

False Negativity of Ziehl-Neelsen Smear Microscopy: Is the Scale-up the Worth It in Developing Countries?

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ABSTRACT

Objective: To evaluate the false negative results of Ziehl-Neelsen (ZN) smear microscopy.

Study Design: Descriptive study.

Place and Duration of Study: Mycobacteriology Laboratory, Allama Iqbal Medical College (AIMC) and Jinnah Hospital, Lahore (JHL), Pakistan, from February 2014 to August 2016.

Methodology: A total of 3,951 (pulmonary 2,773 and extra-pulmonary 1,178) samples were collected from strong TB suspected patients attending JHL Lahore. Follow-up cases were excluded. Every specimen was processed for ZN smear microscopy, Lowenstein Jensen (LJ) culture. SPSS 21.0 was used; false negative and positive results of ZN smear were calculated keeping LJ culture as gold standard.

Results: Out of total 3,951 samples, sputum was most frequently found pulmonary sample 48.4% (n=1915), extra-pulmonary samples, pleural fluid and pus samples were most commonly observed samples 12.0% (n=476) and 8.3% (n=329), respectively. Overall false negativity was 23.1% (pulmonary=19.6%, extra-pulmonary=29.2%) (p<0.001), Maximum false negative results were observed in pericardial, synovial, pleural fluids, and pus samples as 40.0%, 38.0%, 33.0% and 32.0%, respectively.

Conclusion: ZN smear microscopy is not a very efficient tool in case of patients with the low mycobacterial load. Therefore, National TB Control programs should consider extending their diagnostic approaches from ZN microscopy to more advanced techniques.

Key Words: Ziehl-Neelsen (ZN) smear. False negative. Tuberculosis. Microscopy.

INTRODUCTION

Tuberculosis (TB) aptly earned such morbid nicknames like the "White Plague", and "Captain of the Men of Death".¹ The contagious nature of TB is accountable for the heavy burden on the global community for centuries.^{2,3} TB is a serious threat, therefore elimination by 2035,³ is the goal but the commitment to the end of TB is laudable. WHO global tuberculosis reports provide a prospect to think once again about the global TB strategy, and to assess just how much further effort is desired before global TB control can be accomplished.^{2,3}

The emergence of this global dilemma is attributed to

sluggish diagnostic techniques and inappropriate patient management. A single infected case is a threat to 10-15 persons annually.⁴ For the last so many years till to date TB is being diagnosed by multiple methodologies, according to facilities and resources available. Most national TB control programs in the developing countries are implementing direct acid-fast bacilli (AFB) smear microscopy as a primary test for TB case detection,² as it is one of the simplest, oldest, cheapest, rapid, and easily available technique around the globe.⁵ However, it has some limitations, like the low sensitivity of 22-43% and maximum up to 60% under optimal conditions.^{6,7} The sensitivity is even lower in pediatric and human immunodeficiency virus patients who usually present a paucibacillary picture.^{8,9}

The threshold of ZN smear for detection of AFB is 10⁴ to 10⁵ bacilli/ml to give positive ZN smear.¹⁰ The yield is often decreased further due to technical and operational constraints.¹¹ Therefore, cases at initial stages of TB and heaving very low AFB load can be falsely reported as negative.

Lowenstein Jensen (LJ) culture for MTB remains superior to ZN smear microscopy as it can detect MTB as low as 10 to 100 viable bacteria/ml.¹⁰ Although LJ culture has many limiting factors; however, due to its high reliability, WHO recommend culture as the gold standard for tuberculosis diagnosis.

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The present study was conducted to evaluate the false negative results of ZN smear, which can be a significant factor in the resurgence of TB.

METHODOLOGY

This descriptive study was planned and conducted in the Pathology Department (Mycobacteriology Lab) of Allama Iqbal Medical College and Jinnah Hospital (AIMC & JHL) Lahore, Punjab, Pakistan from February 2014 to August 2016. Study protocols were approved from ethical board of the institute.

A total of 3,951 (pulmonary 2,773 and extra-pulmonary 1,178) samples were included. Pulmonary samples

comprise of sputum, broncho-alveolar lavage (BAL) and extrapulmonary samples include pleural fluid, pus, cerebrospinal fluid (CSF), ascitic fluid, pericardial fluid, synovial fluid, and urine specimens. Specimens were collected from strong TB suspected patients (a persistent cough >3 weeks, and/or evening rise of temperature from >2 weeks, weight loss, radiological evidence) attending pulmonology department, medical ward, OPD, MDR clinic of JHL, Lahore, Pakistan. Follow-up cases were excluded. Specimens were collected under expert supervision by following standard WHO guidelines.¹² Every specimen was processed for ZN smear stain,¹³ and LJ culture according to WHO recommendations.⁴ After inoculation on LJ culture, every

Table I: False negativity of Zn smear in pulmonary and extrapulmonary samples.

