

Original Article

Multicenter evaluation of membrane-based smear microscopy for detecting acid-fast bacilli in China

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Received July 5, 2018; Accepted August 23, 2018; Epub November 1, 2018; Published November 15, 2018

Abstract: Objective: To assess the feasibility and reliability of using membrane-based smear microscopy at peripheral laboratories in China. Methods: The clinical case control study was conducted in five tuberculosis (TB) dispensaries from September 2014 to May 2016. The membrane-based microscopy and direct smear microscopy were performed to compare the sensitivity, specificity, and the examination time for both methods was also analyzed. Results: A total of 5359 TB suspects were consecutively enrolled from 5 TB dispensaries, and 9915 specimens were finally analyzed. The sensitivity for membrane-based microscopy and direct smear microscopy was 76.9% (95% CI, 75.4-78.4) and 53.8% (95% CI, 52.1-55.6) respectively, and the specificity was 96.8% (95% CI, 96.4-97.2) and 99.2% (95% CI, 99.0-99.4) respectively. The sensitivity and specificity were both significantly different ($P < 0.001$) between the two methods. The examination time for membrane-based smear microscopy (209.1 ± 112.0 seconds) was significantly shorter than that for direct smear microscopy (253.1 ± 79.4 seconds) ($P < 0.05$). Conclusions: Membrane-based smear microscopy showed higher sensitivity and a shorter examination time in comparison with direct smear microscopy and it could be used at peripheral laboratories in China.

Keywords: Tuberculosis, membrane-based smear microscopy, acid-fast bacilli

Introduction

Tuberculosis continues to be a major health problem in China. Direct smear microscopy is a rapid, simple diagnostic tool used to identify the most infectious cases of TB especially in resource limited countries. It is highly specific but has low sensitivity. The *Global Tuberculosis Report* (2016) reported that the rate of bacteriologically confirmed pulmonary cases was only 31% among total pulmonary tuberculosis cases in China [1]. New diagnosis technologies are therefore needed to increase the proportion of bacteriologically confirmed pulmonary tuberculosis. Indirect smear can improve the detection of bacilli compared to direct smear. A systematic review reported that processing by a several chemical procedures, followed by centrifugation or overnight sedimentation, was more sensitive than direct microscopy, and that the specificity was similar [2]. However, there are few laboratories performing indirect smear microscopy due to its complex process-

es in China. The new membrane-based smear microscopy described in this study simplifies the process by the liquefaction of sputum and by concentrating the bacteria to a specific high polymer membrane at the bottom of the vessel. The bacilli adsorbed onto the membrane can be stained directly in the vessel. The membrane with bacilli is then fixed onto a glass slide and examined under a lighted microscope. This study investigated whether this membrane-based smear microscopy method could enhance the diagnosis of TB in China.

Methods

Study population

Patients suspected to have pulmonary tuberculosis were recruited from September 2014 to May 2016 at the Changchun, Jilin, Tonghua, Meihekou, and Nong'an tuberculosis (TB) dispensaries. Each patient was required to collect 3 sputum samples (spot-night-morning) accord-

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ing to the guidelines [3]. This study was approved by the Ethical Committee of the Chinese Center for Disease Control and Prevention.

Direct smear microscopy

Direct smears were prepared and stained using the Ziehl-Neelsen method [3]. The results were graded as follows: negative, scanty, 1+, 2+, 3+, 4+.

Membrane-based smear microscopy

The membrane-based smears were prepared from the same specimens used for the direct smear microscopy. The sputum specimen was first digested with the digestion reagent in the specific sealed vessel and vortexed for 3 minutes. The vessel was then centrifuged at 4500 RPM for 5 minutes in the centrifuge to concentrate the bacteria. The bacteria were adsorbed to the high-polymer membrane located at the bottom of the vessel after being centrifuged. The supernatant was then discarded, and the membrane was dried by placing the vessel in an oven. The bacteria were fixed onto the membrane by adding ethanol to the membrane and then staining it with 0.8% carbol fuchsin. The vessel was then placed in the oven for 5 minutes at 60°C. Methylene blue was added to counterstain the background for 1-3 minutes after decolorization. The membranes adsorbed with the stained bacilli were ejected with a needle from the bottom of vessel and dried completely in the air. The membrane was then stuck to a glass slide using neutral glue with the side adsorbing the bacilli facing the slide to prevent the objective lens from cross-contamination.

Culture

Two specimens were selected for culture with the simple Petroff's method. In brief, the sputum was digested using 4% sodium hydrate for 15 minutes and inoculated. The slants were incubated at 37°C and observed at 3 days and 7 days after inoculation to examine the contamination and growth of non-mycobacterium tuberculosis, and then the growth of the mycobacteria was observed weekly for 8 weeks.

