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Blinded rechecking of acid-fast bacilli smears by light-emitting diode microscopy

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SUMMARY

BACKGROUND: Blinded rechecking of auramine-stained acid-fast bacilli (AFB) sputum smears using fluorescence microscopy (FM), especially FM using light-emitting diode (LED), is not well understood.

OBJECTIVE: To examine the rechecking of auramine-stained sputum smears without restaining within a month using LED FM.

METHODS: A total of 4799 centrifuged smears of sputum samples were stained by the auramine phenol method and examined using LED FM; 564 systematically selected smears were subjected to blinded rechecking without restaining by controllers. The initial results of the readers were compared to those of the controllers. Discrepancies were resolved by a referee. The quality of LED FM was assessed by the referee using the culture result as gold standard.

RESULTS: Among the rechecked smears, one high false-negative error was made by a reader, while one high false-positive error and 19 high false-negative errors were made by the controllers. The errors were resolved by culture. Smear results for 18 slides were not available due to AFB fading.

CONCLUSION: AFB colour fading using LED FM, which affected the accurate evaluation of blinded rechecking of AFB smears without restaining within a month, is confirmed in this large study.

KEY WORDS: fluorescence microscopy; quality assurance; *M. tuberculosis*; blinded rechecking; LED

ONE OF THE THREE COMPONENTS of external quality assurance (EQA) procedures, the blinded rechecking of basic fuchsin-stained smears of sputum samples, has been successfully implemented in tuberculosis (TB) laboratory networks to facilitate the identification of major problems in laboratories that were performing inadequately.[1–3] However, appropriate rechecking procedures and EQA policies for fluorescence microscopy (FM), especially FM using light-emitting diode (LED) bulbs, are not well developed.[4] Blinded rechecking programmes are a challenge for FM, as laboratory-designed studies have shown that auramine-stained smears fade more rapidly using FM than fuchsin-stained smears in Ziehl-Neelsen microscopy (ZN-M).[5] Moreover, restaining of auramine-stained smears before rechecking increased background fluorescence and led to false-positive errors.[6] Smear restaining can also result in false-negative results, as acid-fast bacilli (AFB) may be washed off when the slides are washed before restaining.[7–9] The present study assesses the rechecking of auramine-stained smears without restaining within 1 month in real-life situations over a 12-month period.

MATERIALS AND METHODS

Study population

A total of 2400 ZN-M smear-positive pulmonary TB patients enrolled in a drug resistance surveillance survey conducted by the National Institute for Research in Tuberculosis (NIRT), Chennai, Tamil Nadu, India, between August 2011 and July 2012, were recruited into the study. Two spot specimens were collected from patients and transported to NIRT for culture and susceptibility studies.

The NIRT Scientific Advisory Committee approved the study; informed consent was obtained from all study patients.

Mycobacterium tuberculosis *culture*

A total of 4799 sputum samples obtained from the survey were transported to the NIRT for mycobacteriological investigation. They were processed using a modified Petroff's procedure and cultured on Löwenstein-Jensen medium per the standard operating procedure (SOP) followed in the laboratory.[10]

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LED fluorescence microscopy

A concentrated smear was made from the processed sediment and stained using 0.3% auramine O (Merck, White House Station, NJ, USA), decolourised and counterstained, using 1% acid alcohol and 0.1% potassium permanganate solution, respectively. Twenty-four batches (two per month) of staining reagents were prepared and used. The quality of each batch of new reagent was checked using control slides (negative and 1+ grade) per India's Revised National Tuberculosis Control Programme (RNTCP) guidelines.[11] The smears were examined by technicians (readers) using an LED microscope (Axioskope 40, Carl Zeiss, Jena, Germany) under 200× magnification (low-power field); AFB were confirmed using 400× magnification (high-power field). Assignment of grades to smears was based on the SOP followed in the laboratory.[10] Briefly, 3+ = ⩾100 AFB in ⩾20 high-power fields (HPF); 2+ = 5–100 AFB/HPF in ⩾50 HPF; 1+ = 4 AFB in 50 HPF or 1–4 AFB/HPF in 50 HPF; negative = no AFB in 50 HPF or <4 AFB in 50 HPF. The slides were stored in slide boxes in a closed cupboard at laboratory temperature (22–25°C).

Blinded rechecking

Each month, 10% of the slides (range 12–68) were selected and supplemented with a few negative slides (range 4–16) prepared from AFB-negative sputum samples for blinded rechecking. The selected slides were relabelled and assigned for blinded rechecking without restaining by the controllers. Discrepant results were resolved immediately by a referee without restaining. The relative sensitivity of the readers and controllers (with respect to the referees' results) was calculated to assess the quality of reading. The quality of the smears was documented by the controllers. The errors were also compared with the culture results to confirm true-positive and -negative smear results.

