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Lessons From a Proficiency Testing Event for Acid-Fast Microscopy*

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Abstract

Objectives—To evaluate the routine performance and the technical parameters of different acid-fast staining methods: Kinyoun, Ziehl-Neelsen (ZN), auramine, and auramine-rhodamine.

Design and participants—The performance of 167 laboratories was analyzed using prestained and unstained slides.

Setting—Laboratories holding New York State permits.

Results—The results revealed that Kinyoun's cold carbol fuchsin method is inferior to both the ZN and fluorochrome (auramine and/or auramine-rhodamine) methods. Even though 91% of the participants used commercial staining kits, the study identified unexpected errors concerning the concentration of carbol fuchsin, time for staining and counterstaining, and the concentration of acid alcohol for decolorization, which may significantly influence the sensitivity. Besides these findings, the present study showed that the examination of < 300 view fields may also decrease the sensitivity of acid-fast microscopy. In addition, we found that the sensitivity and specificity of the ZN and fluorochrome methods are comparable if the procedural standards are followed.

Conclusions—The strict and ongoing quality control of the "simple to perform" acid-fast microscopy and the immediate review of commercially available staining kits are necessary. Because of the rapidity of the fluorochrome method, laboratories with large specimen numbers should use this technique. In all other cases, the ZN method should be used. Moreover, all clinicians should be aware of the method of acid-fast microscopy used and the proficiency of the laboratory in performing the assay.

Keywords

fluorochrome; Kinyoun; microscopy; quality control; tuberculosis; Ziehl-Neelsen

Acid-fast microscopy is the fastest, easiest, and least expensive tool for the rapid identification of potentially infectious tuberculosis patients.^{1,2} The importance of the method is further exemplified by the fact that in low-income countries, the definitive diagnosis of tuberculosis still relies solely on the presence of acid-fast bacilli (AFB) in stained smears.

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Although the specificity of acid-fast microscopy is excellent for mycobacterial species, the sensitivity is not optimal. The sensitivity of microscopy is influenced by numerous factors, such as the prevalence and severity of disease, the type of specimen, the quality of specimen collection, the number of mycobacteria present in the specimen, the method of processing (direct or concentrated), the method of centrifugation, the staining technique, and the quality of the examination.¹⁻⁷ It is recommended that a negative result should only be reported following the examination of at least 100 (in low-income countries) and preferably 300 (in industrialized countries) microscopic immersion view fields (or equivalent fluorescent view fields).^{1-3,8-10} Therefore, when microscopy is performed correctly, it can be time-consuming and laborious. False-negativity due to fatigue may also contribute to decreased sensitivity.

Presently, two types of acid-fast stains are used in clinical mycobacteriology laboratories. One type is carbol fuchsin (Ziehl-Neelsen [ZN] or Kinyoun methods), and the other is fluorochrome (either auramine or auramine-rhodamine). The present ZN method has evolved from Koch's original alkaline methylene blue-based method following significant modifications.¹¹⁻¹⁶ Kinyoun's cold staining is a modification of the classical ZN method that excludes the heating step during staining and therefore uses a higher concentration of carbol fuchsin.^{17,18} Methods applying fluorochromes have been used for acid-fast staining since the introduction of an auramine O-based method by Hagemann¹⁹ and Richards et al.,²⁰ and an auramine-rhodamine based method by Matthaei,²¹ Truant et al.,²² and Morse et al.²³ Because it is easier to detect a fluorescent rod against a darker background, the fluorochrome staining method allows the examiner to scan the slide at a lower magnification and thus observe a larger area than with carbol fuchsin-stained smears. These factors reduce the time for screening and lead to greater sensitivity. Therefore, it is generally accepted that the fluorescent method should be given preference over the ZN and Kinyoun methods.^{2,3,24}

Although several research groups have investigated the clinical validity and differences in sensitivity between various staining methods, to date only a limited number of reports have described the technical and procedural factors of the observed discrepancies.^{4,18,25-31} Since the clinical signs and symptoms of pulmonary tuberculosis are not specific, the accurate performance of acid-fast microscopy is imperative for the early recognition of tuberculosis patients so that adequate treatment, respiratory isolation, and contact investigation can be started as soon as possible. Therefore, to evaluate the routine performance and technical parameters of different acid-fast microscopic methods, we surveyed 167 laboratories that carry New York State permits to perform acid-fast smear examinations.

