

Improving Sensitivity of Direct Microscopy for Detection of Acid-Fast Bacilli in Sputum: Use of Chitin in Mucus Digestion

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In order to try to improve the results of direct smear microscopy, we used the mucus-digesting quality of chitin in tuberculosis (TB) laboratories. For this purpose, a total of 430 sputum specimens were processed by the *N*-acetyl-L-cysteine concentration, sodium hypochlorite (NaOCl) liquefaction, chitin sedimentation, and direct microscopy methods. Then, the smear sensitivity for acid-fast bacillus detection by chitin-treated sputum was compared with the sensitivity of smears prepared by other methods. Our results showed that the chitin solution took less time to completely homogenize the mucoid sputum than did the *N*-acetyl-L-cysteine and NaOCl methods. The *N*-acetyl-L-cysteine concentration method demonstrated sensitivity and specificity levels of 83 and 97%, respectively. In comparison, the sensitivity of chitin sedimentation was 80%, with a specificity of 96.7%. The NaOCl liquefaction method showed a sensitivity of 78%, with a specificity of 96%. Finally, the sensitivity of direct microscopy was lower than those of the other tested methods and was only 46%, with a specificity of 90%. The chitin and NaOCl liquefaction methods are both easy to perform, and they do not require additional equipment (centrifuges). Also, our results demonstrated that the chitin method is less time-consuming than the NaOCl method, since only 30 min of incubation is required to bring complete sedimentation of bacilli in chitin-treated sputum whereas the NaOCl method needs 10 to 12 h to give the same results in the same sputum specimens. Therefore, the chitin liquefaction and sedimentation method may provide better results in TB laboratories of developing countries than the *N*-acetyl-L-cysteine concentration, NaOCl overnight sedimentation, and direct smear microscopy methods.

The microscopic examination of acid-fast bacilli (AFB) remains the main bacteriological tool for tuberculosis (TB) detection (1, 10). Previous studies showed that the technique sensitivity may vary depending on smearing, staining, and smear reading (5, 16). For proper smearing, *N*-acetyl-L-cysteine (CH₃-CO-NH-CH₂-COOH-CH₂SH) with 2% sodium hydroxide (NaOH) is considered the best (15). *N*-acetyl-L-cysteine (NaLc) acts as a strong mucus digester, and the processed smear has less debris and a greater concentration of AFB (15). However, since the method is costly and time-consuming and requires special equipment, it is not applicable in the vast majority of TB laboratories in developing countries (2, 11). In these countries, limited resources allow only direct microscopy (unconcentrated sputum) as the best available option for TB diagnosis (5, 11, 15). The technique has a low sensitivity, because it requires meticulous preparation of smears and is labor-intensive (14). In recent years, interest in improving the direct smear microscopy technique in developing countries has arisen (7, 13). For example, Miorner et al. demonstrated that the simple liquefaction and overnight sedimentation of sputum with sodium hypochlorite (NaOCl) would significantly augment smear sensitivity, up to 70% (13). In the present study, we attempted to improve the efficiency of direct smear microscopy through liquefaction and sedimentation of sputum by chitin.

Chitin, with a formula of (C₈H₁₃O₅N)_n, where *n* may range from 100 to 10,000, is a polysaccharide formed by repeating units of *N*-acetyl-D-glucosamine (3, 9, 17). It has a high molecular weight (1.04 × 10⁶), and it is considered the second-most-abundant natural polymer after cellulose (8, 18).

In fact, chitin molecules resemble cellulose molecules, except that the hydroxyl (OH) groups in position C-2 have been replaced by acetylamino groups (NH-CO-CH₃). A closer look at *N*-acetyl-L-cysteine and chitin reveals that both molecules have an acetylamino group in their structure. And since NH-CO-CH₃ is responsible for the mucolytic effect of cysteine, we thought that chitin might induce the same effect in sputum. Therefore, for the first time we tried to evaluate the usefulness of chitin in TB laboratories. In this regard, the effects of chitin on mucoid sputum were determined. Then, the efficiency of AFB concentration from chitin-treated sputum was compared with that obtained with the *N*-acetyl-L-cysteine method.

