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Assessment of panel slides prepared by phenol ammonium sulphate and NALC methods for proficiency testing

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SUMMARY

BACKGROUND: Existing methods for the preparation of panel slides necessitate handling high-grade acid-fast bacilli positive sputum samples.

OBJECTIVE: To compare panel slides prepared using the phenol ammonium sulphate sediment (PhAS) method with those prepared using the *N*-acetyl-L-cysteine (NALC) method in proficiency testing.

METHODS: Pooled sputum specimens of known smear-positives and -negatives were divided into two parts: one part was used for preparing panel slides using the NALC method and the other using PhAS, a non-hazardous method. Respectively 413 and 384 smears of different grades were prepared in three batches using the PhAS and NALC methods. Smear grade and quality were recorded by 121 microscopists during proficiency testing in different states. Agreement between reference and reported results was analysed using the kappa test.

RESULTS: The overall agreement was 96% for the PhAS method and 91% for the NALC method. There were 37 errors using the NALC method compared to 21 for the PhAS method ($P < 0.223$). Smear quality was equally good in both methods; however, the cell count was significantly higher in the PhAS than in the NALC method.

CONCLUSION: The PhAS method, a non-hazardous procedure with good-quality smears, may be further explored for the preparation of panel slides.

KEY WORDS: microscopy; quality assurance; *M. tuberculosis*; panel testing

SPUTUM MICROSCOPY continues to be the primary tool for the diagnosis of tuberculosis (TB) in health systems worldwide.[1] Its quality is ensured by panel testing of microscopists and quality of staining reagents, blinded rechecking of routine smears and on-site evaluation (OSE) of microscopy centres.[2–6] Panel slides for panel testing are prepared from high-grade acid-fast bacilli (AFB) positive sputum samples using the *N*-acetyl-L-cysteine (NALC) method, the sodium hydroxide method (NaOH) method, or from artificial sputum.[5,7] As samples processed using NALC or NaOH are aqueous in nature, laboratory staff are at risk of exposure to aerosols of *Mycobacterium tuberculosis* when preparing large numbers of slides.[7] In addition, NALC is expensive, and stringent safety precautions should be taken when preparing panel slides.[5] The preparation of slides from artificial sputum containing cells from cell culture, methyl cellulose or polyacrylamide and avirulent *M. bovis* bacille Calmette-Guérin strains needs good laboratory facilities and trained laboratory personnel.[7] A simple and relatively safe method is thus needed for the preparation of large numbers of slides. The phenol ammonium sulphate sedimentation (PhAS) method is shown to be safe and suitable for microscopy.[8] In the present study, we assessed panel slides prepared using the PhAS and NALC methods for proficiency testing.

MATERIALS AND METHODS

Preparation of reagents

The PhAS reagent was prepared by dissolving 5 ml of phenol (Qualigens, Chennai, India) and 4 g of ammonium sulphate (E Merck, Mumbai, India) in 95 ml of distilled water.[8] Fresh NALC reagent was prepared by dissolving 2 g of *N*-acetyl-L-cysteine (Himedia, Mumbai, India) in 100 ml of distilled water and 290 mg of TRIS sodium citrate (Qualigens, Mumbai, India) in 100 ml of distilled water in separate bottles.[9] Formalin (10%) was prepared by adding 10 ml of formaldehyde (Qualigens, Mumbai, India) to 90 ml of distilled water.

Collection and processing of sputum samples

The study was conducted from December 2009 to June 2010 at the National Institute for Research in

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Tuberculosis (NIRT), Chennai, South India. Pooled smear-positive and smear-negative sputum samples (approximately 10 ml) were prepared by mixing high-grade smear-positive and smear-negative samples from different patients. Two to three drops of 10% formalin were added to each specimen immediately after collection to prevent further autolysis, and stored at room temperature until they were processed. The specimens were divided equally into two portions: one was allocated to the NALC method and the other to the PhAS method.

