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Early and rapid microscopy-based diagnosis of true treatment failure and MDR-TB

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SUMMARY

SETTING: Damien Foundation tuberculosis (TB) control project in Bangladesh.

OBJECTIVE: Early diagnosis of true TB treatment failure and multidrug resistance (MDR) for more efficient DOTS-Plus.

DESIGN: Prospective comparison of performance on smear-positive sputum of fluorescein diacetate (FDA) vital staining vs. culture, and of slide drug susceptibility testing (slide DST) vs. the Löwenstein-Jensen (LJ) proportion method.

RESULTS: FDA reached 92% positive and 97% negative predictive value directly on fresh sputum, but only 94% and 62%, respectively, on transported smears. Accuracy on washed cetylpyridinium chloride transported sputum was similar to that on fresh sputum. Slide DST on fresh smear-positive sputum failed less often than LJ DST, with 96% accurate results for rifampicin and MDR-TB diagnosis. Good results were obtained for isoniazid (90% accuracy), but not for ethambutol or streptomycin.

CONCLUSIONS: We can confirm that FDA staining allows rapid screening for viable acid-fast bacilli and true treatment failure in delayed smear converters or smear-defined failures, while slide DST assures fast and accurate confirmation of MDR-TB in selected populations. The tests can be applied safely in resource-poor settings. Their successive use could be an efficient strategy for screening and an early start on standardised regimens of DOTS-Plus candidates.

KEY WORDS: microscopy; Ziehl-Neelsen; vital staining; slide culture

SPUTUM SMEAR MICROSCOPY for acid-fast bacilli (AFB) remains the most important and widely available diagnostic method for tuberculosis (TB) in high-prevalence countries. While its specificity is very high on diagnostic specimens, this is not the case for smears during treatment, as AFB microscopy cannot distinguish between live and dead AFB.[1] With excellent microscopy and highly sterilising treatment regimens, this results in serious over-diagnosis of treatment failure.[2–4] Furthermore, delayed smear conversion (patients still positive at follow-up before treatment failure can be declared) is often due to prolonged excretion of dead bacilli.[5] However, culture would be inefficient for treatment management in control programmes.

We have tried a little-known technique, vital staining with fluorescein-diacetate (FDA), which is reportedly highly predictive of mycobacterial growth in culture, for diagnosis of true treatment failure.[6,7] Slide drug susceptibility testing (DST), another microscopy-based and almost forgotten method, was simultaneously evaluated for rapid diagnosis of multidrug-resistant TB (MDR-TB), defined as resistance to at least rifampicin (RMP) and isoniazid (INH).

The setting is the Damien Foundation (DF) Bangladesh TB control project, under the National TB Control Programme (NTP), featuring excellent AFB microscopy[8] in a population presenting with advanced but generally drug-susceptible disease.[9,10] This caused problems of interpretation for delayed smear converters and for diagnosis of failure, particularly for start and monitoring of standardised second-line treatment.[11] At the end of 2003, the NTP changed from 2(3)EHRZ/6HT* to 2(3)EHRZ/4H_3R_3 for new smear-positive cases (Category 1). The retreatment regimen (Category 2) for previously treated smear-positive cases was 2(3)SEHRZ/1EHRZ/5$E_3H_3R_3$. The project also used standardised regimens for the treatment of

* E, EMB = ethambutol; H = isoniazid; R = rifampicin; Z = pyrazinamide; T = thioacetazone; S, SM = streptomycin. Numbers before the letters indicate the duration in months of the phase of treatment; numbers in subscript indicate the number of times the drug is taken each week.

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MDR-TB, as previously described.[11] As usual in NTPs, Category 1 or 2 failure was declared for repeatedly positive or scanty positive smears at 5 months of treatment or later, and these sputum samples were routinely sent to the culture laboratory.

METHODS

Patients and specimens

Slide DST was performed on freshly sampled sputum from all smear-positive patients presenting at the culture laboratory who had been referred because of delayed smear conversion or treatment failure with suspicion of MDR-TB (retreatment failures, or with delayed conversion and poor clinical evolution). Besides randomly chosen, presumably pan-susceptible controls, a few cases suspected of second-line treatment failure were also included. This report covers all tests done in 2005 after adjustment of the technique in previous years.

