

## Poor Performance of Universal Sample Processing Method for Diagnosis of Pulmonary Tuberculosis by Smear Microscopy and Culture in Uganda<sup>∇</sup>

Adithya Cattamanchi,<sup>1\*</sup> J. Lucian Davis,<sup>1,2</sup> William Worodria,<sup>3,4</sup> Samuel Yoo,<sup>3</sup> John Matovu,<sup>3</sup> John Kiidha,<sup>5</sup> Florence Nankya,<sup>5</sup> Rachel Kyeyune,<sup>5</sup> Alfred Andama,<sup>3</sup> Moses Joloba,<sup>4,6</sup> Dennis Osmond,<sup>7</sup> Phillip Hopewell,<sup>1</sup> and Laurence Huang<sup>1,2</sup>

*Division of Pulmonary and Critical Care Medicine, University of California, San Francisco, California<sup>1</sup>; HIV/AIDS Division, University of California, San Francisco, California<sup>2</sup>; Department of Medicine, Faculty of Medicine, Makerere University, Kampala, Uganda<sup>3</sup>; Uganda Ministry of Health, Kampala, Uganda<sup>4</sup>; Makerere University-University of California San Francisco Research Collaboration, Kampala, Uganda<sup>5</sup>; Department of Medical Microbiology, Faculty of Medicine, Makerere University, Kampala, Uganda<sup>6</sup>; and Department of Epidemiology and Biostatistics, University of California, San Francisco, California<sup>7</sup>*

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**Laboratory methods to improve smear microscopy are an urgent priority for global tuberculosis control. The novel universal sample processing (USP) method has been reported to improve conventional diagnostic testing for tuberculosis while also providing inhibitor-free specimens for molecular assays. However, no studies evaluating the method in the field have been conducted. In this study, we compared the performance of the USP method to that of the standard *N*-acetyl-L-cysteine-NaOH (NALC) method for conventional diagnosis of tuberculosis in 252 adults admitted to Mulago Hospital in Kampala, Uganda, with a clinical suspicion of pneumonia. A single early-morning sputum specimen collected from each patient was divided into two aliquots, each of which was assigned a random identification number. One randomly numbered specimen was processed by the USP method and the other by the NALC method. Mycobacterial cultures were more frequently negative in USP compared to NALC specimen aliquots (58% versus 43%;  $P < 0.001$ ). There was no difference in the proportion of contaminated mycobacterial cultures (12% versus 11%;  $P = 0.87$ ). The sensitivity and specificity of smear microscopy for the USP method were 52% and 86%, respectively, and were not significantly different from those for the NALC method (56% and 86%, respectively) using mycobacterial culture results as a reference standard. These results suggest that the USP method did not provide any significant advantage over the standard NALC method for conventional diagnosis of tuberculosis in our setting and illustrate the importance of well-designed, field-level evaluations of novel diagnostic techniques.**

Direct smear examination by light microscopy is the only diagnostic test for tuberculosis (TB) available in many low-income countries that are most affected by TB. However, only 20 to 50% of the 9 million annual TB cases are estimated to be positive by this method (16, 23). In the setting of human immunodeficiency virus (HIV) infection, the diagnostic sensitivity of direct smear microscopy is further reduced because of a lower bacillary burden in the lungs, a finding confirmed in multiple studies from sub-Saharan Africa (10, 13, 20). Despite these limitations, smear microscopy is rapid, inexpensive, and highly specific and will likely remain the primary diagnostic test for TB for the foreseeable future. Improved smear microscopy is therefore a primary goal for global TB control.

