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Comparative Study on 7 and 14 Days Stored Pot Stained Sputum Smears with Conventional Direct Smears of Acid Fast Bacilli

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Modified staining procedure was tried to evaluate the staining stability of the stored sputum samples until 14 days in comparison with the conventional ZN staining method to detect Acid Fast Bacilli.

Key words: Acid Fast Bacilli, Pot staining, Sputum, Tuberculosis.

Every year, a total of hundred million people get infected with the TB bacilli worldwide. Asia has the heaviest human burden of TB in case members - India has 2.2 million new cases a year, followed by china with 1.4 million cases. 0.5 million people in India die from TB every year, more than 1000 every day (i.e.) 1 every minute. One sputum positive cases infect 10-15 healthy individuals in one year. Presently a number of diagnostic tests ranging from a simple AFB microscopy to complex molecular biological techniques that have become available over a period; to establish or rule out diagnosis of tuberculosis in a given patient. But microscopy is the simplest and most rapid procedure currently available to detect acid fast bacilli (AFB) in clinical specimens by Ziehl Neelsen staining method or its modification. The results of smear microscopy can be influenced by the type of specimens, thickness of the smear, extent of decolourization, type of counter stain used, and training and experience of the person examining the smear. Several approaches are being made to enhance the sensitivity of smear microscopy. Concentration of sputum sample by cytocentrifugation or by Phenol Ammonium Sulphate (PhAS) method has been found to enhance the sensitivity to almost 100% Liquefaction of sputum with sodium hypochlorite followed by concentration of bacilli by overnight sedimentation enhances the sensitivity of smear microscopy close to 70% compared to culture (RNTCP 2005).

Sputum acid-fast bacilli (AFB) microscopy services are established for every 100,000 population by the Revised National Tuberculosis Control Programme (RNTCP) (Technical and operational guidelines for tuberculosis control, 2005). Non availability of this service in many health facilities forces substantial proportion of pulmonary tuberculosis patients to travel long distances to avail diagnostic facilities (Selvam *et al.*,). Safety standards were improved in the laboratories in order to minimize the risk of infection (Mullu *et al.*). Inactivation of Mycobacterium tuberculosis in sputum smears before use is an important safety factor in preventing the potential transmission of tuberculosis (TB) to laboratory workers (Chedore *et al.*).

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A novel method of staining the sputum in the container itself using Carbol Fuchsin with an incubation time of up to 2 hours. Later the smear is prepared from the container stained sputum, heat fixed, decolorized and counterstained with Methylene blue. This was found to have more advantages like less hazardous, simple, less contagious, cost effective and disposal of containers made easy. (Selvakumar *et al.*) Later the pot-method was further explored for storage of sputum samples at ambient conditions in health facilities for detection of AFB and the results were compared with direct smears made by Ziehl-Neelsen (ZN) method (Selvakumar *et al.*). This study further seems to be more effective in revealing that storage of pot stained sputum samples up to 14 days without showing any significant difference in results which are well comparable with that of the direct smear stained

MATERIALS AND METHODS

Sample Procurements

Eighty sputum samples were collected. The samples collected were those discarded after direct smear examinations from patients enrolled for the diagnosis and for follow up examination.. Then those samples were immediately transported through means to Diagnostic Services, Chennai within an hour of collection.

Direct Smears

The direct-smears were prepared and stained by the standard hot Ziehl Neelsen (ZN) method as described in the RNTCP laboratory technician module (ZN-smears) (Technical and operational guidelines for tuberculosis control, 2005).

Pot Staining, Storage and pot smears

Equal volumes of phenol ammonium sulphate (PhAS) basic fuchsin stain was added to the sputum samples (pot method) (Plate I) and stored in a closed carton box at ambient conditions of 22 to 26 degree C for up to 14 days. (Selvakumar *et al.*). The phenol ammonium sulphate (PhAS) basic fuchsin smears made on day 7 and day 14 , were then, decolourized using 25 per cent sulphuric acid for 2 min and counterstained with 0.1 per cent methylene blue for 30 seconds for detection of AFB. The smears of both direct and pot stained were performed with bamboo sticks (Selvakumar *et al*).

Reading of Smears

The ZN stained direct smears and the pot stained slides were initially coded and read as per RNTCP guidelines (Technical and operational guidelines for tuberculosis control, 2005). Later the results were compared with the direct smear. All slides were decoded, the direct smear and their corresponding 7th and 14th day slides showing discrepant results were read again by a second reader. Slides with discordant results were again read by a third reader. The result of 2 concordant readings was considered as final for slides with discordant results.