Samples			LJ (Gold standard)		Total	Fp	Fn	Statistics
			+ve	-ve				
Pulmonary	Zn	+ve	868	8	876	0.9%	19.6%	Chi-square 287.924
		-ve	373	1524	1897			
		Total	1241	1532	2773			
Extra-pulmonary	Zn	+ve	118	1	119	0.8%	29.2%	P-value 0.0001
		-ve	310	749	1059			
		Total	428	750	1178			
Total	Zn	+ve	986	9	995	0.9%	23.1%	P-value 0.0001
		-ve	683	2273	2956			
		Total	1669	2282	3951			

Fn = False negative; Fp = False positive.

Table II: Sample type-wise false negativity of Zn smear.

Samples				(LJ -Gold standard)		Total	Fp	Fn
				Positive	Negative			
Pulmonary samples	Sputum	Zn	+ve	708	5	713	0.7%	23.3%
			-ve	281	921	1202		
			Total	989	926	1915		
	BAL	Zn	+ve	160	3	163	1.8%	13.2%
			-ve	92	603	695		
			Total	252	606	858		
Extra-pulmonary samples	Pleural fluids	Zn	+ve	65	1	66	1.5%	33.4%
			-ve	137	273	410		
			Total	202	274	476		
	Pus	Zn	+ve	26	0	26	0%	32.0%
			-ve	97	206	303		
			Total	123	206	329		
	CSF	Zn	+ve	11	0	11	0%	16.6%
			-ve	17	85	102		
			Total	28	85	113		
	Ascitic fluid	Zn	+ve	7	0	7	0%	15.6%
			-ve	20	108	128		
			Total	27	108	135		
	Pericardial fluid	Zn	+ve	5	0	5	0%	40.0%
			-ve	14	21	35		
			Total	19	21	40		
	Synovial fluid	Zn	+ve	1	0	1	0%	38.2%
			-ve	13	21	34		
			Total	14	21	35		
	Urine	Zn	+ve	3	0	3	0%	24.4%
			-ve	12	37	49		
			Total	15	37	50		

Fn = False negative; Fp = False positive.

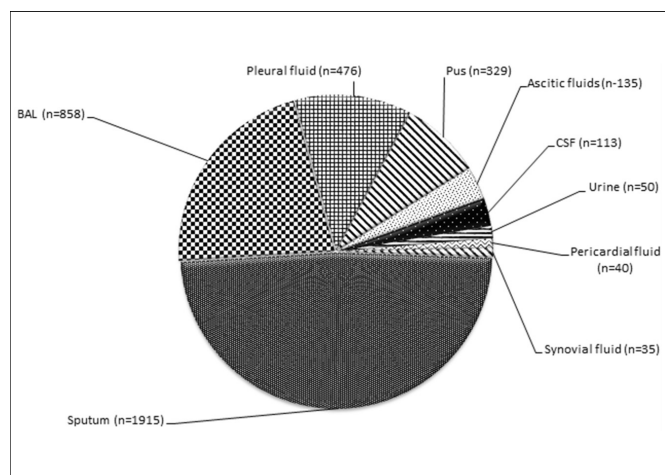


Figure 1: Frequencies of different clinical samples selected for the study (n=3,951).

specimen was kept under observation, for the first 48 hours. Growth was checked after every seven days till eight weeks.

ZN smear: Positive and negative control slides were prepared and stained with every batch of ZN stain. Every slide was checked by two different medical laboratory technologists by using a light microscope. Random positive and negative, doubtful slides were rechecked by highly experienced senior microbiologist for quality assurance purposes. **LJ culture:** Culture media quality and mycobacterial growth were confirmed by using American type culture collection (ATCC) strains of H37rv. LJ media bottle was inoculated with sterile water as negative control.

SPSS 21.0 was used for analysis of demographic characteristics of patient's frequencies and percentages. False negativity and positivity of ZN smear were calculated as followed, keeping LJ culture as gold standard. Chi-square test was used to assess any statistical significance at $p < .05$. (i) $FP = \text{false positive} / (\text{false positive} + \text{true positive}) \times 100$ (ii) $FN = \text{false negative} / (\text{false negative} + \text{true negative}) \times 100$.

RESULTS

The distribution of different samples used in this study was presented in Figure 1. Sputum was most frequently found a pulmonary sample with the frequency of 48.4% (n=1915); while on the other hand, in the category of extra-pulmonary samples, pleural fluid and pus samples were most commonly observed samples with the percentages of 12.0% (n=476) and 8.3% (n=329), respectively.

Table II showed that maximum false negativity was found in pericardial, synovial and pleural fluids with the percentages of 40.0%, 38.2%, and 33.4%, respectively. Among pus samples, the false negativity was found to be 32.0%.