Several methods of smear microscopy that involve sputum liquefaction and concentration have been reported to increase diagnostic sensitivity (2, 17, 21, 22). The *N*-acetyl-L-cysteine-NaOH (NALC) method has been the most widely investigated

and has been reported to increase sensitivity modestly compared to direct smear microscopy (21). More recently, Chakravorty and Tyagi described the universal sample processing (USP) method for preparation of pulmonary and extrapulmonary specimens for smear microscopy, mycobacterial culture, and PCR-based assays (7, 8). In a field study of 571 specimens obtained from predominantly HIV-negative persons with suspected TB in India, the sensitivity of smear microscopy performed using the USP method was 17% higher than that of the NALC method (97% versus 80%;  $P < 0.001$ ) (4). In addition, the USP method was associated with upgrading of the smear result category (e.g., from scanty to 1+, from 1+ to 2+, etc.) and decreased mycobacterial culture contamination. Similar results were reported by the same investigators in two subsequent studies (5, 6). Although these results are promising, field studies are needed to confirm them, particularly in settings with a high incidence of HIV and TB coinfection.

We therefore conducted a blinded evaluation of the USP method compared to the NALC method for the diagnosis of pulmonary TB in Kampala, Uganda, a setting with a high prevalence of HIV and TB coinfection. The primary goal of the study was to determine whether the USP method improved smear microscopy- and culture-based diagnosis of TB compared to standard methods when introduced at the Uganda

\* Corresponding author. Mailing address: San Francisco General Hospital, Room 5K1, Pulmonary Division, 1001 Potrero Avenue, San Francisco, CA 94110. Phone: (415) 206-5489. Fax: (415) 695-1551. E-mail: acattamanchi@medsfgh.ucsf.edu.

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National Tuberculosis and Leprosy Program (NTLP) reference laboratory.

#### MATERIALS AND METHODS

**Clinical specimens.** This study involved collection of single early-morning sputum specimens from 252 consecutive adult patients admitted to the medical wards of Mulago Hospital with a clinical diagnosis of pneumonia between November 2007 and March 2008. The sputum specimens were collected from patients who had cough for more than 2 weeks but less than 6 months in duration, were not on anti-TB treatment, and provided informed consent. Specimens were excluded if either USP or NALC smear microscopy results were not available. The study protocol was approved by the Makerere University Research Ethics Committee, the Mulago Hospital Institutional Review Board, the Uganda National Council for Science and Technology, and the University of California, San Francisco, Committee on Human Research.

**Specimen processing.** Sputum specimens were divided into approximately equal aliquots, labeled with random identification numbers, and delivered to the Uganda NTLP reference laboratory. This random numbering blinded the NTLP staff to the identity of the subject and the knowledge of which aliquots were from the same subject. One randomly labeled aliquot was processed by the NALC method and the other by the USP method, with slight modifications, as previously described (4, 7, 11). The NALC method involved digesting and decontaminating the sputum specimen with an equal volume of 0.5% NALC-2% sodium hydroxide-1.5% sodium citrate solution, followed by centrifugation at  $3,000 \times g$  (11). A detailed protocol for the USP method was obtained from J. S. Tyagi (All India Institute of Medical Sciences, Chandigarh, India), who first described the methodology (8). Sputum specimens were homogenized and decontaminated by treatment with USP solution (4 to 6 M guanidinium hydrochloride, 50 mM Tris-Cl [pH 7.5], 25 mM EDTA, 0.5% Sarkosyl, 0.1 to 0.2 M beta-mercaptoethanol) and then concentrated by centrifugation at  $3,000 \times g$ . For both NALC and USP aliquots, smears were prepared using the concentrated pellets.

**Smear microscopy.** Smear microscopy was performed at the Uganda NTLP reference laboratory. Smears prepared from NALC- and USP-processed specimens were stained with auramine-O (counterstain, potassium permanganate) and read in batches within 24 h of preparation using a fluorescence microscope (magnification,  $\times 200$ ) (19). The presence or absence of acid-fast bacilli in 100 fields was reported using the WHO/IUATLD scale (24).

**Outcome classification.** Mycobacterial cultures were performed on Lowenstein-Jensen slants, one using a portion of the NALC and a second using a portion of the USP-processed sputum pellets (24). Two slants were made for each processed pellet. The reference standard outcome of TB was defined as a positive mycobacterial culture result on either sputum aliquot within 8 weeks of incubation. Specimen pairs were excluded for the calculation of diagnostic sensitivity and specificity if both mycobacterial cultures were contaminated.

