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Performance and acceptability of the FluoLED Easy™ module for tuberculosis fluorescence microscopy

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SUMMARY

SETTING: Tuberculosis (TB) reference laboratory in Bangkok, Thailand, and two health centres in Dar es Salaam, Tanzania.

OBJECTIVES: To assess the performance and user-friendliness of a light-emitting diode (LED) module (FluoLED Easy™) for TB fluorescence microscopy (FM).

DESIGN: Equivalence study vs. conventional FM in Bangkok using blinded re-reading; routine detection in the health centres in Dar es Salaam compared to Ziehl-Neelsen (ZN) over 2 years, with rechecking of FM smears.

RESULTS: For 461 smears re-read, 99.1% concordance with conventional FM was obtained. FluoLED introduction caused a lasting increase in detection in the routine of each of the health centres by on average 20%. Blinded rechecking failed due to unreliable registration. Onsite rechecking of a convenience sample showed absence of false-positive results in one centre and confusion with artefacts that could have been avoided by more training in the other. LED FM was highly appreciated, with both laboratories refusing to revert to ZN as originally intended.

CONCLUSIONS: A simple microscope with a FluoLED module can yield results equivalent to those of conventional FM. Low cost, technical appropriateness and excellent acceptance justify its use in low-income settings, contrary to classical systems. LED FM can lead to increased sensitivity, but for optimal yield good training and quality assurance remain essential requirements.

KEY WORDS: microscopy; Ziehl-Neelsen; auramine; fluorescence; tuberculosis

THERE IS AN URGENT NEED to increase detection of tuberculosis (TB) in high-burden countries, particularly those with a high prevalence of human immunodeficiency virus (HIV). Microscopy remains the most appropriate method in these settings, but it suffers from low sensitivity for paucibacillary samples, found more frequently among immunocompromised patients co-infected with HIV.[1] Moreover, in many such settings the number of suspects coming for examination has tripled, quadrupled or increased even more. This seriously jeopardises the quality of Ziehl-Neelsen (ZN) microscopy, further reducing its yield.[2]

Fluorescence microscopy (FM) would be a highly desirable solution, offering higher sensitivity for low numbers of acid-fast bacilli (AFB), as it allows high quality work for large numbers of specimens.[3] The high costs of the equipment and mercury vapour (HBO) lamps are often thought of as the reason for the scarcity of its use in low-income countries. However, in practice the obstacles seem to be far more diverse.[2] Fluorescence microscopes are actually often available in these countries, but they are frequently not used for long. Some of the reasons for non-use are:

- the need for a stable electricity supply
- a short lifespan and complexity of lamp installation and replacement
- the delicate system can often not be properly maintained or repaired locally
- the emission spectrum extends into ultraviolet, causing fear of blindness, sterility and other perceived hazards
- an intense dislike for working in isolation in a dark, often hot room
- focusing problems with the standard permanganate counterstaining (rendering the background very dark)
- lack of familiarity with the technique, which requires a longer period of initiation.

Light emitting diode (LED) lamps for fluorescence microscopy have recently been introduced. Preliminary evaluations when used in a modified HBO epi-fluorescence microscope have been very favourable.[4] Because of their very narrow spectrum, extremely low power consumption and an extraordinarily long lifespan, they might be able to offer a solution to many

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of the problems described above. We tested the first commercial build-on modules produced by the company Fraen (Fraen Corporation Srl, Settimo Milanese, Italy) for some common types of microscopes.

MATERIALS AND METHODS

For staining, 0.1% auramine-phenol for a minimum of 15 min was used, followed by destaining with 1% HCl in alcohol for 2 min and 0.1% potassium permanganate background quenching for 1 min. One 2 cm length of the smears was screened with both systems at 200× magnification, and doubtful findings were examined at higher magnification. The quantification followed the scale used by the International Union Against Tuberculosis and Lung Disease (The Union) and the World Health Organization (WHO), adjusting for the larger field size.[2]

Equivalence study at a reference laboratory

The Bangkok National Tuberculosis Reference Laboratory Centre evaluated the FluoLED Easy™ module with a Royal Blue LED lamp, an LP510 barrier filter and a special condenser from the kit, mounted on an Olympus CX 31 microscope (Olympus, Tokyo, Japan). One hundred positive (or with scanty AFB) auramine-stained smears obtained successively from routine work with a classic Olympus BX51 (HBO 100 watt) microscope were mixed with a multifold of randomly selected routine negative smears for blinded re-reading with the FluoLED. Two technicians alternated using either system.

