The ReaSLR methodology developed for sputum processing is a novel, low-cost, and simple technique that has improved the sensitivity of smear microscopy for the diagnosis of tuberculosis (TB). Sample processing consists of rapid liquefaction of the sputum specimen with the ReaSLR reagent, followed by syringe filtration, concentration by centrifugation, and use of the sediment for smear microscopy. The performance of the ReaSLR kit was evaluated on 150 sputum samples and was compared with that of the modified Petroff method for sputum decontamination and concentration. Ziehl-Neelsen staining was performed for smear microscopy after processing by these two techniques; simultaneously, culture on Lowenstein-Jensen (LJ) medium was done to evaluate the two methods. The efficiency of smear microscopy was 18/150 (12%) with the modified Petroff method compared to 47/150 (31.33%) with the ReaSLR method, and this difference was statistically significant (P < 0.001). The ReaSLR method for smear microscopy demonstrated a sensitivity and specificity of 90.47% and 91.6%, respectively, whereas the modified Petroff method showed a sensitivity and specificity of 40.47% and 99.07%, respectively, compared to those of culture, which was used as the gold standard. With the newer ReaSLR method, the kappa coefficient (κ) was 0.8, which implies an excellent positive agreement. The ReaSLR method was found to be more sensitive than the conventional method for sputum smear microscopy. The newer ReaSLR method holds promise for adoption in TB control programs across the globe, as it was found suitable for the laboratory diagnosis of pulmonary TB. Further large-scale studies are needed to evaluate other aspects of this method.

Tuberculosis (TB), an airborne infectious disease, continues to be a major global health problem. About 9 million new cases of TB are diagnosed and close to 2 million people die of the disease each year. The majority of cases occur in Africa (30%) and Asia (55%), with India and China accounting for about 35% of all such cases (1). With the emergence of multidrug-resistant tuberculosis (MDR-TB) (around 0.4 to 0.5 million cases each year), the scenario is becoming more challenging (2).

The threat of TB is alarming, and the disease can be controlled only by an effective TB control program. Early case detection is a crucial step in the control of the disease, and this relies solely on the detection of acid-fast bacilli (AFB) in clinical samples. Sputum microscopy with Ziehl-Neelsen (ZN) staining has been a reliable tool for decades, especially in the setting of developing countries, but it has only a 40 to 60% sensitivity for the diagnosis of pulmonary TB (3). It has the advantages of low cost, a need for minimal expertise, rapid processing, and simplicity but has the disadvantage of a lack of sensitivity (4, 5, 6). The methods of decontamination, liquefaction, and concentration before the ZN staining improve the yield and allow identification of the AFB in the sputum sample (7, 8). There is a need to upgrade the existing simple diagnostic techniques without complicating them further to increase sensitivity of case detection (5, 6).

N-Acetyl-L-cysteine (NACL) with 2% sodium hydroxide (NaOH) and the modified Petroff method are among the popular techniques for mucus digestion which allow concentration of AFB in the processed smear, but they are cumbersome and tedious and require special equipment (9). NACL-NaOH and the modified Petroff method have shown improvement, with sensitivities varying between 60 and 86% (6, 10, 11). Various other studies using modifications like the universal sample processing (USP) method (8), household bleach (10), carboxypropylbetaine (12), phenol ammonium sulfate (13), and chitin (14) have reported sensitivities varying from 65 to 85%.

The aim of this prospective blinded study was to evaluate the performance of a new technique called ReaSLR sputum sample processing (ReaMetrix India Pvt. Ltd., Bangalore, India) for its AFB smear sensitivity and specificity for the diagnosis of TB. In this method, ready-to-use tablets with a chaotrope along with reducing agents exert mucolytic activity, which ensures the effective release of the trapped mycobacteria in the sputum sample. A filtration system (using a syringe to remove insoluble particles) improves visualization of AFB in the smear. The diagnostic accuracy and performance of this novel technique for sputum processing were compared to those of the well-established modified Petroff method in a hospital-based setting. The new method described here is a processing technique and not a diagnostic test.