Regarding the applicability of chitin-treated sputum in any peripheral TB laboratories, we compared the sensitivity of smear microscopy in chitin-treated sputum with those obtained with the NaOCl sedimentation and direct microscopy methods.

MATERIALS AND METHODS

Preparation of chitin. Experiments were performed by using both commercially available and home-made chitin. However, since the results obtained were almost similar, the data presented here are those obtained with chitin (C-3641) purchased from Sigma Chemical Company, St. Louis, Mo.

(i) **Liquefying solution made with ready-made chitin.** For each experimental setup 20 mg of chitin was dispersed in 10 ml of hexafluoroisopropanol (CF₃)₂CHOH and stirred slowly for 18 h (18). It was allowed to stand at room temperature until needle-like solid materials disappeared. Then, the solution was

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TABLE 1. Results^a of Ziehl-Neelsen staining of smears prepared by different methods

| Culture results with: | | Microscopy results with indicated method | | | |
|--------------------------|-----------------------|--|----------------------|------------|-------------------------|
| No. of cases detected | NaLc-NaOH Conc method | NaLc-NaOH conc | Chitin sedimentation | NaOCl conc | Direct smear microscopy |
| 36 | + | + | + | + | + |
| 22 | + | + | + | + | - |
| 2 | + | + | + | - | - |
| 2 | + | + | + | - | - |
| 2 | + | + | - | + | - |
| 2 | - | + | + | - | + |
| 2 | - | + | - | + | + |
| 1 | - | + | + | + | + |
| 8 | + | - | - | - | - |
| Total^b | | | | | |
| 77 | 72 (5) | 69 (8) | 65 (12) | 63 (14) | 41 (36) |

^a +, positive; -, negative.

^b The number of smears testing negative is in parentheses.

filtered through sterile gauze and the clear solution was used as a liquefying agent

(ii) **Home-made chitin.** The preparation followed the method suggested by Seiichi et al. (20). Five hundred milligrams of prawn shell (exoskeleton) was ground by a coffee grinder. The ground shell was put in 700 ml of 1 N NaOH solution and was heated at 90°C for 1 h. Once the solution had cooled down, 700 ml of 2N HCl was added to it. The solution was kept stirring slowly for 6 to 8 h at room temperature.

After being washed with water (four to five times), the obtained material was dried in an oven at 50°C for 4 h. The leftover material was approximately 50 to 70 mg. To prepare the chitin-liquefying solution, the same procedure was followed as that described above.

Preparation of slides. Four hundred thirty sputum specimens were collected from suspected tuberculosis patients hospitalized or referred to the National Research Institute of Tuberculosis and Lung Disease, Tehran, Iran. Slides for direct microscopy were prepared by taking a small portion of the purulent part of sputum with a sterile loop (11).

The remaining sputum specimen was divided into 3 equal parts in 10-ml conical screw-cap tubes. The first part of sputum was processed by a standard *N*-acetyl-L-cysteine with 2% NaOH digestion and decontamination method (15).

Briefly, to an NaLc-2% NaOH solution was added an equal amount of sputum, and the mixture was vortexed for 20 to 30 s. To effect decontamination, the tubes were kept at room temperature for 15 min. Then, the tubes were filled with phosphate buffer (pH 6.8) and concentrated by centrifugation at 3,000 × *g* for 15 min. The supernatant was poured off, the resulting sediment was resuspended in 1 to 2 ml of phosphate-buffered saline, and the suspension was used to prepare the concentrated smears.

The second part of sputum was processed by the NaOCl liquefaction and sedimentation method (13). To an equal volume of sputum, NaOCl was added, and the mixture was vortexed for 1 to 3 min (or 10 to 15 min by handshaking), and then the tube was left on the bench at room temperature overnight (12 to 15 h).

Finally, to the third part of sputum specimen (2 ml), approximately 0.5 ml of

chitin solution was added, and after being vortexed for 5 to 6 s (or shaken by hand for 5 to 8 min), the tube was left on the bench at room temperature for 30 min. (To check the effect of chitin, in a separate set of experiments 0.5 ml of hexafluoroisopropanol alone was added to 2 ml of mucoid sputum, but it took 20 to 25 min of shaking by hand to incompletely homogenize the sputum).