Performance and acceptance in field laboratories

This evaluation focused on the appropriateness of the use of FluoLED in the periphery in low-income settings, making a historical comparison of yield and acceptance with ZN microscopy. The same FluoLED modules were used on an Olympus CX31 or CX21 microscope at two health centres in Dar es Salaam, Tanzania. The CX21 microscope did not need a special condenser. Temeke and Mwananyamala laboratories were chosen for their high workload, with an estimated HIV prevalence of around 50% among their TB patients. Classic HBO fluorescence systems had been introduced a few years earlier, but their use was quickly abandoned.

After only 4 days of training, the technicians changed from ZN to LED FM for all their routine work in March 2007. The protocol stipulated returning to ZN after 3 months of FM to allow unbiased comparison, but the technicians in both centres refused to do so. To prevent the impact of seasonal changes on the yield, the data collection period was thus extended to 2 full years.

During their first months of LED FM, Tanzania Central TB Reference Laboratory (CTRL) staff visited the laboratories monthly for supervision and data collection and to obtain a random selection of smears for rechecking (low positives and 20 negatives per month). The protocol required rechecking of these smears at the CTRL after restaining using an HBO epifluorescence microscope. However, the study protocol was not fully adhered to and rechecking by CTRL was done only for FM smears during onsite visits, using the LED microscopes. For this reason a random sample was also rechecked at the Supranational Reference Laboratory (SRL) in Antwerp, Belgium, using a 50w HBO Leitz Laborlux epifluorescence microscope (Leitz, Wetzlar, Germany). This control was done after systematic restaining with auramine, and it included a second control on smears with discordant results. To better understand these results, another onsite control on all positive smears of that week was finally carried out by the principal investigator without prior restaining and using the FluoLED equipment.

RESULTS

Equivalence study

The equivalence study gave a concordance of 99.1% of the results among 461 smears (Table 1). Defining the HBO results as the gold standard, one scanty positive and one 1+ out of 100 AFB-positives were missed, while 2/361 negatives were falsely reported as scanty or 1+ with the LED. There were 49 low positives (scanty or 1+) with the LED compared to 44/100 positive or scanty results with the HBO system.

Field performance and acceptability

The Figure shows the workload and evolution of yield of positive results over the extended study period of 2 successive years. Temeke laboratory processed a monthly average of respectively 1203 and 1540 smears during the ZN and FM periods, with on average 10% and 12% positives. For Mwananyamala, these averages were 1547 (13% positives) and 1392 (16% positives). FM yielded on average 2.0% more positives at Temeke and 2.8% at Mananayamala, or proportionally about 20% over baseline ZN positivity rates in both centres (data not shown). Considerable fluctuations were observed with either system, possibly related

Table 1 FluoLED versus BX51 HBO fluorescence AFB microscopy results*

	BX 51					
FluoLED	Negative	Scanty	1+	2+	3+	Total
Negative	359	1	1	0	0	361
Scanty	1	1	5	0	0	7
1+	1	0	35	4	2	42
2+	0	0	1	9	20	30
3+	0	0	0	1	20	21
Total	361	2	42	14	42	461

*Scanty, 1+, 2+, 3+ refer to The Union/WHO quantification scale for AFB microscopy,[2] adapted to the size of the field.
HBO = mercury vapour; AFB = acid-fast bacilli.

