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COMPARATIVE EVALUATION OF MAGNETIC BEAD METHOD WITH MODIFIED PETROFF METHOD FOR DETECTING TUBERCLE BACILLI

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ABSTRACT **BACKGROUND:** To improve the sensitivity of sputum smear microscopy, a novel magnetic bead based concentration method is evaluated. Magnetic bead solution used here consisted of paramagnetic beads coated with a polymeric ligand that, under selective conditions, is supposed to bind mycobacteria. **METHOD:** Fifty sputum samples were processed. After making direct smears, samples were divided into two equal halves to be processed with two concentration methods followed by making smears. **RESULT:** Seventeen samples were positive and 33 were negative on gold standard culture. The sensitivity of direct smears, smears made after modified petroff method, & magnetic bead method were 58.8%, 88.2%, & 64.7% respectively. Specificities of the same were 84.9%, 96.9% & 93.9% respectively. **CONCLUSION:** Sensitivity and specificity of the novel evaluated method were found to be higher than those of direct smears. The smear microscopy however showed good positive agreement with the gold standard culture results (kappa value of 0.622).

KEYWORDS : Magnetic Beads, Sputum Concentration, Modified Petroff

INTRODUCTION

Tuberculosis (TB) is one of the major global health problems ranking above HIV/AIDS as a leading cause of death worldwide. Almost 90% of cases each year are in 30 high TB burden countries (**1**). In high-incidence, most low and middle income countries, as India where majority of world TB cases and deaths associated with TB occur, TB diagnosis relies on clinical symptoms and radiologic findings or laboratory diagnosis using sputum smear microscopy **(2)**. Culture techniques for the diagnosis of TB are highly sensitive and specific but the cost, technical complexity, and time delay before results are available make culture not suitable for rapid detection and treatment **(3)**. Sputum smear microscopy for the identification of TB bacilli remains the primary tool and mainstay for rapid and cost effective diagnosis of TB in resource-limited countries.

Smear microscopy using Ziehl Neelsen (ZN) staining technique is a conventional method used for TB diagnosis. However the main limitation of the method is its low sensitivity **(4)**. Many researches have been conducted to date to identify sample processing methods, physical as well as chemical, so as to improve the sensitivity of microscopy (**5**). Conventional method for sputum concentration which has been widely tested is modified Petroff's method, involving sputum decontamination and concentration using 4% NaOH and centrifugation respectively. Major hindering factor for using centrifuge at peripheral laboratories in low income countries is irregular power supply, inadequate financial resources and potential biohazards posed by centrifugation **(6)**.

Therefore still there is the need to develop & evaluate newer assays with higher sensitivity and specificity that are simple and can be universally adopted in low resource countries. In the present study, we compared the sensitivity and specificity of smear microscopy done after processing with ligand attached magnetic bead based concentration method with that of widely tested and applied modified Petroff's method for detection of MTB in sputum samples.

METHODS

Fifty sputum samples from clinically suspected pulmonary tuberculosis patients were processed by both - modified Petroff and magnetic bead methods. Sputum samples with a minimum of 4ml volume were included in the study. Direct smear was prepared by taking a small portion of the purulent part of the sputum with a sterile loop and stained by the ZN staining technique. Specimen was then divided into two equal parts for processing of one part by modified Petroff's method and other part by magnetic bead concentration protocol. Sample processing by each method was followed by making smears for ZN staining and inoculation of automated liquid culture medium bottle (BacT/Alert 3D).

Acid fast bacilli (AFB) on ZN stain were identified as red, straight or slightly curved rods, occurring singly or in small groups. If definite AFB were seen in sputum, they were reported as AFB positive and graded quantitatively as follows:1-9 acid-fast bacilli(AFBs) per 100 fields (scanty); 10-99 AFBs per100 fields (1+); 1-10 per field (2+) and >10 per field (3+). However if no AFB was seen, the smear was reported as negative.