The supernatant of each tube was carefully poured off, the sediment was mixed well with the remaining fluid, and 1 to 2 drops were transferred, with a sterile pipette, to a slide. The slides were coded, air dried, heat fixed, and stained by the Ziehl-Neelsen technique. The stained slides were examined under oil immersion (1,000× lens objective), and they were reported negative when no AFB were seen in at least 100 microscopic fields. Smears were reported positive (11) for any of the following observations: when 1 to 9 AFB were seen in 100 microscopic fields (scored as few bacilli), when 10 to 99 AFB were seen in 100 fields (scored as 1+), when 1 to 10 AFB were seen per field in at least 50 fields (2+), and when more than 10 AFB were seen per field in at least 20 fields (3+). The coded slides were examined by two technicians, and their observations were cross-checked by a senior technician. Statistical analysis was performed with the *t* and paired *t* tests.

RESULTS

The results of smear microscopy are summarized in Table 1. Of 430 samples, 69(16%) were positive with the *N*-acetyl-L-cysteine concentration method, 65 (15.1%) were positive with the chitin-treated sputum method, and 63 (14%) were positive with the NaOCl liquefaction method. In comparison, the AFB-positive smear samples detected by direct sputum microscopy amounted only to 41 (9.5%).

The results shown in Table 2 demonstrate that the sensitivity of AFB detection in sputa that contain a high number of bacilli (i.e., scoring 3+ and 2+) would be almost similar to the one obtained with a different smearing method. Of 69 smears detected as positive, 39 (56%) scored 3+ and 2+ by *N*-acetyl-L-cysteine concentration methods. Interestingly, the same number of positive smears, 39, was detected by NaOCl and by chitin sedimentation methods. The number was only 34 (49%) in direct smear microscopy, the difference being not statistically significant (*P* > 0.005).

In a series of 30 specimens having a low number of bacilli (with the *N*-acetyl-L-cysteine concentration method), 24 (80%) and 26 (86%) by NaOCl and chitin sedimentation methods, respectively, could be detected whereas the number detected by direct microscopy decreased to 7 of 30. The difference was statistically significant (*P* < 0.05).

Overall, the sensitivity and specificity of the *N*-acetyl-L-cysteine concentration method were 83 and 97%, respectively. In comparison, the sensitivity of chitin sedimentation was 80%, with a specificity of 96.7%. The NaOCl liquefaction method showed a sensitivity of 78%, with a specificity of 96%. Finally, the sensitivity of direct microscopy was lower than the other

TABLE 2. Correlation of sensitivity to smear preparation and AFB concentration

| Score ^a | No. of smears obtained by: | | | | |
|--------------------|----------------------------|----------------|---------------------------------|-----------------------|-------------------------|
| | NaLc-NaOH conc culture | NaLc-NaOH conc | Chitin sedimentation microscopy | NaOCl conc microscopy | Direct smear microscopy |
| 3+ | 21 | 21 | 21 | 21 | 20 |
| 2+ | 18 | 18 | 18 | 18 | 14 |
| 1+ | 20 | 19 | 17 | 16 | 5 |
| Few bacilli | 13 | 11 | 9 | 8 | 2 |
| Negative | 5 | 8 | 12 | 14 | 36 |

^a For explanation of scoring system, see Materials and Methods.

TABLE 3. Sensitivities and specificities of AFB detection by different smear preparations

| Method | Sensitivity (%) | Specificity (%) | Predictive value of positive smear (%) |
|-------------------|-----------------|-----------------|--|
| NaLc-NaOH | 83 | 97 | 93 |
| Chitin | 80 | 96.7 | 95.4 |
| NaOCl | 78 | 96 | 95.2 |
| Direct microscopy | 46 | 90 | 87 |

tested methods and was only 46%, with a specificity of 90% (Table 3).