**Quality assurance.** The study was performed in conjunction with the Uganda NTLP reference laboratory. The USP method was performed by two laboratory technicians who received specific training by a study investigator (A.C.) over a period of 1 month, and the method was used for 2 weeks prior to starting the study. Smears processed by the USP and NALC methods were read by one of five full-time laboratory technicians who have a median experience of 10 years (range, 5 to 20 years). In addition, the Uganda NTLP reference laboratory has participated in a biannual external quality assurance program for smear microscopy administered by the World Health Organization since 2005.

**Statistical analysis.** All analyses were performed using STATA 9.0 (Stata Corporation, College Station, TX), with the level of significance specified in reference to a two-tailed, type I error ( $P$  value) of less than 0.05. Bivariate analyses were performed using the chi-square test for dichotomous variables and the Mann-Whitney rank sum test for continuous variables. The sensitivity and specificity of smear microscopy performed using specimen aliquots processed by the NALC and USP methods were calculated in reference to a positive mycobacterial culture result (on either specimen aliquot) and compared using McNemar's test.

#### RESULTS

**Study population.** During the study period, 273 eligible patients were identified; 252 (92%) had both NALC and USP smear microscopy results available and were included in the study. Clinical and demographic characteristics of these patients are shown in Table 1. Overall, the study population was

TABLE 1. Demographic and clinical characteristics of patients ( $n = 252$ )

Characteristic	Value
No. (%) female.....	136 (54)
No. (%) male.....	116 (46)
Median age (IQR), yr.....	35 (28–42)
No. (%) HIV negative.....	45 (18)
No. (%) HIV infected.....	207 (82)
Median CD4 cell count (IQR) if HIV infected, cells/ $\mu$ l.....	53 (18–172)

young (median age, 35 years) and had a high prevalence of HIV infection (82%). The median CD4<sup>+</sup> T-lymphocyte count in the HIV-infected subset was 53 (interquartile range [IQR], 18 to 172).

**Mycobacterial cultures were more frequently negative in specimens processed by the USP method.** Mycobacterial cultures were negative in 58% of USP aliquots, compared to 43% of NALC aliquots (difference, 15%; 95% confidence interval [CI], 8 to 22%;  $P < 0.001$ ) (Fig. 1). There were 30 cases in which culture results were positive in the NALC aliquot when results from the USP aliquot were negative. In these cases, culture results from the NALC specimen showed  $< 20$  CFU in 15 (50%), 20 to 100 CFU in 7 (23%), innumerable colonies in 6 (20%), and confluent growth in 2 (7%) cases. There were four cases in which culture results were positive in the USP aliquot (one each with  $< 20$  CFU, 20 to 100 CFU, innumerable colonies, and confluent growth) when results from the NALC aliquot were negative. The proportions of contaminated cultures were nearly identical (11.2% versus 11.7%; difference, 0.5%, 95% CI,  $-7\%$  to  $+6\%$ ;  $P = 0.87$ ) for the NALC and USP methods.

**Smear microscopy results were similar in specimens processed by the NALC and USP methods.** The results of fluorescence microscopy examination of specimen aliquots processed by the NALC and USP methods are shown in Fig. 2. There was no significant difference in the proportion of positive smear examinations (35% versus 33%; difference, 2%; 95% CI,  $-5\%$  to  $+9\%$ ;  $P = 0.54$ ) for the two processing methods. Similarly, there was no significant difference in the proportion of patients within any smear-positive category, suggesting that the USP method did not enhance the smear grade status of sputum specimens.