Preparation of panel slides

PhAS method

Negative slides: from the negative sample, two smears were prepared and stained using the hot Ziehl-Neelsen (ZN) method, and the average number of cells/field was recorded. Specimens with >10 cells/field were selected. This was used as negative stock sputum and for the preparation of negative slides.

Positive slides: the positive aliquot allocated for the PhAS method was added to the PhAS solution, shaken gently at 15 min intervals for 1 h and left to stand at room temperature for another 2 h. The supernatant was discarded without centrifugation and the sediment was vortexed for 2 min. Two smears were stained using the hot ZN method, and the average number of AFB per 100 fields was recorded. Based on the number of AFB/100 fields, the positive suspension was diluted with the negative stock sputum, and smears with 2+, 1+ and scanty grades were made as per India's Revised National Tuberculosis Control Programme (RNTCP) guidelines.[9]

NALC method

Negative slides: the negative aliquot was added to an equal amount of NALC reagent and incubated for 30 min at room temperature. The mixture was vortexed for 5 min, two smears were stained using the hot ZN method and the average number of cells/field recorded. Specimens with >10 cells/field were selected. This was used as negative stock sputum and for the preparation of negative slides.

Positive slides: the positive aliquot allocated for the NALC method was processed and handled in the same way as the negative slides until staining with hot ZN, and the average number of AFB/100 fields was recorded. Based on the number of AFB/100 fields, the suspension was diluted with negative stock sputum, and smears with 2+, 1+ and scanty grades were made as per RNTCP guidelines.[9]

Validation of panel slides

Three batches each of NALC and PhAS slides were prepared and stored until use. The panel slides were validated as per RNTCP guidelines.[9] For each grade, slides from the PhAS and NALC methods were arranged and randomly coded. Date of preparation, number of slides prepared, period of storage and number of slides taken for on-site evaluation (OSE) were documented. During OSE, a set of five slides with different grades, selected randomly, was used for proficiency testing among the participating microscopists. The slides were decoded after OSE.

Panel testing

A total of 413 PhAS and 284 NALC slides were used for OSE in six different Indian states (Chhattisgarh, Goa, Gujarat, Pudhucherry, Punjab and Tamil Nadu). Five microbiologists and five senior laboratory technicians at the intermediate reference laboratories, and 70 senior tuberculosis laboratory supervisors and 41 laboratory technicians at the district tuberculosis centres and designated microscopy centres were assessed. Grade and quality of smears (cell count/field, staining, size, evenness and thickness) were recorded as per RNTCP guidelines.[9] The microscopists and supervising team members were blinded to the identity of the PhAS or NALC smears. Almost every set of five slides contained different numbers of both PhAS and NALC slides, except for six sets with only PhAS and three sets with only NALC slides. One hundred smears each of the NALC and PhAS method were read by a post-graduate student who had received training in smear microscopy for 10 days as per RNTCP guidelines.[9] Discrepant results were resolved by the reference reader during OSE. The results reported for the panel slides were considered for statistical analysis.

Statistical analysis

The statistical significance of the observed difference between PhAS and NALC smears was assessed using the χ^2 test. Agreement between reference and reported results was analysed using the kappa (κ) test.

RESULTS

Of the 413 PhAS smears, respectively 109 (26%), 110 (27%), 89 (21%) and 105 (26%) had 2+, 1+, scanty and negative grades, compared to respectively 89 (23%), 103 (27%), 96 (25%) and 96 (25%) of the 384 NALC smears.

A comparison of the results of the PhAS slides and the reference reading is shown in Table 1: the overall agreement was 96% ($\kappa = 0.96$). The overall agreement of the results of NALC slides with the reference reading was 91% ($\kappa = 0.91$, Table 2). There were 37 errors using the NALC method and 21 using the PhAS method (Table 3). The PhAS method had one high false-negative (HFN) compared to five HFN using the NALC method. The post-graduate student had only one HFN result on PhAS and two HFN and one low false-negative (LFN) on NALC smears. This difference was not significant ($P < 0.52$).