MATERIALS AND METHODS

Preparation of Negative Smears

Briefly, 10 µL loopfuls of *Streptococcus sanguis*, *Corynebacterium diphtheriae*, and *Bacillus cereus* cultures were placed into three tubes containing 5 mL of brain-heart infusion broth and incubated at 37°C for 24 h. A 0.1-mL aliquot of each was then inoculated into each of three vials containing 9.9 mL of diluting fluid (Becton Dickinson Microbiology Systems; Cockeysville, MD). One milliliter of each of these three 1:100 dilutions was added to 100 mL of 1.23% mucin-phosphate buffered saline Solution (pH 6.0) to provide background material. The suspension was mixed on a magnetic stirrer for 30 min, and 50-µl aliquots were pipetted onto slides and spread to cover an area of approximately 1 × 2 cm. The smears were air dried and then heat fixed by passing the slides through the blue cone of a Bunsen burner's flame four times.²⁵ Two negative smears were sent unstained to each participant.

Preparation of Positive Smears

One-half milliliter of a frozen *Mycobacterium tuberculosis* (American Type Culture Collection 2794) stock culture was subcultured into 10 mL of Dubos-Davis broth containing 0.05% Tween 80. The subculture was incubated at 37°C on a rotary mixer until the optical density corresponded to a 1 McFarland nephelometer standard. At that time, the subculture was sonicated for 20 s to break up clumps, and 2 mL was added to 98 mL of 1.23% mucin-phosphate buffered saline solution (pH 6.0). Finally, 1 mL of each of the *S. sanguis*, *C. diphtheriae*, and *B. cereus* 1:100 dilutions (prepared as described above) was added. The suspension was mixed on a magnetic stirrer for 30 min, and 50- μ l aliquots were pipetted onto slides, and spread, air dried, and heat fixed as described above. Three identically prepared positive smears were sent to each participant: one stained by ZN, one stained by the Kinyoun method, and one left unstained.

For prestaining the positive slides by the ZN and the Kinyoun methods before mail-out, the entire slide was flooded with 0.3% (for ZN) or 3.3% (for Kinyoun) carbol fuchsin (Remel Microbiology Products; Lenexa, KS). Staining was carried out using commercially available ZN and Kinyoun staining kits (Remel Microbiology Products) according to the recommendations published in the *Clinical Microbiology Procedures Handbook* (CMPH) by the American Society for Microbiology.⁸

Proficiency Testing Samples Mail-Out

As a part of a proficiency testing sample kit, a set of five smears (one ZN-stained positive, one Kinyoun-stained positive, one unstained positive, and two unstained negatives) was mailed to each participating laboratory by overnight courier. All laboratories were asked to use their routinely applied staining methods (for the unstained smears) and microscopic examination protocols. The participants were unaware of which particular carbol fuchsin staining method was used on the prestained slides. The prestained slides served to evaluate the reading capabilities of the participants, while the self-stained slides were intended to evaluate both reading and staining capabilities.

Analysis of Results

AFB microscopy results with a $\times 1,000$ immersion magnification were interpreted and reported by the participants according to standard recommendations.^{1,3,9,10} Observations made with the fluorochrome smears were converted to a format that equates with the above-listed format by using Smithwick's magnification factors.^{1,3,8}

Follow-up Questionnaire

Subsequently, we requested information about the source of the stain used, the concentration of carbol fuchsin solution, the time of staining, the concentration of acid alcohol solution, the time and method of counterstaining, the magnification and number of view fields examined, and the method of staining preferred (following the examination of the prestained ZN and Kinyoun smears).