**The USP method did not significantly improve the overall diagnostic performance of smear microscopy.** Seven specimen pairs were excluded from the analysis of diagnostic performance because mycobacterial cultures were contaminated for both specimen aliquots. Both NALC and USP smears were considered positive if  $\geq 1$  acid-fast bacilli were identified on microscopic examination. Sensitivity was higher for the NALC method (56% [95% CI, 47 to 66%] versus 52% [95% CI, 43 to 62%]; difference 4% [95% CI,  $-7\%$  to  $+16\%$ ];  $P = 0.42$ ), though the difference was not statistically significant ( $n = 201$ ). There was no difference in specificity (86% [95% CI, 79 to 91%] for both methods;  $P = 1$ ). Similarly, no significant differences in sensitivity or specificity were observed when the

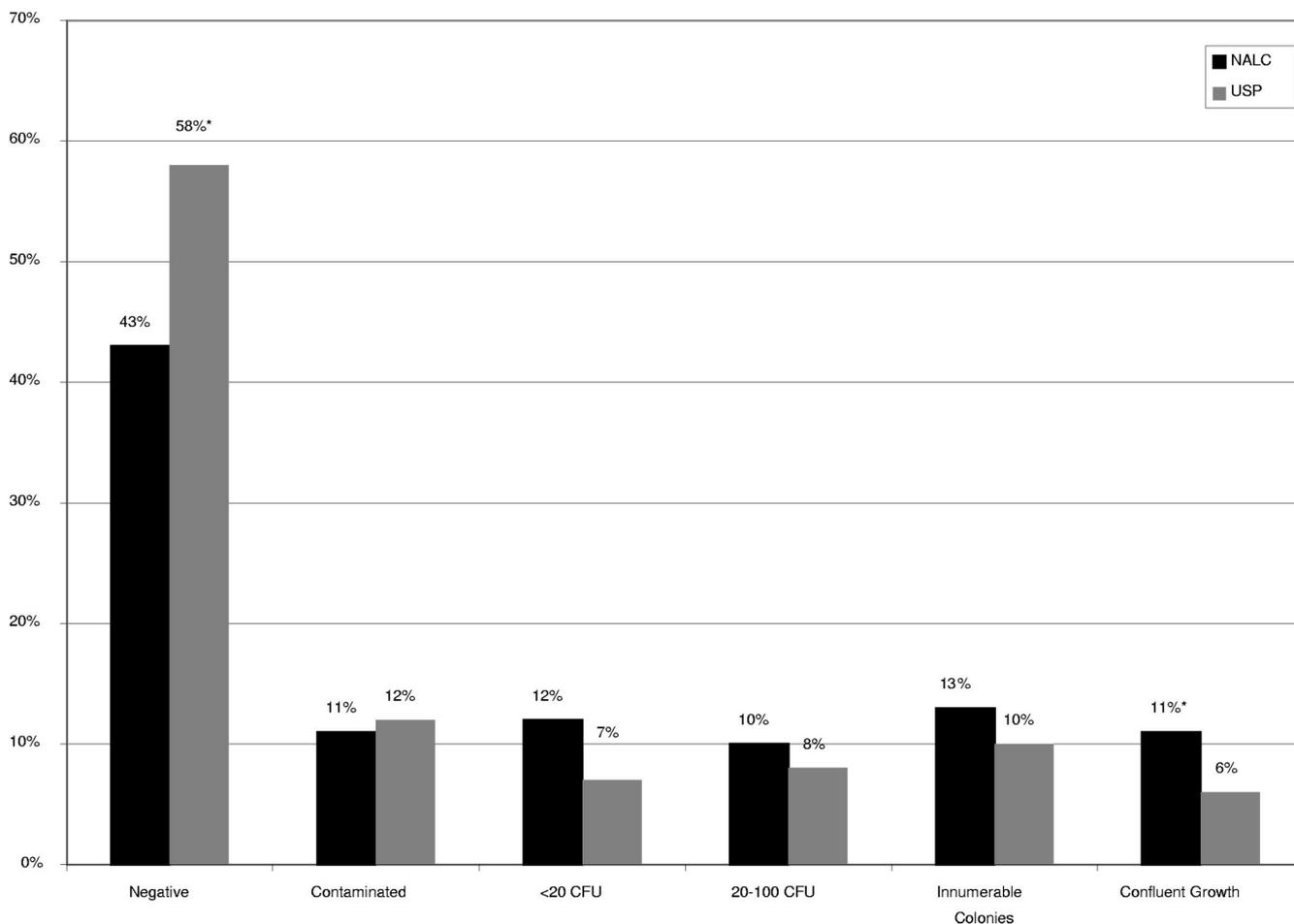


FIG. 1. Mycobacterial culture results ( $n = 207$ ). Mycobacterial cultures were performed on Lowenstein-Jensen slants using specimen aliquots processed by the NALC and USP methods. Specimen aliquots processed by the USP method were significantly more likely to be culture negative (58% versus 43%;  $P < 0.001$ ) and less likely to display confluent growth (6% versus 11%;  $P = 0.02$ ). There were no significant differences in the proportion of each specimen aliquot with any other mycobacterial culture result, including contaminated cultures. \*,  $P < 0.05$  for comparison between the NALC and USP methods.

analysis was stratified by HIV status or restricted to specimen aliquots for which the direct Ziehl-Neelsen smear results were concordant (data not shown).