Sample processing methodology

Modified Petroff's method

Processing steps were as follows **(7)**–

1. Half of the sample was transferred to a 50 ml conical Falcon tube and equal amount of sterile 4% NaOH solution was added, cap was tightened and mixed well using vortex followed by incubation at 37^0C for 15 minutes.
2. After incubation, tubes were centrifuged at 3000 x g for 15 minutes.
3. The supernatant fluid was discarded slowly into a container having 5% phenol solution.
4. The pellet was vortexed and 1N HCL with 2% Phenol red indicator was added drop by drop till the colour changed.
5. From this finally processed sample 20ul was used to make a smear for ZN staining and 0.5ml was inoculated in a BacT/ Alert culture bottle.

Culture reading:

1. The inoculated culture bottles were loaded in a BacT/ALERT 3D mycobacterium detection system for incubation up to a maximum of 6 weeks.
2. When machine flagged a culture bottle positive, it was confirmed by making a smear from the culture bottle and examining ZN stained smear for AFB.
3. Negative culture bottles were discarded only after complete 6 weeks of incubation.

Magnetic Bead based concentration method

Steps of sample processing were as follows:

1. To 20 ml plastic container, remaining 2ml sample was added.
2. Equal volume of thinning reagent ie NALC-NaOH (N-acetyl-L-cysteine NaOH) was added and incubated at 37^0C for 15-20 minutes.
3. After liquefaction, 4mL (equal volume) of 1X TB Beads were added and mixed by vortexing & incubated for 5minutes.
4. The container was placed onto the magnetic stand (**Image 1**) for 30 seconds and the supernatant decanted.
5. The container was then taken outside the magnetic stand and 4mL of washing buffer was added.
6. It was rinsed for 10 seconds and container was again placed onto magnetic stand for 30seconds.

7. Supernatant was discarded.
8. The container was then taken outside the magnetic stand and 100uL of elution buffer was added and incubated for 2-3 minutes.
9. The container was placed onto magnetic stand and eluted cells were collected.
10. Smears were made by using 20uL micropipette and stained with ZN staining.

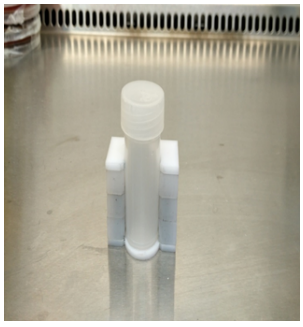

Image 1: Plastic container fitted on magnetic stand

Ethical consideration

The study was approved by the medical education and research's institutional ethical committee.

RESULTS

Results on modified Petroff's concentration method were taken as gold standard. Out of total 50 sputum samples, 17 samples were positive and 33 were negative on gold standard culture.

Smear results

Performance of direct smears

Ziehl Neelsen stained direct smears (direct ZN) detected-

- Fifteen (30%) AFB positive and thirty five (70%) AFB negative smears.
- Among the fifteen positive smears, 7 (14%) were scanty positive, 2 (4%) were 1+, 1 (2%) was 2+ and 5 (10%) were 3+ (**Table 1**)
- Sensitivity of direct ZN staining was 58.8% (10/17).
- Specificity of the same was 84.9% (28/33).

Performance of modified Petroff's method concentrated smears

Out of 50 samples ZN stained modified Petroff's (MP ZN) concentrated smears detected-

- Sixteen (32%) AFB positive and thirty four (68%) AFB negative smears.
- Among the 16 positive samples, 3 (6%) were scanty positive, 2 (4%) were 1+, 5 (10%) were 2+ and 6 (12%) were 3+. (**Table 1**)
- Sensitivity of modified Petroff's ZN staining was 88.2% (15/17).
- Specificity of the same was 96.9% (32/33).