Statistical Analysis

The symmetry test was used to evaluate the differences in the level of positivity between the methods of the different prestained slides, and between the self-stained and the prestained slides. The Mann-Whitney *U* test was applied to analyze the differences in the level of positivity between the different self-stained staining methods. Values of $p < 0.05$ were regarded as significant.

RESULTS

Proficiency Testing Results

Data from 158 laboratories with New York State permits (134 located in New York State, and 24 located in 12 other US states) and 9 reference laboratories (located in 6 US states and in Switzerland) were analyzed in the present study.

Comparison of ZN and Kinyoun Methods Using Prestained Slides (Evaluation of the Reading Capability)—The reported readings with ZN and Kinyoun-prestained positive slides are summarized in Figure 1. The readings (the level of positivity) were compared on a per-laboratory basis, and a statistical analysis revealed that the ZN method was significantly more sensitive (more positive readings with a higher level of positivity) than the Kinyoun method ($p < 0.01$; Fig 1).

Self-Stained Positive and Negative Slides (Evaluation of Both Staining and Reading Capabilities)—Seventy-five of the 167 laboratories (44.9%) applied Kinyoun, 14 laboratories (8.4%) applied ZN, 33 laboratories (19.8%) applied auramine, and 41 laboratories (24.6%) applied auramine-rhodamine staining on the unstained proficiency testing slides. Only 4 of 167 laboratories (2.4%) applied the TB Quick modified Kinyoun method (Becton-Dickinson Microbiology Systems). Because of the low number of users and the significant differences in the reagents of this latter method, these results were excluded from the study. Observations with Kinyoun, ZN, auramine, and auramine-rhodamine self-stained positive slides are listed in Table 1. The results on the self-stained negative slides are summarized in Table 2.

A comparison of readings with the four different staining methods revealed a significantly higher sensitivity of auramine-rhodamine vs Kinyoun ($p < 0.01$), ZN ($p = 0.01$), and auramine ($p < 0.02$). The difference between auramine and Kinyoun ($p = 0.064$), and auramine and ZN ($p = 0.089$) was close to being significant, while there was no significant difference between Kinyoun and ZN ($p = 0.73$).

The readings of the four different staining methods (Kinyoun, ZN, auramine, auramine-rhodamine) performed by the participants were also compared with the results from the prestained (stained according to standard recommendations) ZN and Kinyoun slides (Table 1) on a per-laboratory basis. When comparing the observations of Kinyoun users with the readings of ZN and Kinyoun prestained slides, the statistical test identified a significantly higher sensitivity with the ZN prestained slides ($p < 0.01$) and a slightly higher sensitivity (but not statistically significant, $p = 0.13$) with the Kinyoun prestained slides. Statistical analysis did not find any difference between the ZN readings of the participants and the prestained ZN ($p = 0.12$) and Kinyoun ($p = 0.99$) smears, but both prestained slides yielded a higher sensitivity.

There was a significant difference between the prestained ZN smears and auramine ($p < 0.05$), as the prestained ZN provided a higher sensitivity. Auramine showed a better, however not statistically significant, performance than the prestained Kinyoun ($p = 0.73$). Auramine-rhodamine also showed a higher sensitivity than prestained Kinyoun, and the statistical test revealed a significant difference ($p < 0.05$). Although the difference between the prestained ZN and auramine-rhodamine was not significant ($p = 0.41$), the prestained method provided a higher sensitivity.

Questionnaire

In order to identify which procedural factors and deviations from the standard recommendations (Table 3) may influence the performance and sensitivity of acid-fast microscopy, we sent out a questionnaire to analyze various parameters reported by the participants. All participants sent back the questionnaire. The results of the questionnaire are summarized in Table 4.