## DISCUSSION

In this study, we found that liquefaction and concentration of sputum specimens by the USP method did not improve the diagnosis of TB by smear microscopy or mycobacterial culture compared to the standard NALC method. Mycobacterial cultures were more frequently negative in specimens processed by the USP method, and there was no difference in culture contamination rates. In addition, there was no significant difference in the sensitivity or specificity of smear microscopy when performed using specimens processed by either method, though sensitivity was significantly increased for both processing methods compared to direct Ziehl-Neelsen smears (data not shown).

Our results differ from prior reports of the diagnostic performance of the USP method, all of which have been published by the same group of investigators (4–7). There are several

important differences between the design of this study and that of these previous studies. First, smear interpretation was blinded in this study, which eliminates potential bias in the interpretation of smear results. Second, consecutive patients were enrolled, and exclusion criteria were clearly defined. The absence of these design features has previously been shown to bias estimates of diagnostic test performance (3, 12, 14). Lastly, this was the first evaluation of the USP method with a largely HIV-infected cohort. Though we found no differences in test performance when the analysis was stratified by HIV status, we cannot rule out significant differences in performance between the USP and NALC methods in non-HIV-infected patients because of the small number of such patients in our study.

In addition to study design issues, several technical issues should also be considered when comparing our results to previous evaluations of the USP method. Centrifugation speed has been shown to affect the sensitivity of smear microscopy (15). We therefore centrifuged both USP- and NALC-processed specimens at  $3,000 \times g$ , the standard centrifugation speed recommended by the Centers for Disease Control and Prevention (CDC) (11). In previous studies, centrifugation was

