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Rapid screening of *Mycobacterium tuberculosis* for susceptibility to rifampicin and streptomycin

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SUMMARY

OBJECTIVE: To investigate rapid detection of drug-resistant tuberculosis using the genotypic Inno-LiPA Rif TB assay and a novel, low-cost, bacteriophage-based susceptibility assay.

DESIGN: The performance of the microwell phage replication assay (MPRA) on 18 isolates from suspected multidrug-resistant tuberculosis patients was compared to the LiPA assay performed directly on sputum specimens. Mutations in the *rpoB* gene identified by LiPA that confer resistance to rifampicin (RMP) were confirmed by DNA sequencing, while susceptibilities were confirmed by the proportion method and BACTEC. A further 19 isolates undergoing routine screening for both RMP and streptomycin susceptibility were included for comparison.

RESULTS: Susceptibility to RMP was determined for 17/18 (94.4%) sputum specimens tested by LiPA. Correlation between MPRA, molecular and conventional methods was 100% for the detection of RMP susceptibility. However, for susceptibility to streptomycin one discrepant result was found: an isolate susceptible to streptomycin by the proportion method was found resistant by MPRA to 2 μg/ml of streptomycin. Similarly, an isolate initially resistant by MPRA upon re-testing was found susceptible in agreement with the conventional method.

CONCLUSION: LiPA enables rapid detection of drug-resistant infection, while MPRA offers simple, low-tech testing of drug susceptibilities that may be appropriate for application in low-income countries.

KEYWORDS: bacteriophage; drug susceptibility; rifampicin; tuberculosis

TUBERCULOSIS REMAINS a substantial public health problem; it has been estimated that of the 8 million new cases that occur each year, 95% are in developing countries.[1] Increased levels of resistance to the first line anti-tuberculosis drugs have been reported in some parts of the world, notably in Asia, Eastern Europe, Latin America and Southern Africa,[2,3] and the emergence of multidrug-resistant (MDR) strains (resistant to both rifampicin and isoniazid) pose a serious threat to tuberculosis control. Heightened awareness of drug resistance has resulted in an increased demand for drug susceptibility testing, both for improved management of patients and to monitor levels of resistance, enabling assessment of national control programmes. In most developing countries, current testing methods are slow, involving culture of isolates on solid media containing the drug under test. New, more rapid testing methods based on liquid culture techniques such as BACTEC[4,5] or the mycobacterium growth indicator tube (MGIT)[6] are now available. Molecular methods have also been developed which enable rapid detection of drug-resistant tuberculosis infection directly from clinical specimens. However, the high cost and requirement for sophisticated equipment may prohibit routine use of such methods in low-income countries.

In this study we examine the *rpoB* Line Probe assay (LiPA), a commercially available molecular test, for the determination of resistance to rifampicin (RMP) directly from sputum collected from patients with suspected MDR. Mutations within a variable region of the gene encoding for the beta subunit of DNA dependent RNA polymerase have been identified as conferring resistance to RMP.[7] In the LiPA assay a fragment of the *Mycobacterium tuberculosis rpoB* gene is amplified by polymerase chain reaction (PCR), and the presence or absence of mutations determined by reverse hybridisation to a series of oligonucleotide probes immobilised on a strip of nitrocellulose.[8]

We also examine use of bacteriophage D29 for low-tech, rapid determination of drug resistance in *M. tuberculosis* isolates. Mycobacteria that are sus-

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ceptible to RMP and streptomycin do not support the replication of D29 phages in the presence of critical concentrations of these drugs, while replication is able to proceed in those bacteria that are resistant.[9,10] We have previously demonstrated highly sensitive detection of phage replication in which the chemical inactivation of extraneous phages enables exclusive identification of progeny phage.[11] We report here use of a rapid, microtitre plate based test to assess susceptibility to RMP and streptomycin of *M. tuberculosis* isolates grown on Löwenstein-Jensen (LJ) slopes, a commonly used mode of isolation in low-income countries.

METHODS

Samples

Sputum samples were collected from suspected MDR tuberculosis patients whose clinical response to antituberculosis chemotherapy was assessed with respect to smear, culture and examination of chest radiographs. All patients were hospitalised at King George V Tuberculosis Hospital, Durban, and received various combinations of RMP, isoniazid, pyrazinamide, streptomycin, ethambutol, ciprofloxacin, capreomycin, thiacetazone, kanamycin and ofloxacin. Eighteen sputum specimens, seven smear-positive and 11 smear-negative, were processed immediately and subjected to LiPA, and isolates from these patients were sent for susceptibility testing by microwell phage replication assay (MPRA). A further 19 strains isolated by culture on LJ from sputum at University College Hospital, London, were included in the study.