The vast majority (95.9% of Kinyoun, 71.4% of ZN users, and 89.1% of the fluorochrome users) of the participants were using commercially available staining kits. A review of the package inserts of these commercial kits revealed that only three of eight companies whose products were used apply the recommended carbol fuchsin concentrations (Table 3). This influenced 52 of the Kinyoun users (73.2%) and 6 of the ZN users (46.2%). In addition, although only 14 of the 167 participants (8.4%) are routinely using the ZN method, 129 of the 154 laboratories (83.8%) that answered this question preferred the ZN-prestained smear as opposed to the Kinyoun-prestained smear.

DISCUSSION

Although acid-fast microscopy is more than 100 years old, it still remains the initial and most rapid step in the diagnosis of tuberculosis.² Acid-fast microscopy is simple to perform and therefore could be applied successfully at any laboratory.² However, the findings of the present study, based on the proficiency testing of 167 laboratories, identified unexpected procedural deficiencies that could greatly influence the sensitivity of the acid-fast smear.

In accordance with previous studies, our results also indicate that the sensitivity of Kinyoun staining (either prestained or self-stained) is significantly lower than that of the prestained hot carbol fuchsin-based method (Fig 1, Table 1).^{29,32–34} As summarized in Figure 1, the level of positivity was considerably lower with the prestained Kinyoun compared with the prestained ZN. It is important to note that the comparison of the two prestained methods evaluated the differences in reading capabilities of the participants and excluded the biasing effect of the differences in staining skills.

The two self-stained fluorochrome methods also provided higher sensitivity than the prestained (auramine vs Kinyoun [nonsignificant]; auramine-rhodamine vs Kinyoun [significant]) or self-stained (both fluorochrome vs Kinyoun [significant]) Kinyoun method (Table 1). Moreover, false-negative findings were reported only with Kinyoun (prestained or self-stained) AFB-positive slides (Table 1). Based on these findings, we conclude that the Kinyoun method is inferior to the ZN or fluorochrome methods even if it is performed strictly according to the present recommendations (prestained slides).^{1,3,8} Consequently there is a significant chance that potentially infectious patients who have a lower grade of smear positivity will be reported as smear negative in 46% of the 167 laboratories examined.

Furthermore, the finding that the prestained Kinyoun method showed a better performance than the self-stained method also highlighted the fact that the basic differences between the cold staining method and the ZN or fluorochrome methods were further increased when the participants did not follow the current recommendations for acid-fast microscopy. According to the answers of the questionnaire, the majority of the Kinyoun users (74.7%) applied a much lower carbol fuchsin concentration than the recommended one by Kinyoun, the Centers for Disease Control and Prevention [CDC], and the CMPPH, and the lower concentration may not be sufficient to penetrate the cell wall of mycobacteria at room temperature (Table 4).^{1,3,8,17,18,33,34} The reason that the proper concentration was not used by 73.2% of the Kinyoun users was because several commercial staining kits contained an

inadequate carbol fuchsin concentration. This observation indicates the importance of the review of all commercial acid-fast staining kits.

Although the staining time was longer in 90.7% of Kinyoun users than the recommended 1 to 3 min (Tables 3, 4),^{8,17} this did not improve the sensitivity. It has been shown that cold staining is still inferior after ≥ 2 h of staining as compared to staining with the hot carbol fuchsin method for 5 min.^{35,36} However, the Kinyoun users also did not adhere to the recommended time for counterstaining. A longer counterstaining time was used by 61.3% of Kinyoun users (Table 4). This may have resulted in an intense background that could have hidden the poorly stained mycobacteria.^{1,3,8} Finally, 37.8% of Kinyoun users examined < 300 view fields required by the CDC and the American Thoracic Society when reporting a negative result (Table 4).^{1,3,35} There were laboratories that examined only ≤ 50 view fields. This must have further reduced the sensitivity. In contrast to Kinyoun users, the ZN users followed the recommendations much more appropriately, which resulted in a better performance (Tables 1-4).^{1,3}