MATERIALS AND METHODS

Study population. A total of 150 sputum samples were obtained from patients with clinically suspected pulmonary TB attending either an inpatient or outpatient clinic in our hospital between October 2012 and January 2013. The samples were processed in the Department of Microbiology mycobacteriology laboratory at Sanjay Gandhi Institute of Medical Sciences (Lucknow, India).

Specimen collection. Early-morning mucus expectorate was collected from each patient after proper instructions were given; only one sample from
each patient was included in the study. The procedures were performed in the biological safety level 3 facility in the microbiology laboratory of the Sanjay Gandhi Institute of Medical Sciences. Samples were processed on the same day they were collected and, in case of delay, were stored for less than 24 h at 4°C before processing. Each sample was split into two portions after homogenization, and these portions were transferred into 20-ml plastic conical tubes. Samples with a volume of <4 ml were excluded from the study. A 2-ml volume was used for the ReaSLR method, and the rest of the sample (x ml) was used for the modified Petroff method.

**Specimen processing by ReaSLR methodology.** The ReaSLR reagent kit contains two components, ready-to-use tablets of the ReaSLR reagent, which includes a chaotrope and reducing agents, and a ready-to-use syringe for filtration. In a 15-ml centrifuge tube, one tablet of the reagent was added to approximately 2 ml of the sputum sample. The cap on the sample was closed, and the reagent was mixed by inverting the tube 10 to 15 times. The tube was incubated at room temperature for 10 min for complete liquefaction. The digested sample was poured through the syringe for filtration into another 15-ml centrifuge tube to remove any insoluble particulate matter. This tube was centrifuged at 3,000 × g for 10 min at room temperature. The tube was uncapped, and the supernatant was removed, leaving behind approximately 100 μl of solution. The pellet was not disturbed while the supernatant was removed. The pellet was resuspended, and a suitable volume (1 drop, 50 μl) was transferred onto the glass slide to make the smear for a standard acid-fast staining protocol. The ReaSLR method was used only to visualize AFB by microscopy with ZN staining.

**Specimen processing by the NaOH method and modified Petroff procedure.** To x ml of sputum in McCartney tubes, double the volume of sterile 4% NaOH solution was added aseptically. The caps were tightened, and the contents were mixed well by inverting the tubes. The bottles were then placed in a shaker and kept in a 37°C incubator for 15 min. The tubes were removed, and 15 ml of sterile distilled water was added, mixed well, and centrifuged at 3,000 × g for 15 min. The McCartney tubes were carefully removed from the centrifuge without shaking. The supernatant fluid was discarded slowly into a container with a 5% phenol solution. To the sediment, 50 ml sterile distilled water was added, and the pellet was washed. The supernatant was decanted, and the sediment was used for smears and culture (15). One drop of sediment was used to prepare the slides for AFB microscopy using the standard ZN staining technique.

**AFB smears and ZN staining.** After the slides were processed by the two methods described above, they were given a code number and were heat fixed. Thereafter, ZN staining was performed according to a previously described method (9), and the stained slides were read blindly by a qualified microbiologist. The stained smears were scanned under ×1,000 magnification with a light microscope. AFB were scored as 1+ (10 to 99 AFB in 100 fields), 2+ (1 to 10 AFB per field), 3+ (>10 AFB per field), scanty (1 to 9 AFB per 100 oil immersion fields), or negative (no AFB in 100 fields) according to the Revised National Tuberculosis Control Programme (RNTCP) guidelines (16).

**Culture.** After decontamination/concentration by the modified Petroff method, 200 μl of resuspended pellet was inoculated into Lowenstein-Jensen (LJ) medium. The culture tubes were incubated at 37°C for 8 weeks before being reported as negative. The cultures were read weekly, and the suspect (white or buff-colored) colonies were examined microscopically after ZN staining. The *Mycobacterium* isolates were identified by morphology, growth rate, pigmentation, and ZN staining (9). The colonies were classified as *Mycobacterium tuberculosis* complex (MTC) or *Mycobacterium avium* complex (MAC) colonies (pigmented mycobacteria and rapid growth in <7 days). The MAC colonies were not further characterized to the species level. The cultures that showed any contamination during incubation were further decontaminated with 1% cetrimide agar, reinoculated into the LJ medium, and again incubated for a maximum of 8 weeks (17).