RESULTS AND DISCUSSION

Then direct smears were taken from those samples and stained by conventional ZN method. Then phenol ammonium sulphate basic fuchsin reagent was added to those containers. Smears were taken on 7th day (F7) and 14th day (F14) respectively. The pot slides were decolorized and counterstained. All the slides were coded and read. The results were decoded.

On use of the conventional ZN method 54 slides were found to be positive and 26 were found to be negative.7 flocculate smears positive slides of day 7 were found to be negative in conventional ZN method and 7 flocculate smear negative slides of day 7 were found to be positive in conventional ZN method (Table 1 and corresponding Graph I). Sum of 9 positive slides of flocculate smears of day 14 were found to be negative and 3 negative slides of flocculate smears of day 14 was found to be positive (Table 2 and corresponding graph II). Total of 7 smears were found to be scanty in this method. Number of 1+ smear was found to be 13 in this method. 10 and 24 slides were found to be 2+ and 3+ respectively

Then when the discrepant slides were resolved by the second and third readers, the results improved further. The number of positivity increased from 54 to 61 and the number of negativity decreased from 26 to 18. Number of slides in scanty and 1+ smears increased to 8 and 18 respectively. Number of slides in 2+ smears was found to increase from 10 to 11. But the number of 3+ slides remained the same as 24 only (Table 3 and Table 4 with corresponding Graph III and IV).

On use of the Pot staining method for storage of sputum sample till 7 days , 54 slides were found to be positive and 26 were found to be negative.7 conventional ZN smear positive slides were found to be negative in flocculate smears and 7 conventional ZN smear negative slides were found to be positive in flocculate smears. Total of 8 smears were found to be scanty in this method. Number of 1+ smear was found to be 15 in this method. 16 and 15 slides were found to be 2+ and 3+ (Table 1).

The results improved further on resolving discrepancies.. The number of positivity increased from 54 to 60 and the number of negativity decreased from 26 to 20. Number of slides in scanty and 1+ smears increased to 10 and 18 respectively. Number of slides in 2+ smears was found to increase from 10 to 16. But the number of 3+ slides remained the same as 15 only (Table III).

On use of the Pot staining method for storage of sputum sample till 14 days , 60 slides were found to be positive and 20 were found to be negative. 7 conventional ZN smear positive slides were found to be negative in flocculate smears and 7 conventional ZN smear negative slides were found to be positive in flocculate smears. Total of 9 smears were found to be scanty in this method. Number of 1+ smear was found to be 18 in this method. 16 and 17 slides were found to be 2+ and 3+ respectively (Table II).

Then when the discrepant slides were resolved by the second and third reader, the results improved further. The number of positivity increased from 60 to 65 and the number of negativity decreased from 20 to 14. Number of slides in scanty and 1+ smears increased to 10 and 20 respectively. Number of slides in 2+ smears was found to increase from 16 to 20. But the number of 3+ slides remained the same as 15 only (Table IV).

On resolving the discrepancies it was found that there was no technical error but only reading error. Some direct negative slides were found to be positive in flocculate smears showing the increase in capability of staining on storage. But some direct positive slides were found to be negative in flocculate smears showing the absence of smear material on slide due to increased liquefaction process. The agreement percentage between conventional ZN and flocculate smear day 7 was found to be 82.5%. The agreement percentage

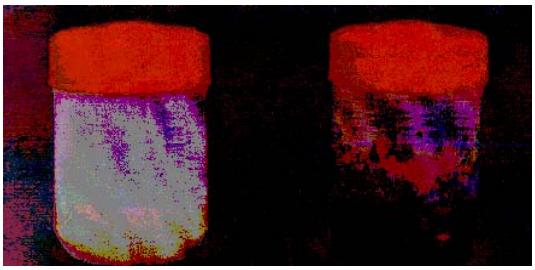
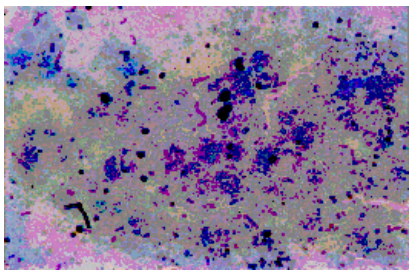
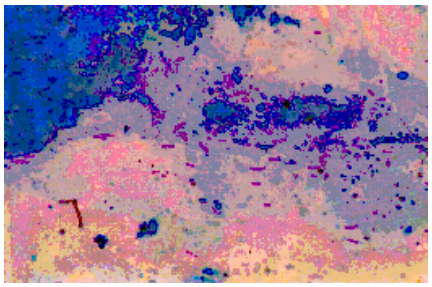


Plate 1. Sputum Containers

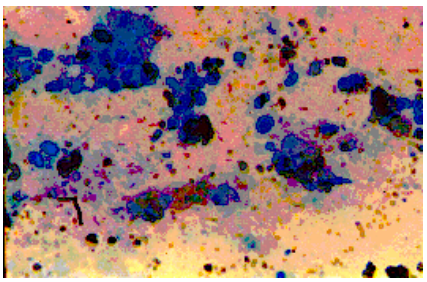
1. Freshly collected sputum sample.
2. Sputum sample after adding phenol ammonium sulphate basic Fuchsin reagent (PhAS).