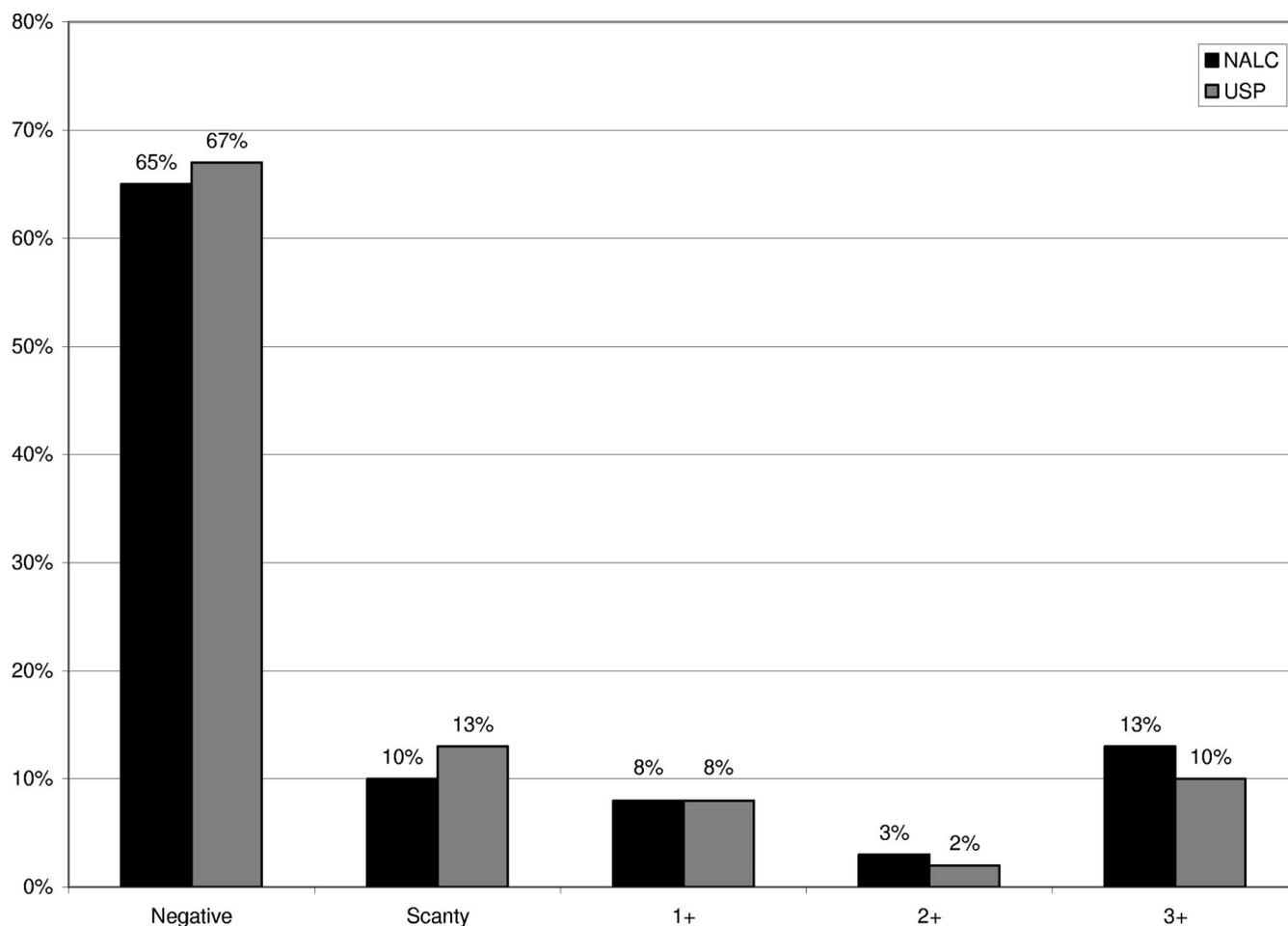


FIG. 2. Smear microscopy results ( $n = 252$ ). Results of fluorescence microscopy examination of smears prepared from specimen aliquots processed by the NALC and USP methods are shown. Smear microscopy results were classified according to the WHO/IUATLD scale. There were no significant differences in the proportion of USP- and NALC-processed specimens within any smear result category.

done at 5,000 to 6,000  $\times g$  for the USP method, whereas the standard CDC protocol was followed for the NALC method (4, 7). Thus, it is possible that the increased centrifugation speed used with the USP method was responsible for the increased sensitivity seen in previous studies. Similarly, the number of fields reviewed during microscopic examination may affect the sensitivity of smear microscopy. Chakravorty et al. reported reviewing 400 to 500 fields in their clinical evaluation of the USP method (4), a greater number than that recommended by international guidelines (24). Though only 100 fields were examined in our study, the same was done for both the USP and NALC methods. Therefore, the sensitivity of both techniques may be lower than previously reported, but this would not affect the comparison of the two methods. Lastly, our results may reflect less experience with the USP method and the use of this method in a field rather than in a research setting. Previous studies of smear microscopy have shown differences when comparing test performance in field versus research settings (1, 9, 18). To minimize the impact of these factors, a detailed protocol was obtained from the investigators who first reported the methodology, and training and

a 1-month trial period were completed prior to starting the study.

