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Comparison of two LED fluorescence microscopy build-on modules for acid-fast smear microscopy

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SUMMARY

SETTING: National Reference Laboratory, Benin.

OBJECTIVES: To compare the performance of Fraen FluoLED™ and LW Lumin™ light-emitting diode (LED) fluorescence microscopy modules.

DESIGN: Acid-fast bacilli (AFB) smears, routinely examined with a classical fluorescence microscope, were blindly re-read with both LED systems at 200× magnification. Smears with discordant results were rechecked on all systems at 200×, and 100 randomly chosen smears were read again at 400×. Confirmed presence of AFB with any system was accepted as a true positive.

RESULTS: A total of 1937 smears were examined by all systems. The Fraen and LW detected 895 (46.2%) and 817 (42.2%) positive and scanty positive smears. After rechecking 201 smears, 15 false-positive and 61 false-negative results were declared for Fraen, against 11 and 135 for LW. The systems had similar false-positive rates (1.7% for Fraen and 1.4% for LW), but differed significantly regarding detection of confirmed microscopy positives (93.5% and 85.6% respectively, $P < 0.00001$). A high correlation between both LED systems was found at 400× magnification.

CONCLUSIONS: The Fraen LED fluorescence microscopy module performed significantly better than the LW LED at the most efficient 200× magnification. It was also more appreciated by all users. The LW module may perform equally well at higher magnification.

KEY WORDS: tuberculosis; light-emitting diode; fluorescence microscopy; auramine; acid-fast bacilli

SPUTUM SMEAR MICROSCOPY for acid-fast bacilli (AFB) remains the most cost-efficient tool available to diagnose tuberculosis (TB) in resource-limited high-burden settings. Many studies have documented increased sensitivity and superior diagnostic performance of fluorescence microscopy (FM) after auramine staining compared with bright field microscopy after Ziehl-Neelsen (ZN) staining.[1] However, routine use of FM is seriously restricted by several factors, such as the short life and high cost of the mercury vapour short arc lamps (HBO) in the classical systems, difficult maintenance and lamp adjustments, the need for a dark room, and strict requirements for electrical power supply. Light-emitting diode lamps (LED) do not have these disadvantages. Moreover, because of the very narrow excitation wavelength band, these FM systems require fewer filters and consume very little power, so that they can run on batteries. The LED life expectancy is said to be 50 000–100 000 h, or 10–20 years of use, and contrary to HBO lamps they do not explode after excessive usage.[2]

The few reports on LED microscopy for TB published so far show that results in terms of agreement or sensitivity and specificity are comparable or even better when the HBO lamp of a classical FM system is replaced by a LED lamp.[3,4]

Several types of LED systems for TB FM are already available on the market. Two modules are meant for conversion of ordinary microscopes. One uses transmitted LED light (FluoLED Easy™, Fraen Corporation Srl, Settimo Milanese, Italy), and the other one epi-fluorescence (Lumin™, LW Scientific, Lawrenceville, GA, USA).

The objective of our study was to compare the performance of these two modules, mounted on the same simple brightfield microscope and used by experienced technicians in a reference laboratory.

METHODS

This study was conducted at the Laboratoire de Référence des Mycobactéries (LRM), Cotonou, Benin. During the study period, all positive sputum smears from the LRM's routine and every negative smear immediately following a positive one were included in the study for rereading with both LED systems, while the remaining negatives were discarded. These smears had been stained with auramine O and counterstained

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with potassium permanganate following international guidelines.[5] Routine readings covered one length of the smears, using a Leica DM 1000 fluorescence microscope (epi-fluorescence, HBO 50 W lamp, Leica Microsystems GmbH, Wetzlar, Germany) at 200× magnification (10× eyepieces and 20× objective). Rereading of the study smears with both LED systems was performed on the same day. To make blinded rereading possible, the original identification numbers were covered and replaced by codes only known to the supervisor. Moreover, the different readers recorded their results in separate notebooks which were kept by the supervisor when not in use.