Microscopic Appearance of Flocculate Smear Day 7



Microscopic Appearance of Direct Smear

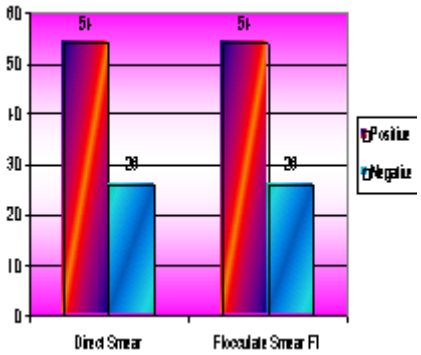


Microscopic Appearance of Flocculate Smear Day 14

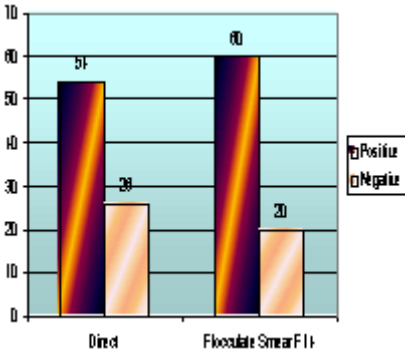
Plate 2. Appearance of AFB

between conventional ZN and flocculate smear day 14 was found to be 85%. Further on resolving discrepancy the percentage improved from 82.5% to 94.9% and 85% to 94.9%.

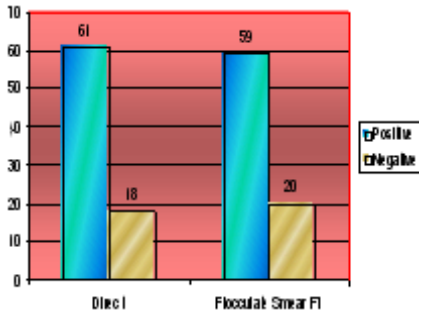
Use of a dye that was capable of staining the cell wall was first introduced in 1882 (Robert Koch). He employed three important factors in staining of tubercle bacilli namely the use of mordant, heating and decolorization. Improved stains were developed, hence the present gold standard Ziehl Neelsen staining procedure has evolved from attempts to improve on Koch's methods. Preparation of direct smears from a sputum sample is quite unpleasant for any technician handling it. Phenol ammonium sulphate sedimentation method was explored by (Selvakumar *et al.*) where the making of sputum smears became acceptable for laboratory



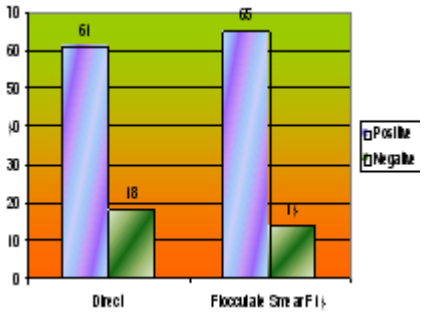
Graph 1



Graph 2



Graph 3



Graph 4

Table 1. Comparison Of Direct Smears With Flocculate – Smears of Day 7 (F7)

		Flocculate Smears Day 7						
		Sc*	1+*	2+*	3+*	Any Pos	Neg*	Total
Direct	Sc*	2	1	1	0	4	3	7
	1+*	2	9	1	0	12	1	13
	2+*	0	0	5	4	9	1	10
	3+*	3	3	7	9	22	2	24
	Any Pos	7	13	14	13	47	7	54
	Neg*	1	2	2	2	7	19	26
	Total	8	15	16	15	54	26	80

*Negative - No AFB per 100 oil immersion fields.
Scanty - 1-9 AFB per 100 oil immersion fields.
1+ - 10-99 AFB per 100 oil immersion fields.
2+ - 1-9 AFB per oil immersion field at least 50 fields.
3+ - more than 10 AFB per oil immersion field at least 20 fields.

