at pH 6.8. It was swirled by the hand to mix.
5. The specimen was concentrated in a centrifuge at a speed of 3,000 x g for 15 minutes.
6. The supernatant fluid was decanted from the pellet.
7. The pellet sediment was resuspended with the phosphate buffer pH 6.8 using a sterile Pasteur pipette to achieve a final volume of 1 to 3 mL.[6]

Staining after concentration of specimen: from the sediment, a thick smear was made, air dried and subjected to ZN and fluorescent staining.

3.6.6. Culture in mycobacterial growth indicator tube (MGIT)

The protocol that is followed was that of Ruhi Bunger et al.[7]

Principle: A MGIT has an oxygen sensor embedded at its silicon bottom which contains 7ml modified Middle brook 7H9 broth which fluoresces following the oxygen reduction which is induced by aerobically metabolizing bacteria within the medium. The amount of fluorescence is inversely proportional to the oxygen level in the culture medium, indicating the consumption of oxygen level in the culture medium, indicating the consumption of oxygen due to growth of the organisms in the vials.

Procedure:

1. The MGIT tubes were labelled with the specimen number.
2. The cap of the tube was unscrewed and 0.5mL of MGIT OADC (Oleic acid, bovine Albumin, Dextrose, Catalase) was added under aseptic precautions.
3. Then 0.1 mL of reconstituted MGIT PANTA (Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim, Azlocillin) antibiotic mixture was added.
4. 0.5 mL of the concentrated specimen – Sputum/BAL fluid/Gastric lavage fluid was added into the tube. Also a drop (0.1mL) of the specimen was added to a 5% blood agar plate to check for purity.
5. Tubes were tightly recapped and mixed well.
6. Tubes were incubated at 37*C in an incubator.
7. The reading was taken daily up to 42 days using MICRO-MGIT system comparing with a control tube provided by the manufacturer.

Detection of positive growth: The MICRO-MGIT system signals a tube positive for growth with an indicator green light showing the reading of 15 or higher. At this point, the tube was removed and scanned outside the instrument. The tube was observed visually for the growth of the M.tuberculosis complex that settles at the bottom of the tube- it appears granular and not very turbid unlike the contaminating bacteria which appear to be less granular and more turbid.

3.6.7. Confirmation of growth

Growth can be confirmed by two methods:

- Microscopy
- Rapid antigen detection

1. The growth of Mycobacterium was confirmed by aseptically pipetting 1 or 2 drops of media and preparing two smears, which were stained by ZN and fluorescent methods. A drop was inoculated into 5% blood agar in order to rule out bacterial contamination.

   Specimens positive for AFB staining and showing no growth on 5% blood agar is considered positive for Mycobacterial growth.
2. Rapid antigen detection by SD TB Ag MPT64 kit was performed to confirm the positive isolates.

This antigen is secreted from tuberculosis bacteria and can help differentiate Mycobacterium tuberculosis complex from non-tubercular mycobacteria. It is based on the principle of antigen antibody reaction.

3.6.8. Drug Sensitivity testing

All the confirmed isolates were subjected to drug susceptibility using MGIT method for four 1^{st} line drugs:

RNTCP ZN staining grading (100x oil immersion objective and 10x eye piece)	Auramine-O fluorescent staining (40X:1length=40fields=200HPF)	Grading
>10 AFB/field after examining 20 fields	>50 AFB/1 field on average after examining 8 fields	3+
1-10 AFB/field after checking 50 fields	5-50 AFB/1 field on average after examining 20 fields	2+
10-99 AFB/field after checking 100 fields	20-199 AFB/1 length after examining 40 fields	1+
1-9 AFB/100 fields	1-19 AFB/1 length after 40 fields	Scanty
No AFB/100 fields	Zero AFB/ length after 40 fields	Negative

Streptomycin, Isoniazid, Rifampicin and Ethambutol. BD BACTEC MGIT 960 SIRE kits were used.

The kit contains lyophilized vials of each of the drugs which is later reconstituted with sterile water/distilled water.

Analysis: Data was entered in Microsoft excel and descriptive statistics like proportion, mean and standard deviation and Informational statistics like the chi-square test and the T-test were used.

Confidentiality: The information collected during the research was protected to maintain the confidentiality of the patients.

Quality control: It was compared with a standard H37Rv Mycobacterial strain.