Though smear microscopy results were similar, the USP method performed poorly for culture-based diagnosis of TB. Previous studies have not found a difference in the yield of mycobacterial culture performed using specimens processed by the USP method or several other methods. In their clinical evaluation of the USP method, Chakravorty et al. reported that USP cultures were compared to cultures performed using specimens processed by either the NALC or the modified Petroff method (4). In other studies, the authors also reported good results when the USP method was compared to direct inoculation onto Lowenstein-Jensen slants and when using liquid culture systems (6, 7). However, in our study, there was a 15% absolute decrease in the proportion of culture-positive specimens compared to the NALC method. As discussed above, the validity of our findings is supported by our study design, which included blinded interpretation of culture results.

In summary, the USP method has been reported as a simple and rapid multipurpose technique that is compatible with both

conventional and PCR-based diagnostic modalities. However, in our setting, we found no advantage over the traditional NALC method for smear microscopy and a decreased likelihood of positive mycobacterial culture results. These results suggest that the USP method is unlikely to replace standard techniques for performing conventional diagnostic tests. Future studies should focus on the reported ability of the USP method to isolate high-quality, inhibitor-free DNA/RNA specimens, a major goal for improving novel molecular assays that can rapidly diagnose TB and detect drug resistance.

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#### REFERENCES

- Angeby, K. A., C. Alvarado-Galvez, L. Pineda-Garcia, and S. E. Hoffner. 2000. Improved sputum microscopy for a more sensitive diagnosis of pulmonary tuberculosis. *Int. J. Tuberc. Lung Dis.* **4**:684–687.
- Angeby, K. A., S. E. Hoffner, and V. K. Diwan. 2004. Should the bleach microscopy method be recommended for improved case detection of tuberculosis? Literature review and key person analysis. *Int. J. Tuberc. Lung Dis.* **8**:806–815.
- Bossuyt, P. M., and J. B. Reitsma. 2003. The STARD initiative. *Lancet* **361**:71.
- Chakravorty, S., M. Dudeja, M. Hanif, and J. S. Tyagi. 2005. Utility of universal sample processing methodology, combining smear microscopy, culture, and PCR, for diagnosis of pulmonary tuberculosis. *J. Clin. Microbiol.* **43**:2703–2708.
- Chakravorty, S., D. Pathak, M. Dudeja, S. Haldar, M. Hanif, and J. S. Tyagi. 2006. PCR amplification of shorter fragments from the devR (Rv3133c) gene significantly increases the sensitivity of tuberculosis diagnosis. *FEMS Microbiol. Lett.* **257**:306–311.
- Chakravorty, S., M. K. Sen, and J. S. Tyagi. 2005. Diagnosis of extrapulmonary tuberculosis by smear, culture, and PCR using universal sample processing technology. *J. Clin. Microbiol.* **43**:4357–4362.
- Chakravorty, S., and J. S. Tyagi. 2005. Novel multipurpose methodology for detection of mycobacteria in pulmonary and extrapulmonary specimens by smear microscopy, culture, and PCR. *J. Clin. Microbiol.* **43**:2697–2702.
- Chakravorty, S., and J. S. Tyagi. 2001. Novel use of guanidinium isothiocyanate in the isolation of *Mycobacterium tuberculosis* DNA from clinical material. *FEMS Microbiol. Lett.* **205**:113–117.