FDA vital staining was compared with culture for randomly selected smear-positive patients, using different sampling techniques successively in time. Unfixed direct smears from a cohort of patients who were smear-positive at the end of the intensive phase were sent by the DF field clinics to the culture laboratory for FDA staining. For failures, fresh unfixed direct smears were prepared at the culture laboratory for referred patients; however, the FDA smears were usually prepared there from sputum sent in cetylpyridinium chloride (CPC).

Staining procedures

Ziehl-Neelsen (ZN) staining was performed as described previously.[8] The FDA staining procedure was adapted from the publication by Tsukiyama et al.[12] After drying, unfixed smears were covered for 30 min with the FDA reagent, which was made up weekly by diluting 100 μl FDA stock (5% w/v fluorescein diacetate [Sigma Chemical Co, St Louis, MO, USA] in acetone, stored in aliquots at −20°C for a maximum of 2 years) in 10 ml phosphate buffer pH 6.8 containing 0.05% Tween 80. After rinsing with water and destaining with 1% acid alcohol for 1–2 min, bacilli were killed using 5% watery phenol for 10 min,[13] followed by a final water rinse and air-drying in the dark. One length of the smear was read under a fluorescence microscope at 1000× magnification. The reader was blinded to the culture results, which would become known only much later.

Slide DST

For DST, we adopted the method described by Dissmann,[14] using Sula liquid medium[15] with 10% locally collected goat serum. Penicillin 200 IU/ml or PANTA (Becton Dickinson, Sparks, MD, USA) antibiotic cocktail 0.1 ml/5 ml medium were added to control contamination. Ten sputum smears were made on one end of autoclaved slides, cut in half longitudinally, and decontaminated in 5% watery sulfuric acid for 15 min followed by a water dip, and placed individually in sterile, heavy glass 28 ml universal bottles containing 7 ml medium. For each sample we used three growth controls besides antibiotic bottles containing INH 0.2 and 1 μg/ml; RMP 1 μg/ml; EMB 2, 5 and/or 10 μg/ml; SM 2 μg/ml. After overnight refrigeration, the bottles were incubated at 37°C for 10 days, and heated at 85°C for 30 min before opening. Dried smears were heat-fixed, stained by ZN and examined at 100× magnification, counting acid-fast micro-colonies. Any number of well-developed colonies in presence of a drug was interpreted as resistance if drug-free controls contained at least one such colony per low-power field. Well-developed colonies included grades 2+ to 4+ per Dickinson et al.,[16] but for EMB only 3+/4+ colonies (with cording) were counted.

By way of sterilisation control, 20 heated fully-grown slide DST controls were inoculated on Löwenstein-Jensen medium (LJ) and incubated for 8 weeks at 37°C.

The critical concentrations and interpretation above represent the protocol applied during the study period, which had been previously adapted from the original publications.[14,16] The correct EMB concentration had not yet been identified. Tests were blinded, as the gold standard results arrived only months later.

Conventional cultures

Sputum transport with CPC, decontamination, primary culture and DST on LJ were performed as previously described.[9] Primary isolation was done locally, but identification and DST on LJ (proportion method) were performed at the TB Supranational Reference Laboratory (SRL) in Antwerp, Belgium.

RESULTS

FDA staining was compared with culture on 422 ZN scanty or positive smears (Table 1). Of these, 127 were from sputum collected at the reference laboratory (Group 1), 102 were sent there as unfixed smears (Group 4), and 193 as sputum in CPC. Of the latter, 153 (Group 2) and 40 (Group 3) were made from centrifuged sediments, respectively without or with prior washing. The bacillary load of the original ZN smears differed considerably. While Groups 1 and 4 contained about equal proportions of low (scanty and 1+) and high (2+, 3+) positives, low positive (mainly scanty) smears made up the majority of Groups 2 and 3. Excluding specimens with contaminated culture (1–11%), sensitivity, specificity and accuracy ranged from 33% to 100%, 82% to 98% and 67% to 97%, respectively. Positive predictive value (PPV) ranged from 80% to 94%, and negative predictive value (NPV) from 62% to 100%. Both freshly examined smears (Group 1) and washed CPC sediments (Group 3) yielded highly reliable results (PPV 92%, NPV 97% and PPV 80%,

Table 1 Performance of FDA vital staining compared to culture, by type of smear studied, with distribution of AFB quantifications in the original ZN smears