**Statistical analysis.** In this study, the efficiency of the test tool was calculated as (total number of positive cases/total number of analyzed cases) × 100. The sensitivity ([Tp/(Tp + Fn)] × 100) and specificity ([Tn/(Tn + Fp)] × 100) where Tp is the total number of true positives, Tn is the total number of true negatives, Fp is the total number of false positives, and Fn is the total number of false negatives) were also determined. In addition, the positive predictive value was calculated as Tp/total number of positives, and the negative predictive value was calculated as Tn/total number of negatives. The positive concordance between the culture and the performed microbiological tests was assessed using the kappa coefficient (κ), where >0.75 indicates excellent agreement, ≤0.75 indicates fair agreement, ≤0.4 indicates poor agreement, and <0.4 indicates poor agreement (18). The significance level was determined by the chi-square (χ²) test with the help of SPSS (version 15.10). A difference was considered statistically significant at a P value of <0.05. The culture on LJ medium was used as the gold standard in the present study.

**RESULTS**

The ReaSLR method was evaluated by using smear microscopy and was compared with the modified Petroff method for sputum processing on 150 sputum specimens from patients suspected of having active pulmonary TB.

**Efficiency of AFB microscopy by the two processing methods.** The overall rate of positivity for smear microscopy was 18/150 (12%) sputum samples with the modified Petroff method compared to 47/150 (31.33%) sputum samples for the ReaSLR method. The numbers of positive samples graded as 3+ (n = 5) were similar for the two methods with no significant difference (P > 0.05). There were 2 additional samples detected in the 2+ category by the ReaSLR method (n = 5) compared to the number detected by the modified Petroff method (n = 3). The ReaSLR method detected a greater number of positive samples graded as 1+ or scanty. The modified Petroff method detected 6 samples in the grade 1+ category, whereas the ReaSLR method detected an additional 6 samples (n = 12) from the same group (Table 1). The most important difference was seen with the smears graded as scanty. Most of the positive samples missed by the modified Petroff method were graded as scanty by the ReaSLR method. The

<table>
<thead>
<tr>
<th>Smear grade</th>
<th>Modified Petroff method</th>
<th>ReaSLR method</th>
<th>Modified Petroff method</th>
<th>ReaSLR method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>1+</td>
<td>6</td>
<td>4</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>2+</td>
<td>3</td>
<td>2</td>
<td>5</td>
<td>3.3</td>
</tr>
<tr>
<td>3+</td>
<td>5</td>
<td>3.3</td>
<td>5</td>
<td>3.3</td>
</tr>
<tr>
<td>Scanty</td>
<td>4</td>
<td>2.7</td>
<td>25</td>
<td>16.7</td>
</tr>
<tr>
<td>Negative</td>
<td>132</td>
<td>88</td>
<td>103</td>
<td>68.7</td>
</tr>
</tbody>
</table>

- Number of positive results on culture/total number of positive results by the modified Petroff method on microscopy.
- Number of positive results on culture/total number of negative results by the modified Petroff method.
- Number of positive results on culture/total number of negative results by the ReaSLR method on microscopy.
- Number of positive results on culture/total number of negative results by the ReaSLR method by microscopy.
modified Petroff method detected 4 samples in the scanty category, whereas the ReaSLR method detected 25 positive samples in the scanty group (Table 1). Differences in grading of the samples by the two methods are shown in Table 2.

**Sensitivity and specificity of the ReaSLR and modified Petroff methods in comparison with cultures.** The overall rate of positivity for cultures in the present study was 42/150 (28%). Out of the 42 culture-positive isolates, 38 were identified as MTC species and 4 were MAC species. In this study, only three LJ slants were found to be contaminated in the course of the inoculation period. After redecontamination with 1% cetrimide and re-inoculation on the fresh LJ slants was done, and after incubation for 8 weeks, there was no visible growth or further contamination seen.