Table 4 shows the quality of the smears prepared

Table 1 Comparison of results of panel slides prepared by the PhAS method with the reference reading

Reported results*	Results of reference laboratory*					
	2+	1+	Scanty	Any positive	Negative	Total
3+	15	1	1	17	0	17
2+	67	33	1	101	0	101
1+	26	60	37	123	1	124
Scanty	1	15	40	56	6	62
Any positive	109	109	79	297	7	304
Negative	0	1	10	11	98	109
Total	109	110	89	308	105	413

*3+ = >10 AFB/oil immersion field at least 20 fields; 2+ = 1–10 AFB/oil immersion field at least 50 fields; 1+ = 10–99 AFB/100 oil immersion field; scanty = 1–9 AFB/100 oil immersion fields; negative = no AFB/100 oil immersion fields.
PhAS = phenol ammonium sulphate sediment; AFB = acid-fast bacilli.

Table 2 Comparison of results of panel slides prepared by the NALC method with the reference reading

Reported results*	Results of reference laboratory*					
	2+	1+	Scanty	Any positive	Negative	Total
3+	51	0	0	51	0	51
2+	27	12	1	40	0	40
1+	11	71	17	99	0	99
Scanty	0	15	61	76	14	90
Any positive	89	98	79	266	14	280
Negative	0	5	17	22	82	104
Total	89	103	96	288	96	384

*See Table 1.
NALC = *N*-acetyl-L-cysteine.

Table 3 Errors reported for panel slides prepared using the PhAS and NALC methods

Method	No of errors					
	LFN	HFN	LFP	HFP	QE	Total
PhAS (n = 413)	10	1	6	1	3	21
NALC (n = 384)	17	5	14	0	1	37

PhAS = phenol ammonium sulphate sediment; NALC = *N*-acetyl-L-cysteine; LFN = low false-negative; HFN = high false-negative; LFP = low false-positive; HFP = high false-positive; QE = quantification error.

using the PhAS and NALC methods. The thickness, evenness, staining and size of smears were similar using both methods, and the differences observed were not statistically significant. Three hundred (73%) PhAS smears had >10 cells/field compared to 173 (45%) NALC smears; the difference was significant ($P < 0.001$).

DISCUSSION

NALC is an expensive chemical that does not kill mycobacteria when sputum is processed for the preparation of smears.[5] As the PhAS solution is reported to kill the tubercle bacilli, this may be a less hazardous method.[8] Rutala et al. reported that 3% phenol

Table 4 Quality of panel slides prepared using the PhAS and NALC methods

Quality of smear, remarks	PhAS (n = 413)	NALC (n = 384)	P value
Cell count			
>10/field	300	173	0.001
<10/field	90	190	
Not available	23	21	
Staining			
Good	372	330	0.6
Poor	20	12	
Overstaining	3	4	
Understaining	3	4	
Not available	15	34	
Thickness			
Good	353	314	0.07
Poor	9	14	
Thin	19	29	
Thick	10	3	
Not available	22	24	
Size			
Good	366	333	0.09
Poor	9	5	
Big	11	15	
Small	4	10	
Not available	23	21	
Evenness			
Even	348	324	0.99
Uneven	41	38	
Not available	24	22	

PhAS = phenol ammonium sulphate sediment; NALC = *N*-acetyl-L-cysteine.

destroyed 10^4 *M. tuberculosis* bacilli in 2–3 h.[10] This method can thus be employed for the preparation of large numbers of panel slides without exposing technicians to hazardous aerosols. In addition, the NALC method requires sophisticated instruments such as biosafety cabinets and shakers, whereas with the PhAS method overnight sedimentation of sputum was used.[8] As smears prepared from overnight sediments showed large clumps of AFB, the sputum sediment obtained within 3 h after the addition of the PhAS reagent was used for preparation of panel smears.