DISCUSSION

Proper identification of cases is the pillar of tuberculosis control programs (10). Currently, this is accomplished mostly by microscopic examination of stained sputum. Thus, technical accuracy and proficiency are of paramount importance (1, 2, 4). Among the methods suggested for smear preparation, the *N*-acetyl-L-cysteine method has been shown to be a sensitive (28 to 87%) and reliable method for microscopy and culture (4, 5). However, due to limitations in funds and equipment, this technique is not being performed in TB laboratories of countries with limited resources (10, 14). In most parts of these countries direct smear microscopy with low sensitivity (25 to 50%) is the only available method for the diagnosis of tuberculosis (6, 7, 10, 22).

In our study, of 430 specimens, 69 (16%) smear-positive cases were detected by the *N*-acetyl-L-cysteine method, whereas with direct microscopy the number of AFB smears detected (with the same sputum specimens examined by the same technical staff) was reduced to 41 (9.5%) from 69. It means that 28 AFB-positive patients were missed when the direct microscopy method was used (Table 1).

Further analysis of the data (Table 2) showed that of the 28 missed smears, 23 (82%) belonged to patients with low numbers of bacilli in their sputa. In other words, fewer bacilli in the sputa would automatically increase the rate of false-negative results by direct microscopy. Therefore, as it has been suggested by others (19, 21), the direct microscopy is a technique of low sensitivity, so there is an urgent need to improve the procedure, especially the steps involved in preparation.

Many reports have suggested that liquefaction and overnight sedimentation of sputum with NaOCl would significantly increase the smear sensitivity, up to 70% (6, 7, 13). Our findings demonstrated the same results (Table 3). The number of AFB-positive smears increased from 41 by direct microscopy to 63 by NaOCl-treated sputum. Indeed, of 28 false-negative reports by direct smear microscopy, 22 (78%) turned out to be positive by the NaOCl method. The increased sensitivity of the NaOCl method is attributed to clearer microscopic fields and higher bacillus concentrations (13, 21).

Chitin and its derivatives, especially deacetylated chitin, called chitosan, are finding increasing uses in different fields such as wound-healing adjuvant, metal ion scavengers, wastewater treatment, and food-processing compounds (12, 17, 18). Chemically, chitin resembles cellulose, except that the hydroxyl group in position C-2 is replaced by an acetylamine (HN-CO-

CH₃) group (8, 9). This new nitrogen group could be responsible for diverse properties of chitin (18, 20).

The presence of NH-CO-CH₃ suggested that chitin might be used as a mucus digester. Therefore, we added chitin to the denser part of the mucoid portion of sputum and noticed the rapid liquefaction of sputum. In fact, this method took less time to homogenize the mucoid sputum than did the *N*-acetyl-L-cysteine and NaOCl methods. The quality of the smears was as good as that of the smears prepared by the *N*-acetyl-L-cysteine concentration method.

Overall, 65 (15.3%) of 430 specimens were detected as smear positive by the chitin-treated sputum. This number was slightly less than that obtained with the *N*-acetyl-L-cysteine (69/430) concentration method, but the difference was not statistically significant ($P > 0.05$). However, the main advantage of the chitin-treated sputum method over the *N*-acetyl-L-cysteine concentration method is that it does not require centrifugation.

The number of smears detected as positive by the chitin-treated sputum method (65/430) was slightly greater than that obtained by the NaOCl liquefaction method (63/430), but the difference was not significant. However, it is important to note that due to the high molecular weight of chitin, only a maximum of 30 min was required to achieve complete sedimentation of bacilli, whereas with the NaOCl liquefaction method at least 10 to 12 h was needed for bacillus sedimentation. Therefore, the chitin method is considerably less time-consuming than the NaOCl method.

Finally, the sensitivity of the chitin-treated sputum method was significantly increased (80%) in comparison to that of direct sputum microscopy (46%). Overall, both the chitin-treated sputum and the NaOCl liquefaction methods are easy to perform and they offer greater sensitivity than that of direct microscopy. In addition, because they don't require any special equipment they can be easily used under existing conditions of TB laboratories in developing countries.

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