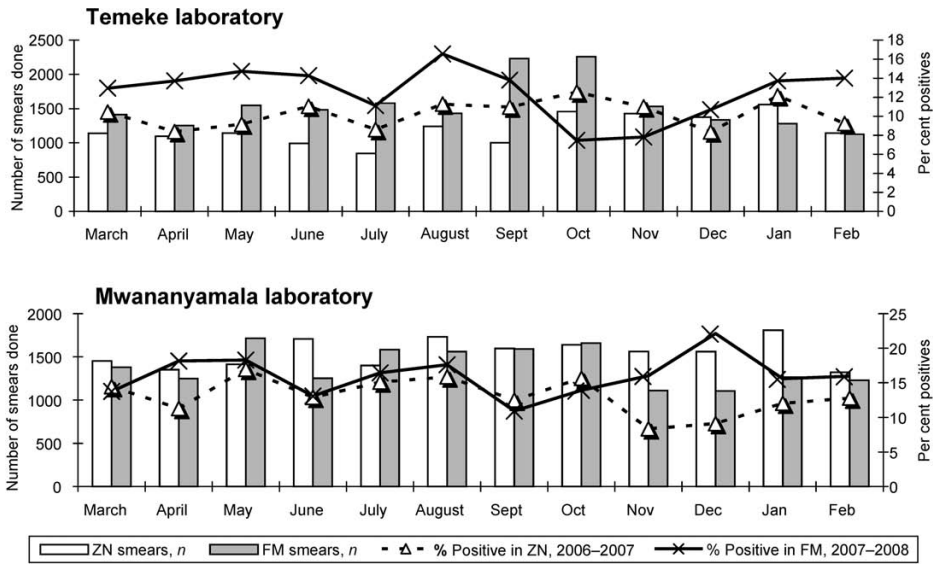

Figure Two-year trends of proportion of positives detected by LED FM vs. ZN in Dar es Salaam health centres. LED = light-emitting-diode lamp; FM = fluorescence microscopy; ZN = Ziehl-Neelsen.

to workload or change of staff, but the Figure shows no evidence for seasonally dependent variations.

Table 2 summarises the results of rechecking at the SRL in Antwerp, classifying errors as defined in the global guidelines.[5] High false-positive errors (HFP) were 21% at Temeke and 7% at Mwananyamala. With two low false-positives each, about half of the scanty results could not be confirmed for either laboratory. False-negatives reached 13% and 20%, with mainly high false-negatives (11%) at Temeke vs. mainly low false-negatives (16%) at Mwananyamala. These results are in stark contrast to those reported from onsite rechecking by CTRL technicians, who reported errors only exceptionally (details not shown). Onsite rechecking by the principal investigator showed no false-positive errors at Temeke (all 20 recent positive slides were rechecked), and four HFP at Mananyamala (among all 24 recent positive slides and two available negatives). Further investigation revealed unreliable recording (serious transcription errors in the registers, complete absence of the required column identification on the slides, excessively homogeneous results for positive cases). These administrative errors were compounded by the confusion with artefacts by an inexperienced technician at Mwananyamala. These artefacts posed no difficulties to the investigator nor to another experienced technician, using the centre's LED fluorescence microscope.

Table 2 Results of rechecking of Tanzania smears

	Temeke laboratory *n* (%)	Mwananyamala laboratory *n* (%)
Smears rechecked		
Positive	47	15
Scanty	4	5
Negative	47	49
Errors detected		
High false-positive	10 (21)	1 (7)
Low false-positive	2 (50)	2 (40)
High false-negative	5 (11)	2 (4)
Low false-negative	1 (2)	8 (16)

DISCUSSION

The first part of our study showed that the performance of the Fraen LED transmitted light fluorescence system, mounted on a simple routine microscope, is very similar to that of a high-quality conventional HBO epifluorescence microscope, at least in expert hands. Slightly fewer AFB were visible, but the overall result was virtually identical, even when considering the HBO as the gold standard, and without further attempts at resolving discordances.

Results of the second part, when we evaluated the FluoLED under routine health centre conditions, convincingly demonstrated user acceptance to the extent that the cross-over design of the study protocol could not be carried out because of the new preference of the technicians. The fact that the technicians refused to revert to ZN, herein supported by their superiors and the responsible medical staff, provides the most convincing proof of its acceptance. Most technicians commented on the pleasant, not too dark background (possibly thanks to the use of transmitted light), while sufficient contrast remains, obviating the need for a dark room. The instruments were thus used on the regular bench, just closing a window curtain, and the technicians never felt isolated. The absence of ultraviolet (UV) radiation may also have played a role in acceptance of the technique, as fear of UV light is frequently mentioned in Tanzania.