- Gebre, N., U. Karlsson, G. Jonsson, R. Macaden, A. Wolde, A. Assefa, and H. Miorner. 1995. Improved microscopical diagnosis of pulmonary tuberculosis in developing countries. *Trans. R. Soc. Trop. Med. Hyg.* **89**:191–193.
- Johnson, J. L., M. J. Vjecha, A. Okwera, E. Hatanga, F. Byekwaso, K. Wolski, T. Aisu, C. C. Whalen, R. Huebner, R. D. Mugerwa, and J. J. Ellner. 1998. Impact of human immunodeficiency virus type-1 infection on the initial bacteriologic and radiographic manifestations of pulmonary tuberculosis in Uganda. *Int. J. Tuberc. Lung Dis.* **2**:397–404.
- Kent, P., and G. Kubica. 1985. *Public health mycobacteriology—a guide for the level III laboratory*. Centers for Disease Control and Prevention, Atlanta, GA.
- Lijmer, J. G., B. W. Mol, S. Heisterkamp, G. J. Bonsel, M. H. Prins, J. H. van der Meulen, and P. M. Bossuyt. 1999. Empirical evidence of design-related bias in studies of diagnostic tests. *JAMA* **282**:1061–1066.
- Mugusi, F., E. Villamor, W. Urassa, E. Saathoff, R. J. Bosch, and W. W. Fawzi. 2006. HIV co-infection, CD4 cell counts and clinical correlates of bacillary density in pulmonary tuberculosis. *Int. J. Tuberc. Lung Dis.* **10**:663–669.
- Pai, M., and R. O'Brien. 2006. Tuberculosis diagnostics trials: do they lack methodological rigor? *Expert Rev. Mol. Diagn.* **6**:509–514.
- Perera, J., and D. M. Arachchi. 1999. The optimum relative centrifugal force and centrifugation time for improved sensitivity of smear and culture for detection of *Mycobacterium tuberculosis* from sputum. *Trans. R. Soc. Trop. Med. Hyg.* **93**:405–409.
- Perkins, M. D. 2000. New diagnostic tools for tuberculosis. *Int. J. Tuberc. Lung Dis.* **4**:S182–S188.
- Perkins, M. D., and J. Cunningham. 2007. Facing the crisis: improving the diagnosis of tuberculosis in the HIV era. *J. Infect. Dis.* **196**(Suppl. 1):S15–S27.
- Peterson, E. M., A. Nakasone, J. M. Platon-DeLeon, Y. Jang, L. M. de La Maza, and E. Desmond. 1999. Comparison of direct and concentrated acid-fast smears to identify specimens culture positive for *Mycobacterium* spp. *J. Clin. Microbiol.* **37**:3564–3568.
- Rieder, H. L., A. Van Deun, K. M. Kam, S. J. Kim, T. M. Chonde, A. Trébuq, and R. Urbanczik. 2007. Priorities for tuberculosis bacteriology services in low income countries. International Union Against Tuberculosis and Lung Disease, Paris, France.
- Samb, B., P. S. Sow, S. Kony, M. Maynard-Badiane, G. Diouf, S. Cissokho, D. Ba, M. Sane, F. Klotz, M. A. Faye-Niang, S. Mboup, I. Ndoye, E. Delaporte, A. A. Hane, A. Samb, J. P. Coulaud, A. M. Coll-Seck, B. Larouze, and J. F. Murray. 1999. Risk factors for negative sputum acid-fast bacilli smears in pulmonary tuberculosis: results from Dakar, Senegal, a city with low HIV seroprevalence. *Int. J. Tuberc. Lung Dis.* **3**:330–336.
- Steingart, K. R., V. Ng, M. Henry, P. C. Hopewell, A. Ramsay, J. Cunningham, R. Urbanczik, M. D. Perkins, M. A. Aziz, and M. Pai. 2006. Sputum processing methods to improve the sensitivity of smear microscopy for tuberculosis: a systematic review. *Lancet Infect. Dis.* **6**:664–674.
- Steingart, K. R., A. Ramsay, and M. Pai. 2007. Optimizing sputum smear microscopy for the diagnosis of pulmonary tuberculosis. *Expert Rev. Anti-Infect. Ther.* **5**:327–331.
- World Health Organization. 2008. *Global tuberculosis control: surveillance, planning, financing*. World Health Organization, Geneva, Switzerland.
- World Health Organization. 1998. *Laboratory services in tuberculosis control*. World Health Organization, Geneva, Switzerland.