The LED modules were mounted on an Olympus CX21 microscope, a type commonly used in low-income country peripheral laboratories. The same 200× magnification (10× eyepieces and 20× objective) and reading of one length were applied as for the HBO routine. Three different experienced technicians examined the same smears with a different FM system. To eliminate reader bias, the technicians alternated using each system (the two LEDs or the HBO fluorescence microscope), and the supervisor ensured that by the end of the study each technician had read about the same number of smears with each of the three systems.

After analysis of the first results, 100 randomly selected study smears were restained with the auramine procedure, as described above, and reread at 400× magnification with the three systems by the supervisor.

Readings for LED modules were performed in a dimly lit room, while the HBO fluorescence microscopy required a more darkened room. Quantification was applied using scales adapted to the lower magnification used (200× or 400×), as shown in Table 1 and recommended by the World Health Organization (WHO).[6]

The smears with discordant results (not all three results were positive or negative or there was a difference of quantification of at least two grades in the scale for at least one smear) were rechecked by a supervisor on each of the three microscopes. Rechecking was performed either on the same day as the first reading or on the following day, keeping the smears refrigerated in the dark to prevent fading and to avoid having to restain. For this reading, three lengths were examined. As none of the systems could a priori be considered to represent the gold standard, the confirmation of presence of AFB with any system was considered to represent a true microscopy-positive result, and their complete absence after rechecking with all systems as a microscopy negative. Errors could thus be allocated to each of the systems for comparison of their performance.

The results were entered in a database and analysed using Epi Info 6.04d (Centers for Disease Control and Prevention, Atlanta, GA, USA), with double data entry and validation. The McNemar χ^2 test was used for comparison of proportions, and the kappa (κ) agreement rate was calculated. However, as only a small part of the routine HBO microscope declared negatives were included in the study, as opposed to all its positives, the LED systems could not possibly detect all the HBO false-negatives. Because of this limitation of the study design, only the performance of the two LEDs was compared.

RESULTS

Of 2009 eligible smears, 72 predominantly positives and scanty positives had to be excluded because of restaining by ZN before all tests could be completed. This left 1937 smears with valid FM results on all three systems, of which 815 (42.1%) had been declared positive and 117 (6.0%) scanty positive by routine HBO reading. Table 2 shows the crude LED results before rechecking of discordant smears. More positive and scanty smears were reported with Fraen (895, 46.2%, 95% confidence interval [CI] 44.0–48.5) than with LW (817, 42.2%, 95%CI 40.0–44.4).

Ten times more positives (1+, 2+, 3+) were detected with Fraen, but not with LW, compared to the number detected only with LW (n = 50 vs. 5). When scanty smears were analysed, 67 of the 116 (57.9%, 95%CI 48.8–66.7) detected by Fraen were negative by LW, while 34 of the 117 (29.1%, 95%CI 20.8–37.3) detected by LW were negative by Fraen.

Of the 1937 smears included in the study, 201 yielded discordant results and were blindly rechecked, resulting in 169 positive smears in at least one vs. 32 negative smears in all systems. Table 3 shows an evaluation after allocating errors as per the final

Table 1 Quantification scale used at 200× and 400× magnification

Result	200× magnification	400× magnification
Negative	0 AFB/1 length	0 AFB/1 length
Scanty	1–29 AFB/1 length	1–19 AFB/1 length
1+	30–299 AFB/1 length	20–199 AFB/1 length
2+	10–100 AFB/1 field	5–50 AFB/1 field
3+	>100 AFB/1 field	>50 AFB/1 field

AFB = acid-fast bacilli.

Table 2 Cross-tabulation of Fraen and LW LED systems crude results

	LW			
Fraen	Negative *n*	Positive *n*	Scanty *n*	Total *n*
Negative	1003	5	34	1042
Positive	50	680	49	779
Scanty	67	15	34	116
Total	1120	700	117	1937

LED = light-emitting diode.