Molecular methods

Line Probe assay (LiPA)

The *rpoB* Line Probe assay has recently been described,[8,12] and was undertaken as performed by De Beenhouwer et al.[8] The assay is based on reverse hybridisation of amplified DNA to oligonucleotide probes from a variable region of the gene encoding for the beta subunit of DNA-dependent RNA polymerase. Each probe is immobilised in a band on a nitrocellulose strip, with each strip holding five wild type probes overlapping the entire variable region and four probes corresponding to specific mutations associated with resistance to RMP. A probe specific for the *M. tuberculosis* complex is also included for confirmation of the presence of *M. tuberculosis* DNA.

The LiPA kits (Inno-LiPA Rif. TB; Innogenetics NV Zwijnaarde, Belgium) were supplied by Murex Diagnostics (Johannesburg, South Africa) and used according to kit instructions.

After decontamination of each sputum specimen, 500 μl to 1 ml of the sediment was sonicated (Gen-Probe, San Diego, CA, USA) for 15 minutes in a microfuge tube containing zirconia silica beads (0.05 g of 0.1 mm diameter Biospec Products Inc, Bartlesville, OK, USA). The *M. tuberculosis* lysates were heat inactivated in a dry heat block (95°C, 30 minutes) and debris pelleted in a microcentrifuge (15 000 g for 15 minutes). The crude supernatant containing *M. tuberculosis* DNA was removed and stored in 100 μl aliquots at −20°C.

Extracted *M. tuberculosis* DNA samples were then subjected to nested PCR using outer primers 5′-GAG AATTCGGTCGGCGAGCTGATCC-3′ and 5′-CGA AGCTTGACCCGCGCGTACACC-3′ followed by a second round of amplification using biotinylated inner primers 5′-GGTCGGCATGTCGCGGATGG-3′ and 5′-GCACGTCGCGGACCTTCCAGC-3′ giving a 257 bp biotin labelled product. Following amplification an aliquot of each reaction was denatured with sodium hydroxide solution before incubation with a LiPA strip in 1 ml hybridisation buffer for 1 hour at 50°C in a shaking water bath. Following washing of the strips, immobilised hybrids were detected via alkaline phosphatase labelled streptavidin and a chromatographic substrate. A positive hybridisation was indicated by the formation of a coloured band at the position of the immobilised probe. Mismatched DNA was unable to hybridise under the stringent conditions used, and thus the absence of a positive signal with one or more of the wild type probes indicated the presence of a mutation, and possibly a resistant strain. A positive signal with all the wild type probes indicated the presence of an RMP-sensitive strain, as no mutations were detected. A positive signal with one or more of the four mutant probes indicated a resistant strain.

With those samples for which no detectable signal on the LiPA test strip was observed, the presence of *M. tuberculosis* DNA was confirmed by PCR amplification using the IS*6110* target.[13] An internal control which co-amplifies with the same primers to yield a 600 bp product was included in each reaction.[14]

DNA automated sequencing on corresponding M. tuberculosis *cultures*

Cycle sequencing was performed in both directions using primers flanking a 305 bp fragment of the *rpoB* gene in *M. tuberculosis*:[14]

Tb Rif 1 5′-CAg ACg TTg ATC AAC ATC Cg-3′
Tb Rif 2 5′-TAC ggC gTT TCg ATg AAC-3′

Sequencing was performed at the South African Sugar Association Molecular Biology Core Facility using an Applied Biosystems (ABI, Perkin Elmer South Africa Pty Ltd, Johannesburg, South Africa) 310 automated sequencer and the Taq FS dideoxy terminator cycler sequencing kit (ABI).

Microscopy, culture and susceptibility testing

The sputum specimens were decontaminated using the standard NALC/NaOH method.[15] Smears were examined after Ziehl-Neelsen staining. Cultures were

done on LJ and liquid (Middlebrook, Difco Laboratories, Scientific Laboratory Supplies, Nottingham, UK) media. RMP resistance was determined by the proportion method on 7H10 agar plates and the minimal inhibitory concentration (MIC) of RMP was assayed by the BACTEC system (Becton Dickinson Microbiology Systems, Sparks, MD, USA).[4,5]

Microwell phage replication assay

Culture of mycobacteria

The 18 *M. tuberculosis* strains isolated from suspect MDR patients in Durban and tested by the molecular methods, the 19 strains isolated in London, *M. tuberculosis* HR37v and *M. bovis* BCG were cultured on LJ slopes. *M. smegmatis* mc²155 and SMR5 were grown in Middlebrook 7H9 broth with a 10% supplement of OADC. *M. smegmatis* SMR5, a streptomycin-resistant strain,[16] was maintained in 10 μg/ml of the drug. Polyoxyethylenesorbitan mono-oleate (Tween 80), a detergent commonly used to prevent aggregation of bacteria in liquid culture,[17] was not included due to its ability to inhibit adsorption of D29 phages to host bacteria.[18]