Performance of magnetic bead method concentrated smears

Out of 50 samples ZN stained magnetic bead concentrated smears (MB ZN) detected -

- Thirteen (26%) AFB positive and thirty seven (74%) AFB negative smears.
- Among the 13 positive samples, 4 (8%) were scanty positive, 3 (6%) were 1+, 1 (2%) was 2+ and 5 (10%) were 3+ (**Table 1**).
- Sensitivity of magnetic bead ZN staining was 64.7% (11/17).
- Specificity of the same was 93.9% (31/33).

Table 1: Distribution of positive and negative smears made directly and after concentration by modified Petroff & Magnetic bead method

		Gold Standard culture		
		Positive	Negative	Total
Direct ZN	Positive	10	05	15
	Negative	07	28	35
	Total	17	33	50
MP ZN	Positive	15	01	16
	Negative	02	32	34
	Total	17	33	50
MB ZN	Positive	11	02	13
	Negative	06	31	37
	Total	17	33	50

ZN- Ziehl Neelsen, modified Petroff method Ziehl Neelsen stained smear (MP ZN), Magnetic bead method Ziehl Neelsen stained smear (MB ZN)

Comparison of grading distribution of direct smears with magnetic bead concentrated smears

Grading of AFB smears made after direct and magnetic bead concentration method was compared **(Table 2)**. Of the 50 specimens, 11 (22%) specimens were positive both on direct and MB ZN smears. Four samples were positive (all culture negative) on direct ZN microscopy but were negative on MB ZN smears. All four samples were of positive scanty grading on direct ZN. MB ZN detected additional one culture positive sample, graded positive 1+ on MB ZN which was negative by direct ZN. Another extra smear of grading positive scanty detected on MB ZN was culture negative and also negative on direct ZN smear.

Comparison of grading distribution of modified Petroff's concentrated smears with magnetic bead method concentrated smears

Grading of AFB smears made after both the concentration methods are compared in **table 2 & 3**. Of the 50 specimens, 11 (22%) specimens were positive by both the methods. Modified Petroff's ZN smear microscopy detected four additional culture positive samples which were positive 2+ on modified Petroff's microscopy but were negative on MB ZN and direct ZN smear as well. One scanty positive modified Petroff's smear not detected by MB ZN was culture negative. MB ZN detected two extra positive smears as scanty but negative by modified Petroff's smear, but these samples were negative on culture.

Table 2: Grading distribution of sputum smears

		MB ZN					
		1+	2+	3+	Scanty	Negative	Total
Direct ZN	1+	2	0	0	0	0	2
	2+	0	0	1	0	0	1
	3+	0	1	4	0	0	5
	Scanty	0	0	0	3	4	7
	Negative	1	0	0	1	33	35
	Total	3	1	5	4	37	50
MP ZN	1+	2	0	0	0	0	2
	2+	0	1	0	0	4	5
	3+	1	0	5	0	0	6
	Scanty	0	0	0	2	1	3
	Negative	0	0	0	2	32	34
	Total	3	1	5	4	37	50

ZN- Ziehl Neelsen, MP ZN- modified Petroff method Ziehl Neelsen stained smear, MB ZN-Magnetic bead method Ziehl Neelsen stained smear

Table 3: Distribution of smear and culture results after processing by both techniques

ZN stained smear grading by:					Smear versus standard culture	
	Modified Petroff method		Magnetic bead method		Modified Petroff method	Magnetic bead method
Smear grade	No (n)	% (n/50 * 100)	No. (n)	% (n/50 * 100)		
1+	2	4	3	6	2/2[a]	3/3[b]
2+	5	10	1	2	5/5[a]	1/1[b]
3+	6	12	5	10	6/6[a]	5/5[b]
Scanty	3	6	4	8	2/3[a]	2/4[b]
Negative	34	68	37	74	1/34[c]	6/37[d]

a Number of positive results on culture/total number of positive results by the modifiedPetroff method on microscopy.
b Number of positive results on culture/total number of positive results by the Magnetic bead method on microscopy.
c Number of positive results on culture/total number of negative results by the modifiedPetroff method by microscopy.
d Number of positive results on culture/total number of negative results by the Magnetic bead method by microscopy

Statistical tests for measurement of agreement & level of association with gold standard culture method

The kappa coefficient for positive agreement between the modified

Petroff method smear microscopy and the gold standard culture and also between magnetic bead method smear microscopy and the gold standard culture was calculated which showed (**Table 4**)-

- Excellent positive agreement between the modified Petroff method ZN stained smear microscopy and the culture (k = 0.864), and
- Good positive agreement between magnetic bead method ZN stained smear microscopy and the culture (k = 0.622).