	Group 1 Fresh smears *n* (%)	Group 2 CPC smears, sediment *n* (%)	Group 3 CPC smears, washed sediment *n* (%)	Group 4 Transported smears *n* (%)
Total number	127	153	40	102
Quantified ZN smear results				
Scanty*	20 (16)	39 (25)	16 (40)	17 (17)
1+	32 (25)	56 (37)	15 (38)	37 (36)
2+	46 (36)	38 (25)	8 (20)	34 (33)
3+	29 (23)	20 (13)	1 (3)	14 (14)
Correlation of FDA staining with culture				
Culture contaminated	11 (9)	17 (11)	1 (1)	5 (5)
True positive, *n*	77	32	4	15
True negative, *n*	31	80	34	50
False positive, *n*	7	4	1	1
False negative, *n*	1	20	0	31
Sensitivity, %	99	62	100	33
Specificity, %	82	95	97	98
Accuracy, %	93	82	97	67
Positive predictive value, %	92	89	80	94
Negative predictive value, %	97	80	100	62

* Scanty, 1+ to 3+: refers to the IUATLD/WHO quantification scale for AFB smears.[17]
FDA = fluorescein diacetate; AFB = acid-fast bacilli; ZN = Ziehl-Neelsen; CPC = cetylpyridinium chloride.

NPV 100%). Transported smears and unwashed CPC sediments showed many false-negative results (respectively 33% and 62% sensitivity). All false positives in Group 1 concerned FDA smears with scanty bacilli, but 10/17 specimens with scanty FDA results did grow in culture.

Slide DST results are shown in Table 2. Slide DST had less false-negative culture or massive contamination than LJ; 20 tests were non-comparable because of failure on LJ compared to eight in slide DST, while 32 failed both LJ and slide DST. Contamination was reduced by using PANTA instead of penicillin, and by improving aseptic techniques. Of two strains identified as *Mycobacterium intracellulare* on LJ, one was reported as NTM in slide DST, while the other was considered as *M. tuberculosis*. DST results were excellent for RMP, reaching 96% accuracy, but only 90% was achieved for INH because of poor specificity. EMB lacked specificity at the lower concentration (2 μg/ml) and sensitivity at 5 or 10 μg/ml, while SM was lacking in both.

Table 3 shows combined FDA and slide DST results, targeting correct diagnosis of treatment failure and MDR-TB by test indication group. All controls (presumably not MDR-TB) were FDA-positive and grew in slide DST (but not always on LJ). Of 41 Category 1 delayed converters (34 smear-positive at 3 months), no FDA-negatives and about half of FDA-positives grew in culture. All but one FDA-positive/slide DST-negative sputum samples were paucibacillary, and only two grew on LJ. Despite some false INH resistance, slide DST MDR-TB classification was correct in both groups. Most Category 2 samples that were smear-positive at 4 months were also FDA-positive and grew in culture, while 2/4 FDA-negatives grew only on LJ. MDR-TB classification was always correct. All 60 Category 2 failures and 11 relapses were FDA-positive, and almost all grew in culture, particularly slide DST. There were six erroneous MDR-TB classifications in these groups. Among these, one

Table 2 Results of slide DST

	H0.2	H1	R1	S2	E2	E5	E10
True resistant, *n*	72	68	71	42	21	26	8
False resistant, *n*	10	10	1	10	3	2	1
True susceptible, *n*	16	18	26	30	11	21	23
False susceptible, *n*	0	1	3	15	6	12	24
Total resistant, *n*	72	69	74	57	27	38	32
Total susceptible, *n*	26	28	27	40	14	23	24
Total good results, *n*	98	97	101	97	41	61	56
Sensitivity, %	100	99	96	74	78	68	25
Specificity, %	62	64	96	75	79	91	96
Accuracy, %	90	89	96	74	78	77	55
Positive predictive value, %	88	87	99	81	88	93	89
Negative predictive value, %	100	95	90	67	65	64	49
Total tests performed, *n*							163
Negative/contaminated in slide DST and on LJ							32
Only LJ contaminated or failed							20
Only slide DST contaminated or failed							8
Slide DST NTM, LJ *M. intracellulare*							1
Slide DST considered *M. tuberculosis*, LJ *M. intracellulare*							1

Totals are not the same for all antibiotics because of individual contaminated bottles, and not all EMB concentrations were used for all strains.
DST = drug susceptibility testing; H0.2, H1 = INH 0.2 and 1 μg/ml; R1 = rifampicin 1 μg/ml; S2 = streptomycin 2 μg/ml; E2, E5, E10 = ethambutol 2, 5 and 10 μg/ml; LJ = Löwenstein-Jensen medium; NTM = non-tuberculous mycobacteria; EMB = ethambutol.