Examination time

To measure the examination time, the examination times were recorded for both methods under routine working conditions. The time

from placing the stained slide under the objective lens to reporting the examination result was measured.

Sterilization effect

Different smear graded sputum samples were used to evaluate the sterilization effect of the digestion process. The sputum was digested with a specific digestion reagent and vortexed for 3 minutes, and the suspension was then inoculated onto the slants of modified acid Lowenstein-Jensen (L-J) medium and modified L-J medium. The slants were incubated at 37°C and observed weekly to examine the growth of mycobacteria for 8 weeks.

End-user appraisal

The appraisal survey, a questionnaire, was conducted among laboratory technicians.

Definition

Smear positive cases were defined as TB patients with at least one positive smear microscopy result (graded scanty, 1+, 2+, 3+, 4+) from the three specimens. Culture positive cases were defined as TB patients with at least one positive L-J culture result from the two selected specimens. Clinical TB patients were defined as patients with clinical symptoms and chest X-rays indicating TB at intake and whose clinical symptoms and chest X-rays were improved after two months of anti-tuberculosis treatment.

Statistical analysis

Data were entered into the database using Excel and analyzed using SPSS version 17.0. The sensitivity and specificity of the two smear microscopy methods were calculated in comparison with culture. McNemar's test or Pearson's chi-square test was used for comparison of the proportions; a t-test was used to compare the differences in examination time between the groups.

Results

5359 cases of suspected pulmonary tuberculosis were recruited during the study period. 17 patients did not receive a membrane-based or direct smear microscopy examination, and 31 patients had contaminated cultures. For

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Table 1. Sensitivity and specificity of direct smear microscopy and membrane-based smear microscopy

Smear microscopy	Culture		Sensitivity % (95% CI)	Specificity % (95% CI)
	Positive	Negative		
Membrane-based smear microscopy	Positive	2403	76.9 (75.4-78.4)	96.8 (96.4-97.2)
	Negative	722		
Direct smear microscopy	Positive	1682	53.8 (52.1-55.6)	99.2 (99.0-99.4)
	Negative	1443		

Table 2. Comparison of grading results between membrane-based smear microscopy and direct smear microscopy

Direct smear microscopy	Membrane-based smear microscopy						
	Negative	Scanty	1+	2+	3+	4+	Total
Negative	7283	273	469	121	25	7	8178
Scanty	2	33	146	43	20	7	251
1+	7	0	219	263	157	113	759
2+	1	0	4	61	114	177	357
3+	0	0	2	7	49	162	220
4+	0	0	0	4	3	143	150
Total	7293	306	840	499	368	609	9915

nificantly lower than the rate of the membrane-based smear microscopy from any sputum specimen, 20.2% (865/4285) for direct smear vs. 25.2% (1078/4285) (spot), 26.7% (1145/4285) (night), 26.7% (1143/4285) (morning) for membrane-based smear microscopy ($P < 0.001$).

Performance of two smear microscopy methods

the remaining 5311 cases, a total of 10031 specimens were examined using the three laboratory methods simultaneously. Out of the 10031 specimens, 116 (1.2%) specimens had contaminated culture results, and 9915 specimens were therefore included in the final analysis.

Detection rates of two smear microscopy methods and culture

Among 9915 specimens, 26.4% (2622/9915), 17.5% (1737/9915) and 31.5% (3125/9915) were positive using a membrane-based smear, direct smear microscopy, and culture, respectively.

80.7% (4285/5311) of the cases had three sputum specimens collected. The positive rates with membrane-based smear microscopy were 25.2% (1078/4285) using the spot specimen, 28.2% (1209/4285) using both the spot and night specimens, and 29.4% (1260/4285) using all three samples, which were significantly higher than the corresponding detection rates using direct smear microscopy (15.8% (675/4285), 18.7% (802/4285) and 20.2% (865/4285)) ($P < 0.001$).

The cumulative positive rate of direct smear microscopy of the three specimens was sig-

The sensitivity for membrane-based microscopy and direct smear microscopy was 76.9% (95% CI, 75.4-78.4) and 53.8% (95% CI, 52.1-55.6) respectively, and the specificity was 96.8% (95% CI, 96.4-97.2) and 99.2% (95% CI, 99.0-99.4), respectively (**Table 1**). The membrane-based method increases the sensitivity up to 23%. The differences of the sensitivity and specificity between the two smear microscopy methods were both significant ($P < 0.001$).

Discrepant results analysis between the two smear microscopy methods

9.1% (905/9915) of specimens had discrepant qualitative results. 98.9% (895/905) of the specimens were positive using membrane-based smear microscopy and negative using direct smear microscopy. The distributions of the two smear microscopy methods results are shown in **Table 2**.