4. Results

Table 1: Age distribution of patients studied

Age range (in years)	Number of Patients	Percentage (%)
1-10	01	0.5
11-20	12	06
21-30	27	13.5
31-40	25	12.5
41-50	46	23
51-60	45	22.5
61-70	38	19
>70	06	03
Grand Total	200	100

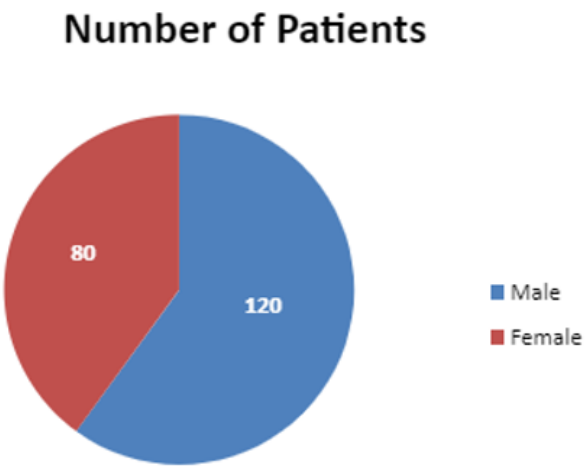
Majority of the patients in the study belonged to age group 41-50 years (23%), followed by 51-60 years (22.5%) and 61-70 years (19%).

Out of 200 patients studied, 120 were male (60%) and 80 were female (40%).

Table 2: Distribution of samples

Specimen Type	Number of Patients	Percentage %
BAL fluid	134	67
Sputum	66	33
Grand Total	200	100

The most common sample processed was broncho alveolar fluid 134 (67%) followed by sputum 66 (33%).



Graph 1: Gender distribution of patients studied

Table 3: Smear positivity – Ziehl Neelson and Fluorescent staining

Method	Criteria	No of cases (N=200)
Ziehl Neelson	Positive	18
	Negative	182
Fluorescent	Positive	21
	Negative	179

Table 4: Findings in MGIT method

MGIT	Number of cases	Percentage
Growth	28	14%
No growth	172	86%

Out of 200 cases, 28 (14%) cases were growth positive and 172 (86%) were negative.

The sensitivity of the direct smears – Ziehl Neelson (ZN) is 57.1% and for Fluorescent stain (FS), it is 64.2%. The specificity for Ziehl Neelson is 98.8% and for Fluorescent stain, it is 98.2%.

Out of 26 isolates of mycobacterium tuberculosis, 20 cases were sensitive to isoniazid, 22 to streptomycin, 24 to rifampicin and 17 to ethambutol. 6 isolates were resistant to isoniazid, 4 to streptomycin, 2 to rifampicin and 9 to ethambutol. There were 2 isolates that were atypical mycobacteria.

Table 5: Correlation of direct smear finding- Ziehl Neelson and Fluorescent Staining with MGIT method:

Method	Criteria	No. of patients (N=200)	MGIT Positive (n=28)	MGIT Negative (n=172)
Direct Smear Finding (ZN)	Positive	18	16	2
	Negative	182	12	170
Direct smear Finding (FS)	Positive	21	18	3
	Negative	179	10	169

Value = <0 0001

Table 6: Correlation of direct smear in relation to MGIT – an observation

Method	True Positive	False Positive	False Negative	True Negative	Total
Direct smear Finding - (ZN)	16	2	12	170	200
Direct smear Finding - (FS)	18	3	10	169	200

Table 7: Correlation of direct smear in relation to MGIT– an evaluation

Method	Sensitivity	Specificity	PPV	NPV
Direct smear Examination - (ZN)	57.1%%	98.8%	88.88%	93.4%
Direct smear Examination - (FS)	64.28%	98.2%	85.7%	94.4%

Value = <0 0001

Table 8: Drug sensitivity of isolates

Drugs	Isoniazid	Streptomycin	Rifampicin	Ethambutol
Resistant	6	4	2	9
Susceptible	20	22	24	17
Percentage Susceptible	76.9%	84.6%	92.3%	65.3%