Table 3 Evaluation of combined use of FDA vital staining and slide DST, by testing indication

Testing indication (*n*)*	FDA result *n*	Slide DST growth *n*	LJ DST growth *n*	MDR-TB correctly classified by slide DST	Non-MDR-TB correctly classified by slide DST
Controls (25)	0 Negative	NA	NA	NA	NA
	25 Positive	25	20	NA	20/20
Category 1 failure? (41)	8 Negative	0	0	NA	NA
	33 Positive	13	8	5/5	1/1
Category 2 failure? (21)	4 Negative	0	2	NA	NA
	17 Positive	13	15	12/12	NA
Category 2 failure (60)	0 Negative	NA	NA	NA	NA
	60 Positive	58	54	47/49	1/2
Category 2 relapse (11)	0 Negative	NA	NA	NA	NA
	11 Positive	10	7	2/3	2/4
MDR treatment failure? (5)	0 Negative	NA	NA	NA	NA
	5 Positive	5	4	4/4	NA

* Controls were non-MDR-TB suspects, including new cases, Category 1 relapses and defaulters.
Category 1, 2 and MDR-TB treatment failure?: early suspect failures of Category 1, 2 or MDR-TB treatment.
Category 2 failure: smear-positive at 5 months or later during Category 2 treatment.
FDA = fluorescein diacetate; DST = drug susceptibility testing; LJ = Löwenstein-Jensen medium; MDR-TB = multidrug-resistant tuberculosis; NA = non-applicable because of zero total.

NTM was considered as *M. tuberculosis*, two RMP-monoresistant strains as resistant also to INH, and one INH+EMB resistant strain as resistant to RMP. Of five delayed converters during MDR-TB treatment, all were FDA-positive and grew in slide DST with a correct MDR-TB result.

Only half of 20 LJ cultures from heated slide DST control medium grew rare colonies of *M. tuberculosis*.

DISCUSSION

False declaration of failure because of non-viable AFB has been described in 75% to 80% of new cases with smear-defined outcome.[3,18–21] Furthermore, secondary infection with NTM should be distinguished from true TB treatment failure.[18,19] Our own data from continuous monitoring of drug resistance in retreatment cases, partially reported earlier,[22] show that 371/824 (45%) failures of 2EHRZ/6HT treatment and 162/216 (75%) of the 6-month regimen remained culture-negative. NTM were isolated from fewer than 0.5% of our new smear-positive cases,[9,10] and from 12/483 (2.5%) Category 1 and 17/352 (4.8%) Category 2 culture-positive failures.

In Lima, around 90% of microscopy-defined Category 1 failures were culture-positive MDR-TB,[23] and delayed smear conversion was strongly associated with Category 1 treatment failure and MDR-TB,[24] but this was not confirmed in other settings.[2,3,18,19] In our own patients, only 4/66 (6%) smear-positive at 3 months of Category 1 were culture-positive; for Category 2 this was 37/124 (30%) at 3 months and 32/75 (43%) at 4 months, vs. 35/39 (90%) at 5 or 6 months (unpublished data). Most (95%) culture-positives were indeed MDR-TB. Although highly desirable to prevent amplification of drug resistance,[25] changing to second-line drugs early due to delayed smear conversion thus remains controversial.

We tried some older techniques to reach an early diagnosis of true failure and MDR-TB. FDA staining has been used to determine the viability of leprosy and other mycobacteria.[26,27] Harada and Numata applied it to TB sputum smears, and found a perfect correlation with growth in culture.[7] We modified the technique, omitting ethidium bromide staining of non-viable bacilli, including an acid-destaining step, and adding a final 5% phenol treatment for safety. Using sputum from ZN-positive patients with delayed conversion or smear-defined failure, correlation with culture depended on transport. Fresh smears yielded high predictive values (92% PPV, 97% NPV), but in transported smears these were low. FDA smears from CPC-transported sputum yielded poor results, except after a washing step. CPC may thus interfere with FDA staining, as has been reported for auramine and ZN staining.[28,29] With fresh sputum, FDA false positives occurred mainly for scanty AFB, but the majority of such smears were FDA true positives.