Among the 895 specimens with membrane-based smear microscopy positive and direct smear microscopy negative results, 30.5% (273/895) were scanty and 52.4% (469/895) were graded 1+. Out of these 895 specimens, 81.3% (728/895) were culture positive and thus favor the membrane-based smear results, and only 18.7% (167/895) specimens were culture negative and consistent with direct smear

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Table 3. Average examination time for direct smear microscopy and membrane-based smear microscopy

Smear result, method	Slides, n	Examination time (seconds)	t	P value
Negative				
Direct smear microscopy	38	291.3±43.0	1.635	0.107
Membrane-based smear microscopy	33	265.5±86.7		
Positive				
Direct smear microscopy	33	209.1±89.0	2.097	0.04
Membrane-based smear microscopy	38	159.1±109.0		
Total				
Direct smear microscopy	71	253.1±79.4	2.701	0.008
Membrane-based smear microscopy	71	209.1±112.0		

Among the 10 specimens with direct smear microscopy positive and membrane-based smear microscopy negative results, 9 specimens were graded scanty or 1+. 7/10 specimens were culture positive and 3/10 specimens were culture negative.

Examination time

The average examination times were shown in

Table 3. The examination time for membrane-based smear microscopy was significantly shorter than for direct smear microscopy (209.1±112.0 s vs. 253.1±79.4 s, $P < 0.05$). Significantly, the difference in the examination times between the two methods was largely attributable to the shortening of the examination time of the positive slides. The shapes of the acid-fast bacilli and the background of the smear prepared with membrane-based smear microscopy are shown in **Figure 1**.

Sterilization study with clinical sputum samples

90 positive specimens were used for the assessment of the sterilization activity of the digestion reagent in the clinical sputum samples. The results of smear grading of positive specimens were as follows: scanty: 8; 1+: 17; 2+: 23; 3+: 24; 4+: 18. All of the samples treated with the reagent did not show any positives for *M. tuberculosis* on the L-J medium after 8 weeks incubation.

End-user appraisal

Of the seven laboratory technicians, five gave positive feedback on the ease of the operation process, good observation effects, and short examination time of the new membrane-based method. All the technicians suggested that this method could be scaled up, but one of them thought that laboratories with heavy daily workloads should not prioritize this method because completing it involves more steps compared with direct smear microscopy.

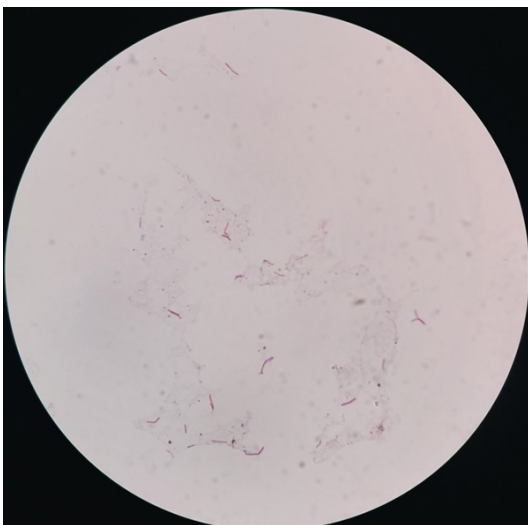


Figure 1. Shapes of AFB and background of smear prepared with membrane-based smear microscopy method.

microscopy. For the 167 specimens with a culture negative result, the results of the other specimens from the same patients were further analyzed. 19.2% (32/167) of the specimens were collected from 31 culture positive patients based on the other specimen, and 16.8% (28/167) of specimens were from 26 culture negative but direct smear positive patients based on other specimens. Among the rest of the culture negative and smear negative specimens, 55.1% (92/167) were from 61 patients clinically diagnosed as having pulmonary tuberculosis. Only 9.0% (15/167) of specimens were collected from 10 patients not diagnosed with pulmonary tuberculosis based on X-ray results and direct smear microscopy and culture.

Discussion

A main drawback of direct smear microscopy is its low sensitivity. First, bacilli were not dispersed evenly in the sputum, so no bacilli were taken out when a small portion of sputum was taken out for direct smear. Secondly, undigested mucoid can superimpose the bacilli, thus masking it during the examination. For the membrane-based smear microscopy method, mucin fibrins were broken down to form a homogenized suspension through liquefaction. A concentration of homogenized suspension could increase the amount of bacilli per milliliter of sputum. The clear fibrin-free background of the smear improved the examination of the bacilli.