As regards the fluorochrome staining methods, the auramine-rhodamine method was significantly more sensitive than any of the other three self-stained (auramine, Kinyoun, ZN) methods (Table 1). The difference between auramine and self-stained Kinyoun and ZN was close to being significant, but the difference between auramine and ZN was smaller. The advantages of fluorescence microscopy are ease, speed, and better contrast due to the dark background. Therefore, several clinical studies^{20,26,29,31} have suggested that more acid-fast smears would be found to be positive by the fluorochrome methods than by the ZN or Kinyoun methods. Nevertheless, the results of the present study and a recent study by Iademaro et al³⁶ prove that the ZN method can be similarly (prestained ZN vs auramine-rhodamine) or even significantly more sensitive than the fluorochrome methods if the slides are prepared and examined according to the standard recommendations.^{1,3,8}

Interestingly, this study also revealed a significant difference between the two fluorochrome methods, as the auramine-rhodamine method showed a better performance (Table 1). The survey revealed that the auramine-rhodamine users applied longer staining times than the recommended 15 min, while the majority of the auramine users followed the recommended time (Table 4).^{1,3,8} This suggests that a longer (20 to 25 min) staining time might increase sensitivity. However, any possible influence of the staining time on the sensitivity of fluorochrome stains needs further investigation.

The questionnaire also revealed another difference between auramine and auramine-rhodamine users. More than one third of the auramine users applied a considerably (two to six times) more concentrated acid-alcohol solution in the decolorization step, which may have resulted an overdecolorization and consequently a decrease in sensitivity (Table 4).^{1,3,8} The majority (82.1%) of auramine-rhodamine users applied the recommended 0.5% acid-alcohol solution.^{1,3,8} Although standard recommendations for carbol fuchsin methods state that mycobacteria cannot easily be de-stained, our results indicate that the concentration of acid alcohol with fluorochrome stains is critical.^{1,8}

As regards the counterstaining, the recommendation of both the CMPH and CDC is that counterstaining for > 2 min with potassium permanganate may quench the fluorescence of AFB and result in false-negativity. In our study, the longer (2 to 5 min) counterstaining time did not influence the sensitivity of the fluorescence microscopy.^{1,3,8} However, in the original report of this counterstaining method by Truant et al,²² loss of brilliance was observed only with a counterstaining time from 4 to 10 min. In addition, while 80.5% of the auramine-rhodamine users examined the recommended 30 view fields at $\times 250$

magnification (or equivalent), 30.3% of auramine users examined fewer view fields in contrast to requirements for reporting a negative result (Table 4)^{1,3,35}

False-positivity did not cause a problem in the study; however, this study did not use clinical specimens. The number of doubtful results (doubtful results are considered as negatives) with the AFB-negative smears prepared in the Wadsworth Center was comparable and was low with all four self-stained methods (Table 2). However, one should keep in mind that less experienced readers have a marked tendency for false-positivity with the fluorochrome methods.^{1,3,25,29,31} Furthermore, it is often forgotten that fluorochrome dyes may stain AFB damaged by antituberculous drugs at a higher rate than carbol fuchsin.^{4,25,37} This should be considered when the specimen is from a patient receiving antituberculous therapy. False-positivity is also pronounced with fluorescence microscopy when blood is present in the specimen, a common contaminant in the sputum of patients with advanced tuberculosis.^{4,25,38} Similar problems do not have an effect on the ZN method. Therefore, it is good laboratory practice to confirm any smear-positive or doubtful result in newly diagnosed patients by a ZN stain or a second examiner.^{1,2,3,8,25}

In conclusion, the further standardization of the present staining guidelines (Table 3), the review of commercial staining kits, and the strict and ongoing quality control of such a “simple to perform” method like acid-fast microscopy is indispensable. Further-more, as clinicians highly rely on the acid-fast smear results in order to choose the adequate therapeutic, isolation, and contact investigation steps, all clinicians should be aware of the method of acid-fast microscopy and proficiency testing used in their laboratory.