Production of mycobacteriophages

D29 phages were produced as previously described.[11] Briefly, phages were inoculated onto a lawn of *M. smegmatis* mc²155 in 1.5% agar in Middlebrook 7H9 supplemented with OADC (Becton Dickinson UK Ltd, Cowley, UK). The phages were harvested by an overnight incubation with 7H9 broth which was then passed through two 0.45 μm disposable filters and stored at 4°C for up to 6 months. The phages were quantified by dilution and plating onto *M. smegmatis* indicator plates where the number of plaque forming units (pfu) produced after an overnight incubation at 37°C were recorded.[19]

Preparation of indicator plates

Indicator plates were prepared by adding a 10% volume of stationary phase *M. smegmatis* SMR5 culture to Luria Broth, Miller (Becton Dickinson UK Ltd, Scientific Laboratory Supplies) containing 1.5% molten agar at a cooled temperature of approximately 45°C. This indicator agar mix was then poured into triple-vented plastic petri dishes and allowed to set. Plates were placed in plastic bags and stored at 4°C for up to 2 weeks until required; prior to use they were dried at 37°C for 30 min to remove excess surface liquid. To enhance visualisation of plaques 10 μl food colouring/ml was added prior to pouring (Egg yellow or Blue, Supercook, Leeds, UK).

Drug susceptibility assay

The drug under test was diluted in assay broth (Luria Broth, Miller, with 0.2% glucose and 1mM $CaCl_2$) to 2× working concentration and 75 μl placed in the wells of a sterile microtitre plate (Greiner Labortechnik Ltd, Stonehouse, UK). Final drug concentrations of 0, 1, 2, 5, 10 and 50 μg/ml were used for both RMP and streptomycin. A 1 μl loop was used to transfer a sample of the isolate under test from the LJ slope to 3 ml of assay broth with 4–7 glass beads in a 7 ml bijou tube. Aggregates were dispersed by vortexing for 20 seconds and the tubes allowed to stand for 3 minutes to allow dispersal of aerosols before 75 μl of each sample was placed in the appropriate well. The plate was sealed in a plastic bag and incubated at 37°C for 24 hours. 50 μl D29 phages diluted in assay broth containing the appropriate drug concentration were added to each well to give a final phage concentration of 2.5×10^7 pfu/ml. The plate was incubated for 1.5 hours at 37°C before 100 μl assay broth containing 30 mM $FeNH_3SO_4$ was added. Samples were mixed by pipetting before 15 μl drops were placed on prepared indicator plates. Plaques were recorded following overnight incubation at 37°C and recorded as 4+ for complete lysis, 3+ nearly complete lysis, 2+ partial lysis, 1+ individual plaques and 0 for no lysis.[19] *M. tuberculosis* H37Rv or *M. bovis* BCG were used as negative controls, and strains supporting phage replication at drug concentrations higher than the controls were classified as resistant.

RESULTS

Use of LiPA for detection of RMP-resistant M. tuberculosis in sputum

Of 18 sputum samples from suspect MDR patients tested by LiPA, results were obtained from seven smear-positive and 10 smear-negative specimens. Sixteen of these were identified as having mutations in the *rpoB* gene conferring resistance to RMP and one to have a wild type hybridisation profile (Table 1). One smear-negative specimen (9839) failed to give a hybridisation signal, which suggested an absence of amplified *M. tuberculosis* DNA in the LiPA test. However, co-amplification of the internal control was observed and the presence of *M. tuberculosis* complex in this specimen was confirmed by IS*6110* target amplification. Confirmation of the susceptibility of isolates obtained from these specimens was provided by BACTEC methodology; the MICs obtained are included in Table 1. Seventeen strains were found resistant to RMP, including an isolate grown from the specimen that had failed to provide a LiPA result; one strain was found susceptible in agreement with the LiPA result. DNA sequencing of the variable region of the *rpoB* gene for these isolates confirmed the presence of those mutations identified by the LiPA test (Table 1). The most frequently observed mutation was replacement of serine by leucine at position 531 of the amino-acid sequence.

Microwell phage replication assay

Isolates from the 18 sputum specimens tested by LiPA and a further 19 strains isolated in London and pre-

Table 1 Sputum specimens examined by Ziehl-Neelsen and tested for resistance to rifampicin by LiPA. Genetic mutations were confirmed by sequencing of the corresponding *M. tuberculosis* cultures. Minimal inhibitory concentrations (MIC) were established with BACTEC.