Table 4: Statistical tests

	McNemar chi square test	Kappa coefficient (k)	
	p value	Value	p Value
Direct ZN	0.774	0.450	= 0.001
MP ZN	1	0.864	<0.001
MB ZN	0.289	0.622	<0.001
Number of samples	50		

ZN- Ziehl Neelsen, MP ZN- modified Petroff method Ziehl Neelsen stained smear, MB ZN-Magnetic bead method Ziehl Neelsen stained smear

DISCUSSION

The results of our study found direct ZN microscopy to be 58.8% sensitive which was in range with a meta-analysis done by Steingart et al **(8)** which showed direct ZN smear sensitivity ranging from 31% to 80%. There were four smears positive as scanty on direct ZN microscopy that did not grow on culture which could be due to presence of dead bacilli in the sample as the samples from patients on anti-tubercular treatment were not excluded.

Sensitivity & specificity of modified Petroff's ZN smear were 88.2% & 96.9% respectively in our study which were comparable to the findings in studies conducted by V Mittal et al in 2014 **(9)** & S Verma et al in 2013 **(10).**

Magnetic bead method ZN microscopy showed sensitivity of 64.71% which was almost similar to the sensitivity of magnetic bead ZN smear (65%) in the study done by Wang X et al in 2013 (**11**). However sensitivity of direct ZN smear (58.82%) observed in our study was higher than sensitivity of direct ZN smear in their study (40%).

This magnetic bead processing method involved an almost similar number of steps, similar level of complexity and laboratory infrastructure as modified Petroff method for sputum concentration & decontamination, and both methods were significantly more time-consuming than performing direct smear preparation. One advantage of magnetic bead processing method is that it does not essentially require power supply as compared to centrifugation processing method. Another significant finding of magnetic bead processing methodology was cleaner smears than direct smear or modified Petroff's smear with less debris and stained artefacts under the microscope which can be the possible explanation of good specificity of magnetic bead processed smears (93.4%). J Liu et al (**12**) also observed similar characteristics of magnetic bead processed smears in their study. In our study NALC NaOH was used as thinning reagent. The significant drawback of using NALC NaOH is that it can be used only till next 24 hours after preparation, so practically NALC NAOH requires daily fresh preparation which is often cumbersome in the daily routine practice of laboratories**.** However there are other reagents available which can be used in place of NALC NaOH for sample digestion and decontamination in conjunction with magnetic bead based concentration technique.

CONCLUSION

The magnetic bead protocol requires only a basic manual magnetic rack, similar in complexity to modified Petroff's protocol, and provides an alternative to centrifugation in processing of sputum specimens. Though the smears made after specimens processed through investigated technique were clearer in microscopic view than those of direct and modified Petroff's method, the magnetic bead specimen concentration technique stood low in performance as compared to widely used modified Petroff's method. However, the results of magnetic bead method's smear microscopy showed good positive agreement with the gold standard culture results with a kappa value of 0.622. Also the sensitivity and specificity of the novel evaluated method were found to be higher than those of direct smears. Magnetic bead processing method did not involve centrifugation and so required no electricity to run the process thus making the use of method feasible in peripheries. This suggests that if the sensitivity of the magnetic bead smear microscopy could be improved in future versions of the technology, this may offer an alternative to centrifugation.

CONFLICTS OF INTEREST

There were no conflicts of interest.

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