RESULTS

In the present study, of the 4799 slides examined, respectively 1448, 1886, 1219 and 246 were reported to have 1+, 2+, 3+ and negative grades. Results of the blinded rechecking are summarised in the Table. Of the 564 smears rechecked (463 positive, 101 negative), one high false-negative (HFN) error was reported by the reader and 19 HFN errors were reported by the controllers. All of the HFN results were found to be culture-positive. One high false-positive error was reported by the controller; this sample was also found to be culture-positive. The relative sensitivity of the controllers and readers was 92%.

Of the 38 HFN slides, the results for 18 slides were not available as the AFB was reported to have faded. Fading was observed during the months of November and December. These slides were restained and found to be AFB-positive using FM; the corresponding samples were positive for *M. tuberculosis* using culture.

DISCUSSION

A total of 4799 smears were examined and 564 smears were rechecked in this study. The relative sensitivity of the readers and controllers was 92%, higher than the stipulated sensitivity of 90%. Generally, controllers made more mistakes than readers. Of the 19 HFN errors, 12 were reported by two controllers during the months of September and January. These controllers were subsequently excluded from smear rechecking. All of the 37 HFN errors (including the 18 smears that faded) made by controllers occurred among 1+ grade smears; the corresponding sputum samples were found to be culture-positive. Other studies have also reported that more errors were made by controllers than by readers.[6] Errors made by controllers were seen consecutively in all except the last 3 months,

Table Results of blinded rechecking

Month	Total slides examined	Slides rechecked, *n*			Major errors				Faded slides *n*
					Readers		Controllers		
		Positive (3+, 2+, 1+)*	Negative*	Total	HFP	HFN	HFP	HFN	
August	344	34	16	50	–	–	–	1	–
September	510	51	8	59	–	–	–	4	–
October	610	62	10	72	–	–	–	2	–
November	460	46	11	57	–	–	–	–	12
December	694	68	10	78	–	–	–	–	6
January	589	57	10	67	–	1	–	8	–
February	284	24	7	31	–	–	–	1	–
March	244	22	6	28	–	–	–	2	–
April	176	13	9	22	–	–	1	1	–
May	250	22	5	27	–	–	–	–	–
June	518	52	5	57	–	–	–	–	–
July	120	12	4	16	–	–	–	–	–
Total	4799	463	101	564	–	1	1	19	18

*Positive: 3+ = ⩾100 AFB/HPF in ⩾20 HPF; 2+ = 5–100 AFB/HPF in ⩾50 HPF; 1+ = 4 AFB in 50 HPF or <4 AFB/HPF in ⩾50 HPF; negative = no AFB in 50 HPF or <4 AFB in 50 HPF.
HFP = high false-positive; HFN = high false-negative; AFB = acid-fast bacilli; HPF = high-power field.

indicating that controllers had difficulty reading smears, although the errors had to be resolved by the referee by extended smear reading to confirm the errors.

Of the 564 slides assigned for rechecking, AFB fading was observed on 18 (3.2%) during the months of November and December; 546 (96.8%) were readable. There were no other observations of AFB fading during the study period. The October–December quarter comprises the monsoon season in the study area, and AFB may have lost colour due to high seasonal humidity, making it difficult for the controllers to read the smears. It is well known that environmental conditions can cause AFB fading.[5] In the study by Minion et al., 21% of the smears faded when the slides were stored at room temperature for 4 weeks.[5] The reduced number of positives in their study could be attributed to the selection of larger numbers of low-grade smears. Minion et al. also mentioned the limitations of microscopy, apart from AFB fading, in reading low-grade smears which might have contributed to the enhanced loss of positivity. Yet another inherent bias in that study was that an additional smear was made from each specimen and smear results compared with the initial results were reported under routine conditions. Repeated exposure of the slides to the LED lamp may also have resulted in faster AFB fading.[5,12] In the present study, we selected greater proportions of 3+ (n = 117) and 2+ (n = 180) smears for rechecking. This could be attributed to the concentrated centrifuged deposit smears made from the smear-positive sputum samples. Moreover, it has already been demonstrated that AFB in high-grade smears retain their colour for longer than low-grade smears and take longer to fade completely.[5]

Another limitation of the present study was the non-inclusion of smear-negative X-ray-positive patients. Only 22 negative slides were selected from the study samples. This limits the quality assessment of smear examination. Nonetheless, only one smear among these slides was picked up as positive by the controller, and was subsequently confirmed to be positive by the referee. According to the records, it was a low-grade smear (1+ = 6 AFB), with an uneven distribution of AFB; the sample was positive for culture.