Abbreviations

AFB	acid-fast bacilli
CDC	Centers for Disease Control and Prevention
CMPH	<i>Clinical Microbiology Procedures Handbook</i>
ZN	Ziehl-Neelsen

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FIGURE 1.

Per-laboratory comparison of results obtained by the prestained ZN and Kinyoun acid-fast staining methods. Comparison of prestained ZN vs prestained Kinyoun for the evaluation of differences in reading capabilities of participants revealed a significantly better performance of the ZN staining method ($p < 0.01$).

Table 1

Per-Laboratory Comparison of Results Obtained by the Different Prestained and Self-Stained Acid-Fast Staining Methods for Positive Smears*

Kinyoun, Self-Stained (n = 75)					ZN, Self-Stained (n = 14)					Auramine, Self-Stained (n = 33)					Auramine-Rhodamine, Self-Stained (n = 41)						
Variables	ne	d	r	f	m	ne	d	r	f	m	ne	d	r	f	m	ne	d	r	f	m	
Kinyoun, prestained																					
ne	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2	—
d	1	1	1	1	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	1	—
r	—	3	15	8	—	—	—	—	4	2	—	—	—	9	8	2	—	—	6	11	6
f	—	—	22	16	5	—	—	—	5	3	—	—	—	4	6	1	—	—	2	7	4
m	—	—	—	1	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	1	1
Total	1	4	38	27	5	—	—	—	9	5	—	—	—	13	17	3	—	—	8	22	11
ZN, prestained																					
ne	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
d	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
r	1	—	—	1	1	—	—	—	1	—	—	—	—	—	2	—	—	—	1	—	1
f	—	1	18	6	1	—	—	—	2	2	—	—	—	9	4	1	—	—	4	9	5
m	—	1	20	20	3	—	—	—	6	3	—	—	—	4	11	2	—	—	3	13	5
n	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

* The evaluation of both staining and reading capabilities revealed the following results; prestained Ziehl-Neelsen (ZN) *us* self-stained Kinyoun, $p < 0.01$, significant; prestained ZN *us* self-stained auramine, $p < 0.05$, significant; self-stained auramine-rhodamine *us* prestained Kinyoun, $p < 0.05$, significant; self-stained auramine-rhodamine *us* self-stained Kinyoun, ZN, and auramine, $p < 0.05$, significant. ne = negative (no AFB observed); d = doubtful (1–2 AFB/300 view-fields); r = rare (1–9 AFB/100 view-fields); f = few (1–9 AFB/10 view-fields); m = moderate (1–9 AFB/view-field); — = 0.

Table 2
Observations With Different Self-Stained Acid-Fast Staining Methods for AFB-Negative Smears*

Variables	Kinyoun		ZN		Auramine		Auramine-Rhodamine	
	ne	d	ne	d	ne	d	ne	nu
Unstained negative smear 1	73	2	12	2	31	1	39	2 0
Unstained negative smear 2	72	3	12	2	29	4	39	1 1

* Per laboratory-based comparison revealed that no laboratory reported a false-positive result from both acid-fast bacilli negative smears. Data are presented as No; nu = numerous (> 9 AFB/view-field). See Table 1 for definition of other abbreviations.