Table 3 Evaluation of Fraen and LW LED systems, using microscopy positivity confirmed by rechecking in any system ($n = 941$) as the gold standard

System	True-positive n	False-positive *n*	True-negative *n*	False-negative *n*	Yield of true-positives % (95%CI)	False-positives % (95%CI)
Fraen	880	15	981	61	93.5 (91.9–95.1)	1.7 (0.9–2.8)
LW	806	11	985	135	85.6 (83.4–87.9)	1.4 (0.7–2.4)

LED = light-emitting diode; CI = confidence interval.

results determined by blinded rechecking, and Table 4 shows the comparison of the presumed true and false results in more detail. Overall positives in any system, including the confirmed HBO positives not shown in this table, reached 941. False-positive results numbered 15 and 11, and false-negative results 61 and 135 for Fraen and LW, respectively. Fraen detected significantly more true microscopy positives (93.5%) than LW (85.6%, $P < 0.00001$), while their false-positive rates were very similar (1.7% vs. 1.4%). The overall agreement between the two systems was high ($\kappa = 0.84$).

Among the 1005 HBO negatives, 25 (2.5%, 95%CI 1.7–3.7) were confirmed false-negatives after reading with the LED systems and resolving discordant results (data not shown).

The second reading of 100 randomly selected study smears, using 400× magnification, yielded 40 positive and scanty results for both Fraen and LW. Of 73 smears with a concordant result at 200× (36 negative, 37 positive or scanty), 65 were concordant also at 400×, and 19 were discordant at 200× but concordant at 400×. Only 16 smears yielded discordant results at 400×, of which exactly half were scanty or positive with either Fraen or LW.

DISCUSSION

Compared to the classical HBO types, LED fluorescent microscopes are more user-friendly and more appropriate to conditions in low-income countries, which may be even more important than the obvious advantages of lower initial as well as recurrent costs and low demands on infrastructure. They do not require warm-up and cool-down times, a considerable advantage when power supply is erratic, and the absence of ultraviolet light makes the systems safer. Furthermore, the epi-fluorescence system always used with HBO lamps may have contributed to the unpopularity of FM. It often creates an extremely dark field of view, with superb AFB contrast after correct focusing, but focusing and keeping focus can be very challenging. With transmitted light FM systems, the AFB contrast is less distinct but the background remains clearly visible and focus is easily made and maintained.

Table 4 Fraen and LW LED system performance, Fraen/LW resolved results

TN/TN	TP/TP	TN/FP	TP/FN	FN/FN	FN/TP	FP/FP	FP/TN
971	777	10	103	32	29	1	14

LED = light-emitting diode; TP = true-positive results; FP = false-positive results; TN = true-negative results; FN = false-negative results.

LED systems developed hitherto for AFB smears consist of either modules that can be fitted to a conventional microscope, such as those evaluated in our study, or a complete microscope with built-in LED as the light source. Partec (Münster, Germany) and Cytoscience (Fontaines, Switzerland) have developed complete LED FM microscopes. However, these microscopes seem less appropriate for TB since they are monoculars, causing excessive eye strain when more than just a few smears have to be read. Using a camera and monitor might be a solution in affluent countries.[7] A binocular FM microscope with built-in LED lamp for epi-fluorescence was recently marketed (Primo Star iLED™, Carl Zeiss, Oberkochen, Germany), which has given very good results in reference laboratories.[8] Other renowned microscope manufacturers are also now developing LED FM microscopes.

Our study is the first direct comparison of the two build-on LED modules on the market. So far very few reports on these systems exist, showing their excellent performance compared to conventional HBO FM, or increased detection compared to ZN microscopy.[9,10] The Lumin™ kit includes an objective with integrated filter cube and LED illuminator as well as a universal power supply. The kit is easily attached to a standard microscope by exchanging an objective, resulting in an epi-fluorescence FM system. The LED add-on kits produced by Fraen have been designed for different common types of bright field microscopes, and consist of several parts. As the LED light source is attached to the microscope base for use with transmitted light, the barrier filter has to be inserted separately below the head of the microscope. The complete installation is not difficult, but it requires slightly more time and care. On the other hand, contrary to the LW, it is easy to switch to a higher power objective, e.g., for confirmation of doubtful AFB. A safety device has now been built into the barrier filter changer, switching off the blue lamp when the correct filter is not in place, such as when changing to conventional brightfield microscopy. All technicians rated the Fraen module more favourably because of the