Because reverting back to the ZN technique was no longer feasible, the evidence for the increased yield with LED FM must remain less certain. The duration of evaluation was considerably extended to demonstrate the increased detection of cases in comparison to historical controls. No evidence of seasonal variation was seen over the 2 full years, strongly suggesting a true increase in the yield, in the order of 20%. This is indeed plausible, as these are extraordinarily busy laboratories with only one technician responsible for all AFB microscopy, and with often difficult specimens from suspects with a high HIV prevalence. The gross fluctuations observed may at least in part be due to changes in technicians occurring in both laboratories; at Temeke, the most severe drop partly coincided with an extreme overload (due to temporary nonfunctionality of a nearby laboratory). This finding seems to confirm that FM on its own is not a sufficient guarantee for higher sensitivity of AFB microscopy.[6]

The main problem in the field study was the interpretation of rechecking results, because the study protocol was breached. Onsite rechecking did not show excessive error rates, but there was insufficient guarantee for its technically correct execution (possible lack of blinding and restaining, and certain use of the same LED equipment). Rigorously executed rechecking at the SRL, taking all these factors into account, yielded surprisingly high error rates, particularly considering the over 20% HFP at Temeke. However, further onsite rechecking of a convenience random sample of recent positives, which could not yet have faded, did not show any false-positive results for the same Temeke technician, and it proved that the FluoLED system posed no problems in distinguishing artefacts from true AFB. The extremely high false-positive rates found earlier were due to a variety of factors, mostly related to poor identification of slides and unreliable registration. Furthermore, the Tanzania NTP practice of preserving only the first slide of a suspect series for rechecking may have been a cause, as these are spot-specimen smears that are more often negative or scanty positive in a smear-positive case series. At Mwananyamala, confusion with artefacts was linked to a very new technician. As has been suggested earlier,[7] FM requires a longer period of training to yield results as reliable as those of ZN. In our interpretation, the most serious problem was thus not false-positive results, but very numerous false-negatives approaching the prevalence of positives. This would imply that the sensitivity relative to the controllers was only about 50% in both laboratories, despite its considerable increase since the introduction of FM. However, given the Tanzania guidelines to keep only the first smear of a series, the sample cannot be considered representative. Contamination with environmental mycobacteria, visualised only after restaining and thus not seen during onsite rechecking, cannot be excluded as another possible explanation of this high (although not impossibly high) proportion of false-negatives.[8,9] Pleading against this hypothesis, the false-negatives reviewed in Antwerp by the principal investigator did contain AFB morphologically compatible with *Mycobacterium tuberculosis*.

Because of their very low requirements as regards power supply, correct installation, maintenance and spare bulbs, LED FM systems may be far more suitable for use under the conditions prevailing in low-income countries for which the two selected centres are highly representative. An additional benefit is the avoidance of the toxic waste from worn out HBO lamps. Our results show that the FluoLED™ module is very user-friendly. This is of utmost importance, as poor acceptance has so far been the main barrier to the introduction of the classic FM systems. The cost of a build-on module is affordable, and the possibility to run on dry batteries for hours offers good prospects for widespread implementation. Adding an interchangeable white LED would be a solution for other routine microscopic tests in brightfield if the power supply is very erratic. An optional 50× dry no-coverslip objective is recommended to confirm doubtful AFB without reverting to the 100× oil immersion objective, as its image is far sharper than that of a standard 40× dry objective.

Our results also suggest that this low-cost FM could make a large difference for TB detection, particularly in settings with high HIV prevalence where more patients present with paucibacillary sputum, because of the about 10 times larger area examined at 200× compared to 1000× magnification. With more experience and regular quality assurance, sensitivity might further improve considerably, as shown by the high rate of false-negatives detected in spite of the already clearly increased rate of detection with FM. With appropriate equipment, FM offers many advantages. Staining quality may be easier to control, as the quality of commercial auramine powder is less variable than that of basic fuchsin,[10] the solution is easier to prepare, and the cold staining technique easier to control.[11] Both the immersion oil and the xylene used for cleaning off the oil needed with ZN are expensive, and often cause damage to the objectives through misuse or poor quality.[12] The initial investment is a consideration, but the running costs are lower than for ZN (no need for replacement bulbs or objectives, immersion oil or xylene, and smaller quantities of dye needed).[13] More importantly, it would guarantee trouble-free use for a long time if the electrical and mechanical parts also prove to be of high quality. Considering the possible 20000 h lifespan of the LED light source, the idea of saving on the initial investment may therefore be misguided.[12]

More research is now needed to compare the different LED systems recently introduced on the market, not only regarding their effectiveness, but even more for their acceptance in peripheral laboratories and for their durability. Next, the considerable planning, train-

ing and logistics necessary for large-scale replacement of ZN by LED FM warrants a phased introduction with careful evaluation.