DST directly from smear-positive sputum smeared on slides has been used since the early 1940s. Dickinson et al. simplified the technique and reported almost perfect agreement with the absolute concentration method on LJ after 7 days for RMP, SM and EMB in a selective medium based on human blood.[16] They could not obtain satisfactory growth in artificial media enriched with animal serum, used by Dissmann and others.[14] For safety reasons we preferred Dissmann's medium and unbreakable universal bottles, besides heat-killing of unopened grown cultures. Ten days incubation yielded satisfactory growth, usually with cording, even for some MDR-TB strains that failed on LJ. Contamination was the main problem, but excluding

NTM and tests that failed in both systems, 94% of samples yielded valid results in slide DST compared with 84% on LJ.

Our results with the original method using acid decontamination, malachite green and penicillin were very good only for RMP. False INH resistance was greatly reduced with better contamination control, particularly by replacing penicillin with PANTA, an antibiotic cocktail containing polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin. Overall agreement reached 96% for RMP but only 90% for INH, and remained poor for EMB and SM. EMB results were concentration-dependent, and might still improve by adopting a critical concentration between 2 and 5 μg/ml. This seems unlikely for SM, as the considerable disagreement with the reference laboratory is related to the very different test environment (medium, atmosphere, container). Preliminary results (not shown) suggest that excellent agreement may also be reached for kanamycin. We have learnt that results should be considered doubtful, and tests repeated (on the remaining refrigerated sputum), in case of scanty or negative growth of controls, or with rare colonies growing in drug-containing tubes while the controls show massive growth.

Considering only patients suspected of MDR-TB, FDA always predicted growth failure in slide DST (but missing 2/12 growing on LJ), while a positive test was highly predictive for growth, except for Category 1 delayed conversion with scanty AFB. With few exceptions, slide DST proved accurate for MDR-TB diagnosis in suspected Category 1 and 2 patients. Half of these over-diagnoses would have been at high risk for development of MDR-TB during standard Category 2 treatment.

As suggested by our slide DST results, suboptimal LJ cultures were a limitation of our study. However, both were performed in the same laboratory and on the same samples, and in our experience these LJ results are representative of many reference laboratories in low-income countries. Furthermore, genuine superiority of slide DST was likely with some dysgonic MDR strains. Use of PANTA improved the results, but may not be indispensable with strictly aseptic techniques. With culture as gold standard, FDA specificity and PPV may thus have been underestimated, and sensitivity and NPV overestimated, particularly for paucibacillary samples. In routine, it might even be preferable to examine more than one FDA smear (preferably from more than one sputum), as a missed true failure is the more serious mistake.

Another limitation was the gold standard used for slide DST, with the results obtained in a single SRL using the LJ proportion method. Use of an SRL quality assurance panel with known judicial results would have been preferable, but its adaptation to the slide DST test format failed. Our slide DST evaluation is thus not quite correct, and its performance might in fact be underrated.

In recent years, several rapid DST methods have been described. Nitratase detection DST[30] requires very little change from standard LJ-based methods, while the colorimetric tests[31] and MODS (microscopic observation drug susceptibility)[32,33] using cheap, in-house prepared reagents and media may be more appropriate for low-income countries, although MODS requires an invert microscope. However, their liquid media in microtiter plates impose higher level safety precautions, often a weak point in resource-poor settings. MODS also allows morphological identification of *M. tuberculosis*,[32,34] but sloppy colorimetric DST could easily lead to false results due to unrecognised contamination. The accuracy of these liquid medium tests may be similar, typically yielding excellent agreement for INH and RMP, but not for SM and/or EMB.[31–33,35]

CONCLUSIONS

FDA vital staining on fresh or CPC-transported smear-positive sputum can be used for early and accurate diagnosis of true TB treatment failure. Slide DST may be a suitable system for rapid diagnosis of MDR-TB in resource-poor settings, requiring minimal equipment and only smear microscopy level safety precautions. With good contamination control and sufficient experience, a reasonably accurate MDR-TB diagnosis can be made in smear-positive cases. It may be a particularly useful test for small-volume MDR-TB referral centres without sophisticated laboratories. Prior FDA screening could then be used to avoid unnecessary referrals and excessive numbers of failing slide DST, particularly when targeting early diagnosis in delayed smear converters.