quality of the image and ease of focusing, which can be explained by the transmitted light system used. As acceptance by inexperienced microscopists seems to be the main obstacle to use of FM outside referral laboratories, this may prove to be a major advantage of transmitted LED light FM, as reported earlier from Tanzania for the Fraen module.[9] It also remains to be seen whether complete binocular LED microscopes using epi-fluorescence rather than transmitted light will meet with the same acceptance level with the progressive decentralisation of FM to peripheral hospitals and health centres.

Our study was conducted in a reference laboratory setting, where HBO FM has been in routine use for many years, and the technicians are highly experienced with the technique.[11] Smears from the laboratory's routine were selected for blinded rereading with both LED systems, taking care to avoid observer bias by ensuring that each technician read an equal number of smears on the different systems. Fading and the need for restaining were avoided by fast processing and appropriate storage precautions. We chose a low 200× magnification as it results in a large field of view, permitting fast processing of the smears while the AFB remain clearly visible, at least in the classical FM systems. Kubica has reported that scanning at this magnification results in the highest sensitivity and efficiency, with preserved high specificity, provided that presence of rare AFB is confirmed at a higher magnification.[12] A good LED system should therefore also meet these requirements for optimal use. Culture could not be used as a gold standard, as our successive positive smears included many treatment follow-ups, known to yield a high proportion of smear-positive/culture-negative results.[13] The harsh Petroff decontamination technique routinely used would have added more culture false-negatives and thus seemingly FM false-positives, particularly among the paucibacillary diagnostic specimens, the most sensitive group to reveal differences in yield of microscopy systems. Instead, we considered all results concordant with the three systems as true. In case of discordance between the first readings, the true result was considered to be positive if a supervisor confirmed the presence of AFB with at least one system after rechecking using all three systems. The true result was considered as negative only if none of the systems detected AFB on rechecking. Using a consensus result as the gold standard would have resulted in false declaration of smears with very few AFB as false-positives, due to the inherent low reproducibility of microscopy in this group.

Our results showed a significantly higher yield of microscopy positives with the Fraen than with the LW system (93.5% vs. 85.6%), for almost the same false-positive rates. The superior AFB detection capacity of Fraen LED was apparent also from the quantified results. While virtually the same number of scanty smears was found with the LW, more clear positives were reported with Fraen, suggesting a shift towards higher quantifications.

Considering these surprising results, we rechecked a sample of the study smears with both systems using a replacement LW module, for fear that the first was damaged during transport, and taking utmost care in its correct installation. The results did not improve. As the LW was originally designed for the 400× magnification, we then reread another sample at this magnification with both LED microscopes, which now resulted in an equal number of positives detected by either system.

The impossibility of comparing the performance of the LEDs and HBO is a limitation of our study. To arrive at a more precise comparison of microscopy positive detection by the two LEDs, all the positives but only about 1/5 negatives detected in routine work with an HBO fluorescence microscope were included. In this small fraction of HBO negatives, a few per cent false-negatives were detected by the LEDs and confirmed by resolving discordances between the three systems, and it seems reasonable to assume that more HBO false-negatives would have been detected if all of the negatives had been reread by the LEDs. On the other hand, all of the LED false-negatives could be identified, as all smears positive by either one of the LEDs or the HBO had been read also by the other LED, and missing a positive detected by another system would have resulted in a false-negative. Because of these different challenges, comparison of detection capacity was thus only possible between the LEDs, but not between a LED and the HBO.

CONCLUSION

In our study, the Fraen FluoLED Easy™ build-on FM module performed at least as well as the conventional HBO FM microscope. At the most efficient 200× magnification it was significantly better than the LW Lumin™ LED, which may reach the same performance level only at 400× magnification. The Fraen module also excelled in terms of user-friendliness and user-acceptance.