Table 2. Comparison Of Direct Smears With Flocculate – Smear of Day 14 (F14)

		Flocculate Smears Day 14						
		Sc*	1+*	2+*	3+*	Any Pos	Neg*	Total
Direct	Sc*	3	2	2	0	7	0	7
	1+*	1	8	2	0	11	2	13
	2+*	0	0	7	3	10	0	10
	3+*	3	4	4	12	23	1	24
	Any Pos	7	14	15	15	51	3	54
	Neg*	2	4	1	2	9	17	26
	Total	9	18	16	17	60	20	80

*Negative - No AFB per 100 oil immersion fields.
Scanty - 1-9 AFB per 100 oil immersion fields.
1+ - 10-99 AFB per 100 oil immersion fields.
2+ - 1-9 AFB per oil immersion field at least 50 fields.
3+ - more than 10 AFB per oil immersion field at least 20 fields.

Table 3. Comparison Of Direct Smears With Flocculate – Smears Of Day 7 (F7) After Resolving Discrepancy

		Flocculate Smears Day 7						
		Sc*	1+*	2+*	3+*	Any Pos	Neg*	Total
Direct	Sc*	4	2	1	0	7	1	8
	1+*	3	10	3	1	17	1	18
	2+*	0	1	4	6	11	0	11
	3+*	2	5	8	8	23	1	24
	Any Pos	9	18	16	15	58	3	61
	Neg*	1	0	0	0	1	17	18
	Total	10	18	16	15	59	20	79

*Negative - No AFB per 100 oil immersion fields.
Scanty - 1-9 AFB per 100 oil immersion fields.
1+ - 10-99 AFB per 100 oil immersion fields.
2+ - 1-9 AFB per oil immersion field at least 50 fields.
3+ - more than 10 AFB per oil immersion field at least 20 fields.

Table 4. Comparison Of Direct Smears With Flocculate – Smears Of Day 14 (F14) After Resolving Discrepanc

		Flocculate Smears Day 14						
		Sc*	1+*	2+*	3+*	Any Pos	Neg*	Total
Direct	Sc*	3	2	3	0	8	0	8
	1+*	2	11	4	1	18	0	18
	2+*	0	0	8	3	11	0	11
	3+*	2	6	5	11	24	0	24
	Any Pos	7	19	20	15	61	0	61
	Neg*	3	1	0	0	4	14	18
	Total	10	20	20	15	65	14	79

*Negative - No AFB per 100 oil immersion fields.
Scanty - 1-9 AFB per 100 oil immersion fields.
1+ - 10-99 AFB per 100 oil immersion fields.
2+ - 1-9 AFB per oil immersion field at least 50 fields.
3+ - more than 10 AFB per oil immersion field at least 20 fields.

technicians as the phenol ammonium sulphate solution kills the tubercle bacilli within 30 minutes. The present study is based on the previous studies like the novel pot methodology and storage of sputum using cetylpyridinium chloride till 7 days demonstrated by (Selvakumar *et al*.) and storage of sputum in cetylpyridinium chloride till 20 days (Manuela Pardini et al.). Pot method for storage of sputum demonstrated by (Selvakumar *et al*.) up to 7 days was a breakthrough for this study. Performing this study enabled us to demonstrate that storage of sputum sample using phenol ammonium sulphate basic fuchsin up to 14 days produced comparable results with that of the conventional Ziehl Neelsen method.

Summary

The various advantages of this method includes the following: (i) smearing of these flocculate pot stained samples was much easier than the direct smears as the mucous was digested, (ii) Sputum samples can be stored and transported from remote collection centres to the district laboratories and national reference laboratories safely, (iii) Smears can be made at any convenient time as the morphology of the bacilli remains unaffected till 14 days of storage, (iv) There is no risk of aerosols infecting the environment even on accidental breakage of the containers during transportation, (v) Phenol acts as a potential disinfectant making the sputum sample sterile and non-hazardous. Therefore there is no risk of infection and such treated containers can be discarded as the normal waste, (vi) Ammonium sulphate acts as a protein precipitating and mucous digesting agent helping in flocculation of sputum samples. Moreover they render good fixation of smears on the slide, (vii) Basic fuchsin dye in the reagent stains the bacilli effectively thereby enabling us to view the bacilli as pink rods, (viii) Use of this method simplifies the staining procedure as it does not involve heating of carbol fuchsin, (ix) The morphology of the bacilli remains unaffected even after storage in this procedure whereas the risk of disfiguration and drying of smears due to overheating can occur in conventional ZN method. Only limitation found was that the acid fast bacilli in flocculate smears were not as bright as that of direct smears proving the gold standard to be the best.

CONCLUSION

This study revealed that the flocculate smears results made on 7th and 14th day were comparable with that of direct smear results without showing any significant difference. Therefore the sputum samples can be stored for 14 days by addition of phenol ammonium sulphate basic fuchsin solution directly to the sputum container. This renders the sample sterile and non-hazardous.

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