In the present study, membrane-based smear microscopy had a significantly higher sensitivity than direct smear microscopy (76.9% vs. 53.8%). Previous studies reported that the sensitivity range of direct smear microscopy was 50%-57% and the sensitivity range of concentrated smear microscopy was 63% to 89% [4-8]. A study conducted by Peng et al. showed that the sensitivity of the same membrane-based smear microscopy increased (97.3% vs. 55.2%) without a loss of specificity (100% vs. 100%) [9]. That study was conducted in only one high level hospital, and the number of positive specimens was small. In contrast, our study enrolled samples from 5 TB dispensaries and collected more specimens. So our results were more accurate compared with the previous study. The increase in sensitivity with the new membrane-based method is believed to be the result of liquefaction and subsequent concentration with the membrane as an efficient adsorption system. The specificity was 99.2% and 96.8% for the direct and the membrane-based methods. It was comparable with other studies (96%-99%) [4-6]. A possible explanation for specimens with positive smear microscopy results but negative culture results was that these specimens were collected from patients who had taken some anti-tuberculosis drugs before the specimens were collected. So the nonviable bacteria remaining in the sputum were detected by smear microscopy but did not grow in L-J media. Another possible explanation was that this result could have been caused by chance, with only the AFB-containing portion of the sputum used to make a direct smear.

17.5%, 26.4% and 31.5% were found to be positive by direct smear microscopy, membrane-based smear microscopy, and culture, respectively. The smear positive rate was increased from 10.4% to 16% with the concentration method compared to direct smear microscopy [10]. The different positive rate in the different studies was possibly associated with different populations, the different methodologies used, and different levels of proficiency in conducting smear microscopy. Three sputum specimens are required for smear microscopy for the diagnosis of tuberculosis in China. Only 80.7% of the suspected cases had three specimens collected in this study, however. The positive rate of the membrane-based smear microscopy from any sputum specimen was significantly higher than the cumulative positive rate of direct smear microscopy of the three specimens. It indicated that the new smear microscopy method based on only one specimen can detect even more positive patients than direct smear microscopy based on three specimens. It will be very useful, especially in areas where most of the patients cannot return to collect the second and third specimens on the following days. Collection of one single spot sputum specimen combined with the new membrane-based smear microscopy method will reduce patient burden in terms of the number of visits to TB dispensaries and could also shorten the delay between the presentation of clinical symptoms and the initiation of treatment.

It was shown that 80.2% of specimens with membrane-based smear were positive, but direct smear negatives were also found to be culture positive. The membrane-based smear microscopy method detected more scanty and 1+ smears which were negative by direct smear microscopy, an indication that there were a small number of bacteria in the sputum, and direct smear microscopy did not detect the bacilli due to the method's low sensitivity. The bacilli may have been lost during the staining process. Another possible explanation is that the membrane-based method made it easier for laboratory technicians to examine slides with fewer bacilli against a uniform and clear background. Among the 167 specimens that were positive with the membrane-based method but negative with both direct smear and culture, we found that 19.2% and 16.8% of the specimens were collected from culture positive

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patients and smear positive patients respectively based on the results of other specimens, and 55.1% of the specimens collected from 61 clinical diagnosed TB patients. Only a very small proportion (9%) of specimens were collected from patients who were negative for pulmonary tuberculosis based on X-ray result and direct smear microscopy and culture, which indicated that this membrane-based smear microscopy detected more positive results especially for specimens containing few bacilli. 7/10 specimens with a positive result by direct smear but negative by the membrane-based smear microscopy method were also found to be positive by culture. It may be possible that the only AFB-containing portion of the sputum was used to make the direct smear by chance. Anyway, the absolute number is very small in comparison with specimens that are membrane-based smear positive but direct smear negative.

The feasibility of using membrane-based smear microscopy was further explored. Membrane-based smear microscopy could decrease examination time by 23.9% compared with direct smear microscopy, when reporting positive smear results. Some operation procedures, including smear preparation, vortex, and centrifugation, can generate potentially infectious aerosols from a sputum sample containing bacteria. Liquefaction with a chemical reagent efficiently sterilizes bacilli in the sputum. The new membrane-based smear microscopy poses a smaller biohazard risk and was accepted by most of the laboratory technicians.

Conclusions

The membrane-based smear microscopy showed higher sensitivity and shorter examination time in comparison with direct smear microscopy, and it could be used at peripheral laboratories in China.

Acknowledgements

The authors acknowledge staff in five study sites in Jilin Province for their hard work in completing this project. This work was supported by the National Science and Technology Major Project [grant number 2014ZX10003002]; Major State Basic Research Development Program of China [grant number 2014CB-744403]. The study sponsors had no roles in the study design, in the collection, analysis and

interpretation of data; in the writing of the manuscript or in the decision to submit the manuscript for publication.

Disclosure of conflict of interest

None.

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