CONCLUSIONS

The FluoLED™ FM module mounted on a simple routine microscope can yield results that are equivalent to those of a conventional, higher-class HBO epifluorescence microscope for AFB microscopy. It offers the advantages of low cost and appropriateness even for use in peripheral laboratories in low-income settings, and contrary to the HBO systems it is very well accepted by the users. Particularly with high workload, FM can lead to increased sensitivity, but for optimal results it also needs careful training and good quality assurance.

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RÉSUMÉ

CONTEXTE : Laboratoire de référence de tuberculose (TB) à Bangkok, Thaïlande, et deux centres de santé à Dar es Salaam, Tanzanie.

OBJECTIFS : Evaluer la performance et l'agrément d'utilisation d'un module d'une diode émettant la lumière (LED) (FluoLED Easy™) pour l'examen microscopique par fluorescence (FM) pour la TB.

SCHÉMA : Etude de l'équivalence de cette technique avec la microscopie par fluorescence conventionnelle à Bangkok en utilisant la relecture à l'aveugle ; détection en routine dans les centres de santé à Dar es Salaam par comparaison avec le Ziehl-Neelsen (ZN) au cours de deux années avec relecture des frottis FM.

RÉSULTATS : Sur 461 frottis relus, la concordance avec la fluorescence conventionnelle a été de 99,1%. L'introduction de FluoLED a entraîné une augmentation durable de 20% en moyenne du taux de détection en routine dans chacun des centres de santé. Le recontrôle en aveugle a échoué en raison d'un enregistrement non fiable. Le recontrôle sur site d'un échantillon de convenance a montré l'absence de résultats faussement positifs dans un centre et la confusion avec des artéfacts qui pourrait être évitée par une amélioration de la formation dans l'autre centre. La LED FM a été très appréciée, les deux laboratoires refusant de retourner au ZN comme cela avait été initialement prévu.

CONCLUSIONS : Un simple microscope monté avec le module FluoLED peut obtenir des résultats équivalents à ceux de la microscopie de fluorescence conventionnelle. Le faible coût, la technicité appropriée et l'excellente acceptation justifient son utilisation dans les contextes à faibles revenus à l'opposé des systèmes classiques. La microscopie par fluorescence LED peut entraîner une augmentation de la sensibilité, mais pour obtenir un rendement optimal, une formation valable et une assurance de qualité restent des exigences essentielles.

RESUMEN

MARCO DE REFERENCIA : El laboratorio de referencia de tuberculosis (TB) en Bangkok, Tailanda, y dos centros de salud en Dar es Salaam, Tanzania.

OBJETIVOS : Evaluar el rendimiento y la facilidad de uso de un módulo de diodo emisor de luz (FluoLED Easy™) con el microscopio de fluorescencia en el diagnóstico de TB.

MÉTODO : Estudio de equivalencia entre el módulo FluoLED y la microscopia de fluorescencia convencional mediante una relectura con anonimato ; comparación con la detección sistemática mediante coloración de Ziehl-Neelsen (ZN) en los centros de salud en Dar es Salaam durante 2 años, con nueva verificación de las laminillas de microscopia de fluorescencia.

RESULTADOS : En la relectura de las 461 laminillas de microscopia de fluorescencia convencional se obtuvo un 99,1% de concordancia. La introducción del módulo FluoLED condujo a un aumento durable de la detección sistemática en cada uno de los centros de salud, en promedio del 20%. La segunda verificación con anonimato no fue útil a causa de un registro poco fiable. La verificación de una muestra de conveniencia en cada establecimiento puso en evidencia la ausencia de resultados positivos falsos en un centro y en el otro, resultados confusos y artefactos, que se habrían podido evitar mediante una mejor capacitación. El sistema de microscopia de fluorescencia con LED tuvo tan alta aceptabilidad en los laboratorios que ambos rechazaron el regreso a la técnica de ZN como estaba planeado.

CONCLUSIÓN : Un microscopio sencillo dotado con el modulo FluoLED puede ofrecer resultados equivalentes a los de la microscopia de fluorescencia convencional. Su bajo costo, comodidad técnica y excelente aceptabilidad justifican el uso de este sistema en medios con escasos recursos, en lugar de los sistemas clásicos. La microscopia de fluorescencia con LED puede aumentar la sensibilidad, pero con el fin de alcanzar un rendimiento óptimo se precisa un entrenamiento adecuado y un buen control de la calidad.