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Short Communication

Microscopic detection of *Mycobacterium tuberculosis* in direct or processed sputum smears

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Abstract

Introduction: Microscopic identification of active pulmonary tuberculosis (PTB) from direct smears of sputum (DS) is widely used for detection, but has limited sensitivity. Here, we assessed the yield of acid-fast bacilli (AFB) detection in processed sputum smears (PSS). **Methods:** Sputum samples were simultaneously analyzed by direct sputum smearing and after chemical treatment and spontaneous sedimentation. **Results:** Of the 1,719 samples analyzed, 16.4% were positive for AFB in conventional DS and 21.4% in PSS, corresponding to a 30% increase in detection. **Conclusions:** Increased sensitivity from analyzing PSS and better safety protocols will contribute to improved detection and control of the disease.

Keywords: Tuberculosis. Sputum processed. Smear microscopy.

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) is one of the oldest transmitted diseases in humans. Pulmonary TB (PTB) transmission occurs from inhalation of MTB-containing aerosols produced by a TB-infected person through coughing, sneezing, or exhaling[1].

Identifying active PTB cases is crucial for reducing disease transmission and strengthening disease control. Screening for acid-fast bacilli (AFB) by conventional sputum smear microscopy of direct smears of sputum (DS) is the first line test used for PTB laboratory diagnosis. Analysis of DS is easy to perform, quick, and inexpensive, but has limited sensitivity, with a detection threshold of 5000 to 10,000 AFB/mL of sputum[2].

Bacterial culture is the gold standard for diagnosis; it is highly sensitive and detects as low as 10 AFB/mL of sputum. However, it is laborious and time-consuming, requiring up to 12 weeks to provide results. Complex laboratory facilities are also required because handling of MTB organisms requires conditions of biosafety containment level 3 (BSL3)[2,3].

A rapid test (Xpert MTB/RIF® assay) developed by Cepheid (Sunnyvale, CA, USA) based on real-time quantitative polymerase chain reaction (qPCR) allows, in up to two hours, the detection of MTB and identification of mutations that are associated with rifampicin resistance, in the *rpoB* gene region. This technology is expensive, however, and is therefore underused and even inaccessible in some places[2].

Considering the importance of microscopic detection of AFB for PTB diagnosis, several modifications to the conventional DS procedure were carried out, aiming to improve the sensitivity, with varied results[4,5]. Modifications included those in the concentration of bacilli by sedimentation or centrifugation and liquefaction of the samples or chemical treatment of the sputum. While the modifications improved sensitivity, they considerably raised biohazardous risks[6].

Because MTB is very hazardous, there is a need in public health services for the implementation of effective, low-cost methodologies for identifying active PTB cases that are devoid of occupational hazards. This is especially true in remote and rural areas and in low income, high PTB-burden regions. In this work, we assessed the incremental yield of AFB detection using a modified sputum smear microscopy protocol.

Single sputum samples from individuals with signs and symptoms of PTB were obtained from July 2015 to March 2016 from the *Laboratório Municipal de Saúde Pública* (LMSP), Recife, PE, Brasil. After routine diagnosis, samples were transferred and analyzed at the *Instituto Aggeu Magalhães* of the *Fundação Oswaldo Cruz*, Recife, PE. The samples were stored at 4°C to 10°C for 6-7 days between collection at LMSP and analysis at our biosafety containment level 2 (BSL2) laboratory. At the time of screening, only the samples ≥1mL and of suitable

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consistency were enrolled for the study. The samples were analyzed simultaneously by direct smearing of the sputum and after processing by chemical treatment with bleach [2.0-2.5% commercial sodium hypochlorite (NaOCl)] and concentration by spontaneous overnight sedimentation. The difference between the two methods was tested using the chi square test[2,7].

Direct smears of sputum were prepared directly from the patient samples prior to processing. In a biological safety cabinet (Class II-B2), the sputum samples were macroscopically observed and the thicker mucous portions were picked with bamboo applicator sticks and thoroughly smeared on glass microscope slides. The smears were air-dried at room temperature (RT), heat-fixed using a spirit lamp flame and stained using the standard Ziehl-Neelsen (Z-N) method[8,9].

After the conventional smear was prepared, a volume of bleach that was twice the volume of sputum remaining in the container was added and gently mixed to avoid spilling. The mixture was then left for approximately 10 minutes to inactivate the MTB or other contaminating bacteria. The mixture was then poured directly into a 12-mL screw cap conical tube, mixed thoroughly to improve the chemical digestion of the organic material, and maintained at RT overnight (12 to 18 hours) for spontaneous sedimentation.

Next, the supernatant was discarded. The remaining deposit was vigorously mixed, the lid was discarded, the open tube was inverted and placed in the center of a glass slide, and left until its contents were completely drained onto the slide surface. The samples were then smeared over the slide using the mouth of the tube. The smears were air-dried at RT, and the aforementioned steps were performed, except for the following minor modifications: fuchsine staining 3 minutes, alcohol-acid solution staining for 30 seconds, and methylene blue staining for 30 seconds.

The readings were obtained using bright-field microscopy with a 100x oil objective lens, and the results were recorded based on the standard scoring scheme for AFB identification as follows: negative (no AFB observed in 100 fields), 1-9 AFB (1-9 AFB in 100 fields), 1+ (10-99 AFB in 100 fields), 2+ (1-10 AFB per field, after checking 50 fields), and 3+ (more than 10 AFB per field after checking 20 fields)[8,9].