Table 3**Acid-Fast Staining Procedures***

Variables	ZN	Kinyoun	Auramine	Anramine-Rhodamine
Stain/temperature	0.3% carbol fuchsin/steaming	3.3% carbol fuchsin/room temperature	0.1% auramine O/room temperature	1.1% auramine O and 0.55% rhodamine/room temperature
CDC	5 min	5 min	15 min	15 min
CMPH	3–5 min	2 min (1–3 min)	15 min	15 min
WHO	3–5 min	Not detailed	15 min	Not detailed
IUATLD	At least 5 min	Not detailed	15 min	Not detailed
Decolorization	3% HCl in 95% ethanol	3% HCl in 95% ethanol	0.5% HCl in 70% ethanol	0.5% HCl in 70% ethanol
CDC	2 min	2 min	2 min	2 min
CMPH	Until no more color drains	Until no more color drains	2 min	2 min
WHO	Maximum 3 min	Not detailed	2 min	Not detailed
IUATLD	3 min	Not detailed	2 min	Not detailed
Counterstain time	0.3% methylen blue	0.3% methylen blue	0.5% KMnO ₄	0.5% KMnO ₄
CDC	1–2 min	1–2 min	2 min	2 min
CMPH	20–30 s	20–30 s	2 min	2 min
WHO	1 min	Not detailed	2 min	Not detailed
IUATLD	1 min with 0.3% or 30 s with 0.1% solution	Not detailed	2 min	Not detailed
Magnification	× 1,000 immersion oil	× 1,000 immersion oil	Minimum × 250 fluorescent	Minimum × 250 fluorescent
No. of view fields				
CDC	Minimum 300	Minimum 300	Minimum 30	30
CMPH	Minimum 100	Minimum 100	Not detailed	Not detailed
WHO	Minimum 100	Not detailed	Minimum 30	Not detailed
IUATLD	Minimum 100	Not detailed	Minimum 30	Not detailed
Appearance of AFB	Red rods	Red rods	Yellow rods	Yellow-orange rods

* HCl = hypochloric acid; CDC = Centers for Disease Control and Prevention^{1,3}; CMPH = Clinical Microbiology Procedures Handbook⁸; WHO = World Health Organization¹⁰; IUATLD = International Union Against Tuberculosis and Lung Disease.⁹

Table 4

Results of the Questionnaire To Analyze the Acid-Fast Staining Parameters That Influenced the Sensitivity of Acid-Fast Microscopy*

Users		Carbol Fuchsin Concentration	Carbol Fuchsin Staining Time	Fluorescent Staining Time	Decolorizer Acid- Alcohol Concentration	Counterstaining Time	No. of Viewfields
ZN	Correct	5/13 (38.5)	13/14 (92.9)	NA	11/13 (84.6)	7/14 (50.0)	11/14 (78.6)
	Higher/longer	5/13 (38.5)	0	NA	0	7/14 (50.0)	0
	Lower/shorter	3/13 (23%)	1/14 (7.1)	NA	2/13 (15.4)	0	3/14 (21.4)
Kinyoun	Correct	17/71 (23.9)	7/75 (9.3)	NA	67/74 (90.5)	28/75 (37.3)	44/74 (59.5)
	Higher/longer	1/71 (1.4)	68/75 (90.7)	NA	3/74 (4.1%)	46/75 (61.3)	2/74 (2.7)
	Lower/shorter	53/71 (74.7)	0	NA	4/74 (5.4)	1/75 (1.3)	28/74 (37.8)
Auramine	Correct	NA	NA	23/33 (69.7)	21/33 (63.6)	17/27 (63.0)	23/33 (69.7)
	Higher/longer	NA	NA	6/33 (18.2)	12/33 (36.4)	6/27 (22.2)	0
	Lower/shorter	NA	NA	4/33 (12.1)	0	4/27 (14.8)	10/33 (30.3)
Auramine-rhodamine	Correct	NA	NA	15/41 (36.6)	32/39 (82.1)	11/41 (26.8)	18/41 (43.9)
	Higher/longer	NA	NA	25/41 (61.0)	7/39 (17.9)	29/41 (70.7)	15/41 (36.6)
	Lower/shorter	NA	NA	1/41 (2.4)	0	1/41 (2.4)	8/41 (19.5)

* Data are presented as No./No. of users (%); NA = not applicable.