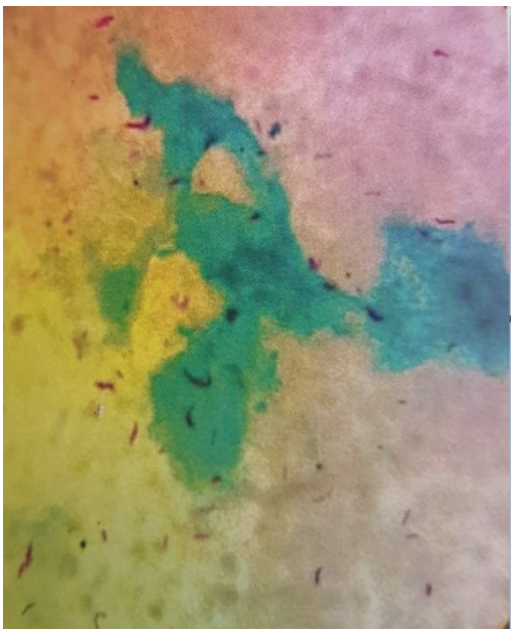


Fig. 1: Ziehl Neelsonstaining – Direct smear showing acid fast bacilli

Fig. 2: Fluorescentstaining – Direct smear showing acid fast bacilli

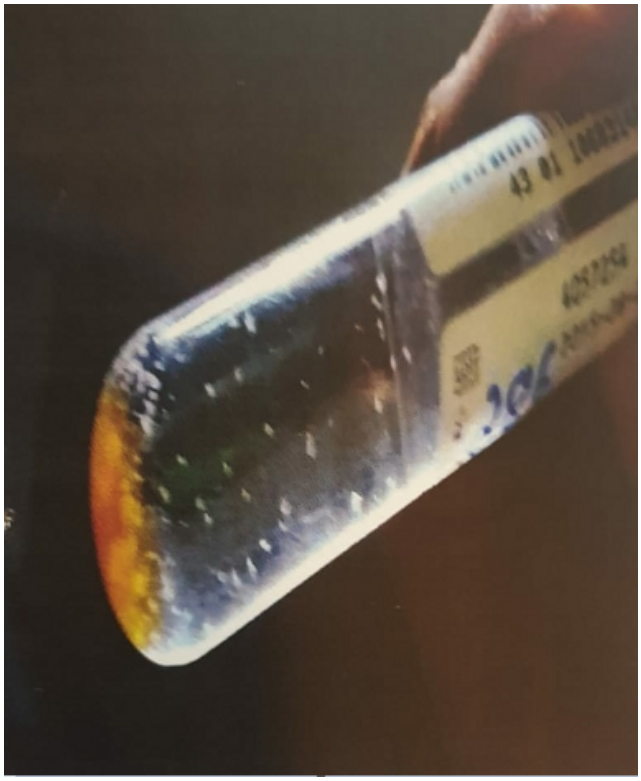


Fig. 3: BBLMGIT tube shwoing growth of Mycobacterium tuberculosis

Fig. 4: BACTEC Micro MGIT showing positive reading

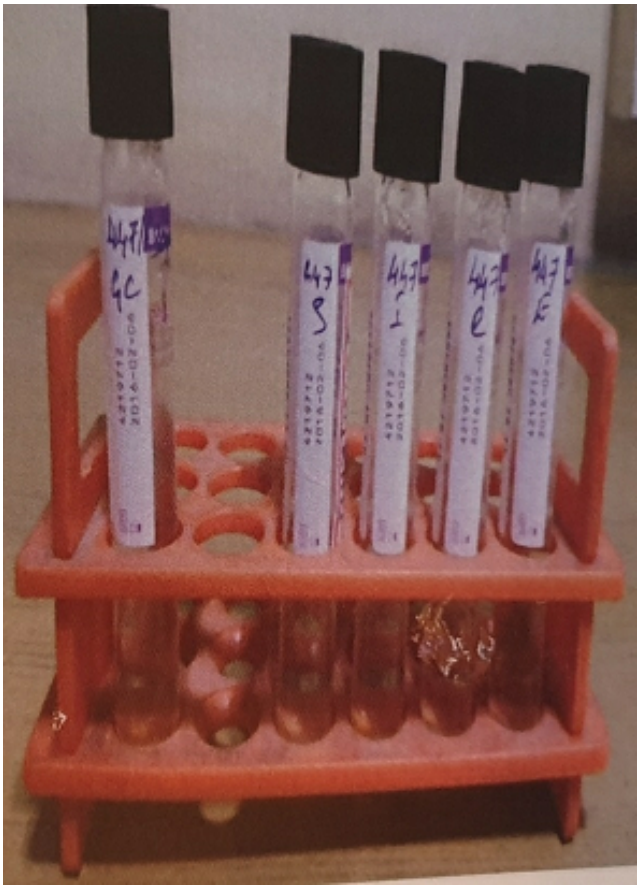


Fig. 5: Drugsusceptibilty testing

5. Discussion

5.1. Age

Majority of the patients in the study group belonged to the age group of 41-50 years (23%), followed by 51-60 years (22.5%) and 61-70 years (19%). The youngest patient in the study group was of age 8 years and the eldest was 80 years. The results were similar to the results obtained by Bansal R et al, where the mean (±1 SD) age of the patients studied was 49.03.[8] In one study by Dhanaraj B et al, the maximum prevalence of bacteriologically positive Pulmonary tuberculosis was found in the age group of 55–64 years in male. A number of factors underlie the high and rising rates of tuberculosis seen among older adults. Increased life expectancy changes the demographic profile.[9] With increased numbers of older people the proportion of both tuberculosis-related deaths and DALYs among older adults is likely to rise. But it is not only about relative numbers, older adults tend to be more vulnerable as a group than younger adults. This may be ascribed to compromised immune responses resulting from increased co-morbidity,[10] due to a range of chronic diseases (e.g. diabetes or chronic lung disease) and immunosuppressive

therapy (with arthritis, organ transplants or cancer etc.) and age-related immune-senescence.

5.2. Gender

Out of the 200 patients studied, 120(60%) were males while 80(40%) were females. The male to female ratio was 1.5:1. Narang.P et al have reported that 61.03% of their subjects were male while 38.97% were female.[10] An increased ratio was also noticed globally (1.6:1)[11] as well as by Sukesh Rao.[12]

The likely reason can be because in developing countries and countries with high prevalence, due to various socioeconomic reasons, males may be the earning members and there are higher chances of them being employed in the unorganized sectors, lesser chances of awareness about diseases, differential severity in males and females and probability of completing treatment is low.[13] WHO gender and health 2002.