DISCUSSION

Inspite of the steps taken to control the spread of TB under the supervision of international organizations like WHO, still it is a serious global dilemma, poses a significant challenge for clinicians and diagnostic services, especially in developing countries like Pakistan.¹⁴ Although, last decade was the era of modern and innovative diagnostic technologies in ground TB diagnosis, multiple modalities (LIPA, GeneXpert, MTBDR plus, MGIT) have been introduced with high accuracy precision and low turnaround time. But these are not easily reachable for everyone in developing countries due to its high cost and unawareness. According to WHO, maximum burden of TB is present in developing countries.^{2,3} As for new case detection, we are still dependent on conventional diagnostic techniques, like ZN and LJ culture. This upcoming threat of the spread of TB may be attributed to the limitation of these conventional techniques, like false negativity of ZN smears and long incubation time of LJ culture. According to standard instructions, diagnosis of MTB by LJ culture has been taken as gold standard.

The results of present study highlighted the critical issue of the false negativity of ZN smear microscopy for the screening of TB cases as it is a most widely used technique in Pakistan as well as in other developing countries. An alarmingly high false negativity was observed in EP samples 29.2% followed by pulmonary samples 19.6% with an overall false negativity of 23.1% (Table I). This is a threat to the community as these misdiagnosed patients are the potential source to spread TB in the general community.^{12,13} This is the major reason behind the resurgence of TB in devolving countries. Multiple studies have been reported false negativity of ZN smear in the last decades ranging from 6-36%;¹⁵⁻¹⁸ but still, this less sensitive technique is being used due to its cost-effectiveness, simplicity, and rapidity.

A study from Pakistan reported a comparatively low false negativity 11.7%.¹⁵ Similarly, Irum *et al.* reported 10.6%.¹⁹ In the same way, an Indian population-based study from New Delhi Tuberculosis Centre (NDTC) also reported 12.4% false negativity.²⁰ Agrawal *et al.* reported 16.3%.¹⁸ Furthermore, some studies reported high rate of false negativity as compared to present study, Negi *et al.* from India reported 29.7%, Nagpal *et al.* compare ZN smear with PCR and reported false negativity 26.9% and Caws *et al.* reported 37.0%.¹⁶⁻¹⁸

The differences are attributed to many factors such as sample size, technical expertise of lab technologists and the availability of professional microbiologist, which influence directly on the results of ZN smear. In case of extra-pulmonary samples, present study reported a high false negativity 29.2%, which is supported by multiple national and international research articles where false negativity is reported up to 79%.^{4,6,21-23} High false

negativity has been supposed to be due to scantiness and paucibacillary nature of samples. The present study also confirms Yam *et al.*, statement that AFB smear of fluids is rarely positive.²²

False negativity among ZN smear microscopy is attributed to various factors, which include patients' selection criteria, type of samples, concomitant medical conditions like HIV, neoplasia, and immune-compromised status of patients and technical faults. Smear microscopy cannot be solely relied upon for diagnosis and its results must be correlated with additional clinical information and other diagnostics due to a considerable amount of false negativity, especially in extra-pulmonary tuberculosis. However, if there is no bacteriologic evidence, the existence of extra-pulmonary tuberculosis could not be denied.

Above mentioned outcomes of this study imply that in a high-burden country like Pakistan, special care must be taken in smear-negative suspects before declaring them TB-free. The best option is to refer all suspicious smear-negative suspects for culture, but conventional culture technique leads to considerable delay, not only for diagnosis but also in starting treatment which may contribute to increasing morbidity-mortality and drug-resistance. Therefore, for improved case detection and better patient management, the need of the day is to take a step forward from conventional to latest diagnostic techniques. This is also recommended by WHO, both for pulmonary and extra-pulmonary TB, especially in smear-negative cases, which recommend the implementation of GeneXpert and other molecular-based techniques.^{2,3,23} As these are very rapid techniques so that diagnostic services can facilitate clinicians by confirming the diagnosis within few hours to days. According to WHO recommendation, every suspect of TB should be screened by using GeneXpert in developing countries as these are the high burden areas and need a speedy diagnosis under high qualified and technical expertise.^{2,3} WHO recommended implementation of this rapid PCR-based technique as a screening marker for MTB. Therefore, for better case detection the need of the day is on spot accurate diagnosis, in time treatment, and management of patients to control the spread of disease to break off the chain of TB. Any misdiagnosed TB infected case can be a source of potential threat for the community.

CONCLUSION

Although smear microscopy remains the backbone of diagnostic services in any TB screening programs, it is not a very efficient tool in case of patients with the low mycobacterial load. Therefore, National TB Control programs should consider extending their diagnostic approaches from ZN microscopy to more advance single test-based molecular technology "GeneXpert",

especially for strong TB suspected new cases with smear-negative results. The cost of GeneXpert is the only prohibitive factor to the implementation of GeneXpert in developing countries.

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