The sensitivity of the modified Petroff method was 40.47%, and the specificity was 99.07%, with positive and negative predictive values of 94.4% and 81.06%, respectively, compared with those for the culture, which was the gold standard for diagnosis. The kappa coefficient for positive agreement was calculated (κ = 0.48) and showed good agreement between the modified Petroff method and the culture. When the newer ReaSLR method was compared with the culture, the sensitivity improved to 90.47%, with a specificity of 91.6% and positive and negative predictive values of 80.83% and 96.12%, respectively. The positive agreement also showed an improvement, and the κ value was 0.8, which indicates excellent positive agreement for the new method (Table 3).

Most of the samples (n = 16) identified as positive by the conventional method were also positive by the ReaSLR method, but 2 samples were positive only by the modified Petroff method. Out of these 2, only 1 was positive by culture. This might have been due to an unequal distribution of the sample by the technical staff (Table 2).

**Comparison of the ReaSLR and modified Petroff methods.** Out of the 132 samples deemed negative by the modified Petroff method (smear-negative samples), the ReaSLR method detected 22 additional samples that were also positive by culture, thereby showing an enhancement in the sensitivity of 45.66%, which was statistically significant (P < 0.001). Among the additional 22 ReaSLR smear-positive and culture-positive samples, 14 were graded as scanty, 5 had a score of 1+, 2 had a score of 2+, and 1 had a score of 3+, suggesting that the modified Petroff method can lead to false results in specimens with lower bacterial loads due to a lack of technical expertise during smear preparation or faulty reading of the slides.

The ReaSLR method showed an enhancement in the sensitivity of 50% (P < 0.001) over that of the Petroff method (90.47%) and 40.47% sensitivities for the ReaSLR and modified Petroff methods, respectively. On the contrary, there was a decrease in specificity with the ReaSLR method (91.6% specificity) compared to that with the modified Petroff method (99.07% specificity).

**DISCUSSION**

Microscopy for AFB has been considered the key to diagnosis of TB and for monitoring the progress of TB patients (19). The major constituent of a TB sputum sample is mucin protein; mycobacterial cells in the sputum are localized in mucin monomers, which readily polymerize to develop highly complex hydrophobic structures (12). Decontamination and concentration methods using various types of substances and their modifications have been reported to show improvements in the yield of AFB by microscopy, but the high cost and specialized training needed have been limiting factors for their widespread use (11). Smear sensitivities vary among laboratories, and there is a need to evaluate newer methods which can easily be adapted for large-scale use (20).

The technique described here was evaluated in a setting with a high prevalence of TB and low resources. An increase in the positivity rate from 12% to 31.33% in smear microscopy was seen when samples were processed by the ReaSLR method over that of the modified Petroff method. The 50% increase in overall sensitivity (P < 0.001) with the ReaSLR method (90.47%) over that of the modified Petroff method (40.47%) was the most significant finding of this study. The increase with the new method is believed to be the result of rapid liquefaction and subsequent concentration of the mycobacterial cells along with the use of a syringe as an efficient filtration system. The clear debris-free background after processing by the ReaSLR method improved the visualization of AFB, and as a result, samples with a lower bacillary load were easily detected.

The smear-negative cases have significant importance, as 30% of pulmonary TB cases are negative for AFB on microscopy (21, 22). Several investigators have used different techniques which have indicated that smear-negative TB cases pose a serious threat to the effective functioning of TB control programs (23). Among the 132 smear-negative samples, the ReaSLR method detected 22 additional samples which were subsequently positive by culture, with an enhancement in sensitivity of 45.66% (P < 0.001). The salient finding was that the new ReaSLR method detected 25 positive samples in the scanty group, whereas the modified Petroff method detected only 4 samples in the same group.