A total of 1,719 sputum samples were enrolled in the study. Of these, 283 (16.5%) were positive, and 1,436 (83.5%) were negative for AFB by analysis of DS. Using PSS, 368 (21.4%) samples were positive, and 1,351 (78.6%) were negative (**Table 1**).

There was agreement in the number of AFB-negative samples in the 1,351 samples using both procedures. Eighty-five sputum samples that were negative by DS analysis were positive for AFB in PSS analysis, corresponding to the 30% increase in positivity. The difference in the percentage of AFB-positive samples detected by the two methods was statistically significant ($p < 0.001$)[7].

Comparing the scores recorded in the two procedures, similar results of the AFB counts were observed in 148 samples and in 212 samples the number of AFB per microscopic field increased up to 10-fold regarding the DS counts (**Table 2**).

Nineteen paucibacillary samples (with 1-9 AFB in 100 fields) by PSS analysis were negative by DS.

In 104 PSS samples with 10-99 AFB in 100 fields, categorized as 1+, similar results were observed in both procedures in 45 (43%) samples, which were also categorized 1+ by DS analysis. Fifty-three (51%) samples were negative by DS analysis. However, five (4.8%) samples showed a higher number of AFBs by DS analysis. Among these exceptions, four samples were categorized as 2+, and one sample was categorized 3+.

In 137 PSS samples with 1-10 AFB in 50 fields (2+), similar results were observed by both procedures in 48 (35%) samples, which were also categorized 2+ by DS analysis. Thirteen (9.5%) samples were negative by DS analysis and 74 (54%) were categorized 1+, and two (1.4%) samples (1.4%) were categorized 3+.

There were 108 PSS samples with >10 AFB in 20 fields (3+) also positive by DS analysis. However, the AFB counts were consistent in both procedures in only 55 (51%) samples; 21 (19%) samples were only 1+, and 32 (30%) were 2+ by DS.

Regarding the positive results from the two procedures, the AFB count was consistent among 148 samples. However, in 212 samples, the number of AFB in the PSS were up to 10 times higher than in the conventional DS. Therefore, false-negatives in DS analysis were identified after simultaneous analysis of PSS, mainly among the paucibacillary samples, showing a considerable increase in sensitivity.

In addition to increasing AFB detection, the use of NaOCl in PSS preparation fulfills laboratory biosafety. The release of free chlorine from NaOCl inactivates the microorganisms present in the clinical sample. Furthermore, NaOCl promotes the dissolution of particles from the sputum, such as mucus and saliva, favoring the spontaneous precipitation of the MTB bacilli while maintaining the morphology and binding of dyes to the bacterial

TABLE 1: Analysis of 1,719 sputum samples from individuals with signs and symptoms of PTB, using direct smears of sputum and processed sputum smears.

Procedure	Positive*		Negative	
	n	%	n	%
DS	283	16.5	1,436	83.5
PSS	368	21.4	1,351	78.6

PTB: pulmonary tuberculosis; **DS:** direct smears of sputum; **PSS**: processed sputum smear; **n:** number; **%**: percentage. *The difference between the percentage of positive samples is statistically significant ($p < 0,001$) between the procedures.

TABLE 2: Acid-fast bacilli score in 1,719 sputum samples from individuals with signs and symptoms of PTB, using direct smears of sputum and processed sputum smears.

	Direct smear of sputum					
PSS	**Negative**	**1-9 AFB**	**1+**	**2+**	**3+**	**Total**
Negative	1,351	0	0	0	0	1,351
1-9 AFB	19	0	0	0	0	19
1+	53	1	45	4	1	104
2+	13	0	74	48	2	137
3+	0	0	21	32	55	108
Total	1,436	1	140	84	58	1,719

PTB: pulmonary tuberculosis; **PSS**: processed sputum smear; **AFB:** acid-fast bacilli.

cell wall. The digestion of particles from the sputum results in a cleaner microscopic field, which favors bacterial identification[10,11].

However, NaOCl digestion hampers adherence of the smear to the slide, which can be easily washed away during staining[12]. We circumvented this problem by decreasing the fuchsine staining time to 3 minutes, without impairing the staining results.

Another drawback of NaOCl treatment is that experimental time is increased by 24 hours, which is in disagreement with TB control strategies[2]. However, the procedure has advantages of both, the increased safety of laboratory personnel and increased sensitivity for the patients.

In conclusion, PSS preparation is easy to perform, inexpensive, and allows for increased AFB detection than from conventional DS. When comparing the results of the AFB count using both procedures, the poorest yield was obtained by DS analysis in the paucibacillary samples. PSS screening of AFB was superior to the conventional screening of DS; therefore, the procedure will be valuable for remote and rural areas in low-income, high TB-burden regions that lack automated diagnosis. Increased safety and sensitivity of MTB microscopic detection in processed sputum smears will contribute to improving the detection of active PTB cases and, consequently, better control of the disease.

Ethical considerations

The research project was approved by the Ethics Committee of the *Instituto Aggeu Magalhães* (CEP-CPqAM) - *Fundação Oswaldo Cruz*, Recife, PE (CAAE 57175616.9.0000.5190) with consent from the *Unidades de Saúde da Secretaria Municipal de Saúde*, Recife, PE.

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Conflict of interest

The authors declare that there is no conflict of interest.

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