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RÉSUMÉ

CONTEXTE : Laboratoire National de Référence au Bénin.

OBJECTIFS : Comparer les performances des modules de microscopes à fluorescence à diodes émettrices de lumière LED (Fraen FluoLED™ et LW Lumin™).

SCHÉMA : Des frottis comportant des bacilles acido-résistants (BAAR), examinés en routine par un microscope à fluorescence classique, ont fait l'objet d'une relecture aveugle avec les deux systèmes LED à un grossissement de 200×. En cas de résultats discordants, les frottis ont été recontrolés avec tous les systèmes à un grossissement de 200× et 100 lames choisies au hasard ont été relues à 400×. On a considéré comme « vrais positifs » les frottis où la présence de BAAR était confirmée par n'importe lequel des systèmes.

RÉSULTATS : On a examiné 937 frottis par tous les systèmes. Les système Fraen et LW ont détecté respectivement 895 (46,2%) et 817 (42,2%) frottis positifs ou très légèrement positifs. Après recontrôle de 201 frottis, avec Fraen on a considéré 15 résultats comme faux positifs et 61 comme faux négatifs, alors qu'avec LW il y avait 11 faux positifs et 135 faux négatifs. Les taux de faux positifs sont similaires avec les deux systèmes (1,7% pour Fraen et 1,4% pour LW), mais ces systèmes donnent des résultats significativement différents en matière d'examen microscopique confirmés comme positifs (respectivement 93,5% et 85,6%, $P < 0{,}00001$). Au grossissement 400×, on a trouvé une forte corrélation entre les deux systèmes LED.

CONCLUSIONS : Le module de microscope à fluorescence LED de type Fraen a des performances significativement supérieures à celles du système LED de type LW au grossissement le plus efficient, c'est à dire 200×. Il a été également plus apprécié par tous les utilisateurs. Avec un grossissement plus important, le module LW peut avoir des performances égales.

RESUMEN

MARCO DE REFERENCIA: El laboratorio nacional de referencia de tuberculosis en Benín.

OBJETIVOS: Comparar el rendimiento de dos módulos de fluorescencia con sistemas de iluminación basados en diodos emisores luz: el Fraen FluoLED™ y el LW Lumin™.

MÉTODOS: Se practicó una relectura anónima de las baciloscopias examinadas en forma sistemática con un microscopio convencional de fluorescencia, utilizando ambos módulos de iluminación, con un objetivo 200×. Los frotis con resultados discordantes se verificaron de nuevo con todos los sistemas con un aumento de 200× y se escogieron aleatoriamente 100 de ellas, a fin de leerlas de nuevo en 400×. La confirmación de la presencia de bacilos acidorresistentes con cualquiera de los sistemas se aceptó como un resultado positivo verdadero.

RESULTADOS: Se examinaron 1937 frotis con todos los sistemas. Los módulos Fraen y LW detectaron 895 (46,2%) baciloscopias positivas y 817 (42,2%) baciloscopias positivas con escasos bacilos. Tras una nueva verificación de 201 baciloscopias, se detectaron 15 positivos falsos y 61 negativos falsos con el módulo Fraen y 11 positivos falsos y 135 negativos falsos con el módulo LW. Ambos sistemas ofrecieron una tasa equivalente de positivos falsos (1,68% con Fraen y 1,35% con LW), pero difirieron en forma significativa con respecto a la detección de casos positivos confirmados (93,5% con Fraen y 85,6% con LW; $P < 0{,}00001$). Se observó una alta correlación entre ambos sistemas en la lectura con el objetivo 400×.

CONCLUSIÓN: El modulo de fluorescencia con diodos luminosos Fraen ofreció un rendimiento significativamente superior al módulo LW en la lectura más eficiente con el objetivo 200× y también fue el mejor apreciado por todos los usuarios. Es posible que el módulo LW tenga un rendimiento equivalente en lecturas con un mayor aumento.