5.3. Direct microscopy

Out of 200 cases, 18 cases (9%) were positive with Ziehl Neelsen staining and 21(10.5%) were positive by Fluorescent staining. In a study by Dzodanu et al, of the 200 samples analyzed, 71(35.5%) and 46(23.0%) were positive for pulmonary tuberculosis when Fluorescent and Ziehl Neelson stains were used, respectively. The mean reading time of Fluorescent microscopy was three times faster than the technique with very good acceptance.[14]

5.4. MGIT culture

In our study, out of 200 cases, 26 cases (14%) were found positive for Mycobacterium tuberculosis and 2 positive for non-tubercular mycobacteria and 172 cases (86%) were negative. The culture positivity reported by 2 other studies: -Akos somoskovi et al[15] and Ruhi Bunger et al[7] was 14% and 10.10% respectively.

5.5. Drug isolates

Out of 28 isolates, 2 isolates were atypical mycobacteria. Out of the other 26, 20 cases were sensitive to isoniazid (76.9%), 24 to streptomycin (92.3%), 24 to rifampicin (92.3%) and 17 to ethambutol (65.3%). 6 isolates were resistant to isoniazid (23.0%), 4 to streptomycin (15.3%), 2 to rifampicin (7.6%) and 9 to ethambutol (34.6%). In a study by Sunil Sethi et al, there was 75% sensitivity to isoniazid, 69% to streptomycin, 69% to rifampicin and 88% to ethambutol.[16]

In our study, there were 2 cases of MDR-TB.

6. Conclusion

1. Out of 200 clinically or radiologically suspected cases of tuberculosis, 18 were positive by Ziehl Neelson staining, 21 by Fluorescent and 28 cases by MGIT. We found, through the study that, the sensitivity of Fluorescent staining is higher than that of Ziehl-Neelson staining.(Sensitivity of Ziehl Neelson was 51.7% and that of Fluorescent staining was 64.28%).
2. Out of 28 cases that had a positive MGIT growth, 26 were Mycobacterium tuberculosis and 2 were Non-tubercular mycobacteria.
3. Mean duration of isolation in MGIT was 18.1 days.
4. Out of 28 isolates, 22 cases were sensitive to isoniazid, 20 to streptomycin, 24 to rifampicin and 17 to ethambutol. 6 isolates were resistant to isoniazid, 4 to streptomycin, 2 to rifampicin and 9 to ethambutol. There were 2 isolates that were atypical mycobacteria.

7. Conflicts of Interest

All contributing authors declare no conflicts of interest.

8. Source of Funding

None.

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