In many settings that have implemented ZN-M, particularly India's RNTCP, rechecking of slides is carried out within 1 month.[11,13,14] To simulate this setting, the slides in this study were stored and rechecked within a month to examine the rechecking procedures for LED FM. Nevertheless, the following conditions might have favoured the blinded rechecking procedure for LED FM: 1) all the slides were stacked in slide boxes and kept in a closed cupboard at stable laboratory temperatures (22–25°C), and 2) the slides were not transported, but were re-examined within a month. As the controllers and referee were present at the same site, there was no need for the controller to restain the smear to resolve discrepancies. Such favourable conditions may not be available at microscopy centres in peripheral laboratories, and the performance of blinded rechecking procedures using LED FM remains to be studied in large-scale field studies.

CONCLUSION

Our study reconfirms that AFB stains using LED FM are likely to fade, especially in conditions of high humidity such as during the monsoon season, and this may affect the accurate assessment of blinded AFB smear rechecking without restaining.

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RÉSUMÉ

CONTEXTE : On ne connait pas bien les résultats d'un recontrôle aveugle des frottis à la recherche des bacilles acido-résistants (BAAR) lorsqu'il s'agit de frottis de crachats colorés à l'auramine et examinés par microscopie à fluorescence (FM), surtout lorsqu'on utilise des lampes du type diode émettrices de lumière (LED).

OBJECTIF : S'informer sur le recontrôle dans le premier mois par LED FM des frottis de crachats colorés à l'auramine sans recoloration.

MÉTHODES : On a coloré par la méthode auramine-phénol et examiné par LED FM 4799 frottis de culots de crachats centrifugés. Au total, 564 frottis sélectionnés de manière systématique ont été soumis sans recoloration à un recontrôle aveugle par les contrôleurs. Les résultats originaux des lecteurs ont été comparés avec ceux des contrôleurs. Les discordances ont été résolues par un arbitre. La qualité du LED FM a été évaluée sur base d'un standard reposant sur l'arbitre et les résultats des cultures.

RÉSULTATS : Parmi les frottis recontrôlés, on a constaté une erreur grave faussement négative d'un lecteur, et une erreur grave faussement positive et 19 erreurs graves faussement négatives chez les contrôleurs. Les erreurs ont été vérifiées par culture. Les résultats des frottis n'ont pas été disponibles pour 18 lames en raison de la décoloration des BAAR.

CONCLUSION : La décoloration des BAAR par LED FM qui a affecté l'évaluation précise d'un recontrôle aveugle des frottis BAAR sans recoloration dans le mois est confirmée dans cette grande étude.

RESUMEN

MARCO DE REFERENCIA: La relectura anónima de las baciloscopias de esputo (BAAR) con tinción fluorescente de auramina en microscopios de fluorescencia (FM) es muy poco utilizada, sobre todo con los microscopios que incorporan la tecnología lumínica LED.

OBJETIVO: Obtener mayor información sobre procedimiento de relectura de las baciloscopias de esputo teñidas con auramina, en el lapso de 1 mes, sin nueva tinción y mediante un FM que utiliza la tecnología LED.

MÉTODOS: Se examinaron 4799 frotis de muestras de esputo teñidas por el método de fenol auramina, mediante un LED FM. Se escogieron de manera sistemática 564 baciloscopias para relectura por los controladores, sin practicar una nueva coloración. Se compararon los resultados originales de los lectores con los resultados de los controladores. Un árbitro resolvió las discrepancias que se presentaron. La evaluación de la calidad de la FM se basó en la opinión del árbitro y los resultados de los cultivos como criterios de referencia.

RESULTADOS: En la relectura se notificó un error alto negativo falso en un lector y un error alto positivo falso y 19 errores altos negativos falsos notificados por controladores. Los errores se resolvieron según los resultados de los cultivos. No se pudieron obtener resultados de relectura en 18 baciloscopias, debido a la decoloración de la tinción de los BAAR.

CONCLUSIÓN: En un amplio estudio se confirmó de nuevo la atenuación de la intensidad de la tinción de los BAAR por el LED FM, la cual obstaculizó una evaluación precisa en la relectura con enmascaramiento de los extendidos de BAAR en el lapso de 1 mes, sin practicar una nueva tinción.