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RÉSUMÉ

CONTEXTE : Projet de lutte antituberculeuse de la Fondation Damien au Bangladesh.

OBJECTIF : Un diagnostic précoce des vrais échecs du traitement de la tuberculose (TB) et de la TB à germes multi-résistants (TB-MDR) afin d'arriver à un DOTS-Plus plus efficient.

SCHÉMA : Comparaison prospective de la performance de la coloration vitale des expectorations par le diacétate de fluorescéine (FDA) versus la culture, ainsi que des tests de sensibilité aux médicaments sur lame (slide DST) versus la méthode des proportions de Löwenstein-Jensen (LJ).

RÉSULTATS : La valeur prédictive positive de la FDA a été de 92% et la valeur négative de 97% sur des expectorations fraîches, mais seulement respectivement de 94% et 62% sur des frottis après envoi. La précision des expectorations transportées sur du chlorure de cétylpyridium lavé a été similaire à celle des expectorations fraîches. Le DST sur lame sur des expectorations fraîches à bacilloscopie positive a échoué moins souvent que le LJ DST, avec des résultats exacts de 96% pour le diagnostic de la rifampicine et de la TB-MDR. De bons résultats ont été obtenus pour l'isoniazide (précision 90%), mais pas pour l'éthambutol ou la streptomycine.

CONCLUSIONS : Nous pouvons confirmer que la coloration FDA permet un dépistage rapide des bacilles acido-résistants vivants et des vrais échecs de traitement chez les sujets dont les frottis, sont négativés avec retard ou dans les échecs définis par les frottis, alors que la DST sur lame assure une confirmation rapide et précise de la TB-MDR dans des populations sélectionnées. Les tests peuvent être appliqués avec sécurité dans des contextes à faibles ressources. Leur utilisation successive pourrait constituer une stratégie efficiente pour le dépistage et le démarrage précoce du traitement chez les candidats à des régimes standardisés de DOTS-Plus.

RESUMEN

MARCO DE REFERENCIA : Proyecto de control de la tuberculosis (TB) de la Damien Foundation en Bangladesh.
OBJETIVO : Alcanzar una mayor eficiencia de la estrategia DOTS-Plus, mediante el diagnóstico precoz de los verdaderos fracasos del tratamiento antituberculoso y de la TB multidrogorresistente (MDR).
MÉTODO : Estudio prospectivo de comparación del rendimiento diagnóstico en esputos positivos para bacilos acidorresistentes, entre la baciloscopia mediante coloración vital con diacetato de fluoresceína (FDA) y el cultivo y entre las pruebas de sensibilidad a los medicamentos (DST) sobre láminas y el método proporcional en medio Löwenstein-Jensen (LJ).
RESULTADOS : La coloración vital demostró un valor diagnóstico para un resultado positivo del 92% y para un resultado negativo del 97% en esputo fresco, pero sólo 94% y 62% respectivamente en los frotis de esputo transportados. La exactitud para las muestras de esputo transportadas descontaminadas con cloruro de cetilpiridinio fue equivalente a la exactitud en las muestras de esputo fresco. Las DST en frotis frescos de esputo, positivos para bacilos acidorresistentes fallaron menos que las pruebas en LJ, con un 96% de resultados exactos en el diagnóstico de TB con resistencia a rifampicina y MDR. Se obtuvieron buenos resultados para isoniacida (exactitud del 90%), pero no así para etambutol ni estreptomicina.
CONCLUSIONES : El presente estudio confirma que la coloración con FDA constituye un medio rápido de detección sistemática de bacilos acidorresistentes viables y de los fracasos terapéuticos verdaderos en pacientes con retraso de la conversión de la baciloscopia o fracasos definidos por la baciloscopia y que las DST sobre las láminas del frotis de esputo proporcionan una confirmación rápida y exacta de la TB-MDR en poblaciones escogidas. Las pruebas pueden aplicarse en forma segura en medios con escasos recursos. Su uso continuo podría representar una estrategia eficaz de detección y de inicio precoz de pautas estandarizadas en pacientes candidatos a DOTS-Plus.