The quality of panel slides prepared using both methods was found to be equally good, except for the cell count, which was significantly higher in PhAS smears than in NALC smears (Table 4). While the sputum is diluted with the addition of the reagent in the NALC method, it is partially sedimented using the PhAS method. This partial sedimentation might have contributed to the higher cell count in PhAS smears. In the study, 85% of the PhAS smears were of satisfactory thickness and 90% had good staining, a finding that was similar to the quality reported for artificial sputum smears prepared in a Japanese study.[7] The higher number of false-negative and false-positive errors in the NALC method could be due, respectively, to unequal distribution and insufficient AFB staining or to the presence of artifacts in the smears.

In conclusion, given the good agreement with the reference results (κ = 0.96) and the good quality

smears, PhAS, a non-hazardous procedure, may be explored further for the preparation of panel slides.

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RÉSUMÉ

CONTEXTE : Les méthodes existantes pour la préparation de séries de lames exigent le traitement d'échantillons de crachats à taux élevés de positivité pour les bacilles acido-résistants.

OBJECTIF : Evaluer pour leur utilisation dans les tests de compétence, les séries de lames préparées par la méthode de sédimentation au sulfate d'ammonium phénolé (PhAS) par rapport à celles reposant sur la *N*-acétyl-L-cystéine (NALC).

MÉTHODES : Les échantillons groupés de crachats provenant de cas connus comme ayant des frottis positifs et négatifs ont été séparés en deux groupes. Une partie a été utilisée pour la préparation de séries de lames par la méthode NALC et l'autre partie par la méthode PhAS, une méthode sans risque. On a préparé respectivement 413 et 384 frottis de différents degrés de positivité par les méthodes PhAS et NALC dans trois lots ; 121 microscopistes ont enregistré le degré de positivité et la qualité des frottis à différentes étapes au cours des tests de compétence. On a analysé par le test kappa la concordance entre les résultats de référence et les résultats enregistrés.

RÉSULTATS : Le taux global de concordance a été de 96% pour la méthode PhAS et de 91% pour la méthode NALC. On a noté 37 erreurs pour la méthode NALC en comparaison avec 21 pour la méthode PhAS ($P <$ 0,223). Les frottis ont été d'une qualité égale dans les deux méthodes ; toutefois, le décompte des cellules a été significativement plus élevé pour la méthode PhAS que pour la méthode NALC.

CONCLUSION : La méthode PhAS, une procédure sans risque et produisant des frottis de bonne qualité, peut être explorée davantage en vue de la préparation de séries de lames.

RESUMEN

MARCO DE REFERENCIA: Los métodos actuales de preparación de los paneles de láminas de baciloscopia requiere la manipulación de muestras de esputo con un alto contenido de bacilos acidorresistentes.

OBJETIVO: Se buscó evaluar los conjuntos de láminas preparadas por el método del sedimento con solución fenicada de sulfato de amonio (PhAS) y con *N*-acetilcisteína (NALC) destinadas a las pruebas de competencia.

MÉTODOS: Las mezclas de muestras de esputo con baciloscopia positiva y negativa conocidas se dividieron en dos alícuotas. Una parte se usó en la preparación del panel de láminas por el método NALC y la otra parte se preparó por el método seguro PhAS. Se prepararon cerca de 413 frotis con PhAS y 384 con NALC de diferentes grados de positividad, en tres lotes. Durante las pruebas de competencia, 121 microscopistas en estados diferentes registraron el grado y la calidad de los extendidos. La concordancia entre la referencia y los resultados notificados se analizó mediante el índice κ.

RESULTADOS: La concordancia global fue de 96% con el método PhAS y 91% con el método NALC. Se observaron 37 errores con el método NALC, en comparación con 21 errores con PhAS ($P <$ 0,223). La calidad de las baciloscopias fue igualmente buena con ambos métodos, pero el recuento de bacilos fue más alto con PhAS que con NALC.

CONCLUSIÓN: El método de preparación de frotis para baciloscopia con PhAS es un procedimiento seguro, que ofrece baciloscopias de buena calidad. Se podría investigar más su uso en la preparación de los paneles de láminas para baciloscopia.