In this study, only one case was reported negative by the ReaSLR method, whereas the ReaSLR method detected 22 positive samples in the scanty group. This might have been due to an unequal distribution of the sample by the technical staff. Sensitivity and specificity, positive and negative predictive values, and positive correlation of AFB microscopy results by both decontamination methods compared with culture are shown below.

**Efficiency of New ReaSLR Method**

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Positive Predictive Value (%)</th>
<th>Negative Predictive Value (%)</th>
<th>χ² Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroff</td>
<td>40.47</td>
<td>99.07</td>
<td>80.83</td>
<td>96.12</td>
<td>0.48</td>
</tr>
<tr>
<td>ReaSLR</td>
<td>90.47</td>
<td>91.6</td>
<td>94.4</td>
<td>81.06</td>
<td>0.8</td>
</tr>
</tbody>
</table>

In conclusion, the ReaSLR method is shown to be a promising tool in the diagnosis of TB, especially in settings with limited resources and high smear negativity.
method but graded as 1+ by the modified Petroff method, and this case was found to be culture positive also. This difference in results might be due to unequal sample distribution during processing, as it is known that mycobacteria tend to form aggregates, clumps, and cords (24). It was observed that 9/42 (21.4%) smear-positive cases detected by microscopy using the ReaSLR method did not eventually grow on culture after 8 weeks. This can be explained by the fact that the microscopy-detected AFB were either nonviable or dead due to the effect of antitubercular drugs, as patients undergoing treatment were not excluded from the study. This may also be the reason for the lower positive predictive value of the new test (80.85%) compared to that of the modified Petroff method (94.4%).

Another published study reported a sensitivity as high as 85% and a specificity of 97% with the use of a combination of phenol and ammonium sulfate for sputum liquefaction (13). With the hypertonic saline-sodium hydroxide (HS-SH) method, the sensitivity value for microscopy was 73.5% and without concentration was 58.3% (4). The USP method compatible with culturing on solid and liquid media demonstrated a sensitivity and specificity of 98.2% and 91.4%, respectively, compared to 68.6% and 92.6% for the direct method (8).

The significant drawback of the modified Petroff method is the requirement for daily preparation of the reagents, which is often cumbersome in the daily routine practice of laboratories (9). In contrast, the advantage of the ReaSLR kit is that the reagent tablets can normally be stored at room temperature without any deterioration in their physical appearance. The proposed method uses a filtration system which efficiently removes debris, making the background clearer for interpretation of the results. The average cost of the NALC-NaOH/modified Petroff method is about $1.00 per clinical specimen, and the cost of a commercial kit is $3.50. The average cost of each tablet along with the syringe used for the ReaSLR method is about $0.50 per sample. The limitation of the ReaSLR method is that the test cannot be performed with sputum samples of <2 ml, as the tablets require a ≥2-ml volume for dissolution. In the present study, the ReaSLR method was not evaluated for its usefulness in culture. Therefore, further studies are needed to determine important issues like its effects on culture and to directly perform susceptibility testing in the presence of the ReaSLR reagent.

Smear microscopy is the most important weapon in TB control programs in developing countries, like India. In most of the resource-limited countries where treatment is initiated on the basis of smear microscopy results alone because culture is time-consuming, the new method might be very useful for the early initiation of antitubercular treatment (ATT). The ReaSLR method has a high sensitivity and can identify TB infections in patients efficiently, which would help to curb the transmission of TB and ensure an effective cure. Therefore, it is suggested that the ReaSLR method for smear preparation be adopted in the future by TB control programs in developing countries for the detection of AFB.

In conclusion, we found an increased difference in sensitivity between the ReaSLR method and the modified Petroff concentration method for sputum smear microscopy when performed in patients with clinically suspected TB. The method described here is simple and easy, requires a minimal infrastructure, and is a better alternative to conventional methods for sputum processing. We believe that this new technique for sputum processing is a breakthrough milestone for the diagnosis of TB. In the future, further field evaluations are needed to demonstrate the benefits of the ReaSLR method before it can be widely recommended for sputum processing on a large scale.

ACKNOWLEDGMENT

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REFERENCES