Specimen	AFB	LiPA result	Genotypic mutation*	MIC μg/ml
8989	Negative	Resistant	Ser531Leu	>8
9838	Negative	Resistant	Leu 511 Pro, Asp 516 Gly	>8
9839	Negative	Not available†	Ser531Leu	>8
9840	Positive	Resistant	Ser531Leu	>8
9841	Negative	Resistant	Ser531Leu	>8
9842	Positive	Resistant	Ser531Leu	>8
9843	Negative	Resistant	Leu533 Pro	>8
9853	Positive	Resistant	Ser531Leu	>8
9854	Negative	Resistant	Leu533 Pro	>1
9855	Negative	Resistant	Ser531Leu	>8
9857	Negative	Resistant	Ser531Leu	>8
9861	Positive	Resistant	Ser531Leu	>8
9865	Positive	Resistant	Ser531Leu	>8
9869	Positive	Resistant	Leu 511 Pro, Asp 516 Gly	>8
9871	Negative	Resistant	Leu 511Pro	>8
9874	Positive	Resistant	Ser531Leu	>8
9877	Negative	Resistant	Leu533 Pro	>8
9899	Negative	Susceptible	wild type	<1

* Numbering according to Telenti et al.[7]
† No hybridisation product was observed from sample 9839.
AFB = acid-fast bacilli.

viously subjected to drug susceptibility testing (Public Health Laboratory Service, Mycobacterium Reference Centre, Dulwich, UK) were grown on LJ slopes and tested for susceptibility to RMP and streptomycin by MPRA. Previously determined susceptibility results were not available to the laboratory performing MPRA. Results from the assay were obtained within 48 hours. In susceptible strains replication of phages was inhibited in the presence of the drug and plaques were not observed above critical concentrations, whereas resistant strains supported phage replication and plaques were seen at high drug concentrations. Laboratory strains of *M. tuberculosis* and BCG were used as susceptible controls. Experiments containing up to three times the number of cfu (colony forming units) yielded consistent results, demonstrating that the concentration of bacteria was not critical for determination of resistance (results not shown). Isolates sub-cultured and grown at 37°C for between 2 and 13 weeks gave consistent results, however slopes stored at 4°C did not provide sufficient pfu to allow assessment of drug susceptibilities (results not shown).

Correlation of results from the 18 patients previously tested by LiPA, BACTEC and the proportional method showed that 100% of resistant strains were detected by MPRA (Table 2, Study 1). One discrepant result was obtained where a strain found sensitive to streptomycin by the proportional method was found resistant to 2 μg/ml but sensitive at 10 μg/ml by MPRA.

In a separate study of 19 isolates, 100% of strains resistant to RMP or streptomycin were detected by MPRA (Table 2, Study 2). One discrepant result was obtained where a strain found resistant to streptomycin by MPRA had previously been found sensitive by routine screening; however, on re-testing by MPRA the strain was found sensitive at 2 μg streptomycin/ml.

Table 2 Summary of susceptibilities obtained by MPRA compared to culture-based methods

	RMP^S	RMP^R	SM^S	SM^R
Study 1 (n = 18)				
MPRA	1	17	4	14*
Culture	1	17	5	13
Study 2 (n = 19)				
MPRA	19	0	13	6†
Culture	19	0	14	5

* Discrepant strain resistant at 2 μg/ml, but sensitive at 10 μg/ml.
† On re-testing, the discrepant strain was found to be sensitive to streptomycin.
MPRA = microwell phage replication assay; RMP^S = susceptible to rifampicin; RMP^R = resistant to rifampicin; SM^S = susceptible to streptomycin; SM^R = resistant to streptomycin.

DISCUSSION

Increased levels of drug-resistant tuberculosis have been reported from low-income countries.[20] The high level of drug resistance is associated with poor treatment programmes in this setting, where the most frequently reported resistance to anti-tuberculosis drugs is against RMP.[21] Resistance to RMP is often associated with resistance to other anti-tuberculosis drugs, and strains resistant to at least rifampicin and isoniazid are classified as MDR. Infection with such strains may render standard anti-tuberculosis therapy inappropriate.[22] This study was conducted in response to requests from clinicians for a rapid assessment of suspected MDR cases of tuberculosis, as validation of

the sensitivity of the assay would facilitate in patient management. The high sensitivity of the LiPA test enabled susceptibility to RMP to be assessed directly in all smear-positive and 10/11 smear-negative specimens, suggesting that the test would be a useful tool both for detection of primary RMP-resistant infection and for the rapid assessment of secondary resistance resulting from poor treatment regimens. In this study all RMP-resistant strains were MDR, being also resistant to at least isoniazid, which would suggest that RMP resistance could be considered a surrogate marker for the detection of MDR in this setting.

In accordance with other studies[8,23] and our own previous observations,[24] the most frequently observed mutation was the Ser 531 Leu mutation. It has previously been suggested that the level of resistance to RMP may be dependent on the type of mutation involved;[25] however, in agreement with previous reports,[8] no correlation between mutation and MIC was observed during this study. Similarly, there was no apparent correlation between the mutation present and susceptibility as determined by MPRA (results not shown).

One specimen that was found resistant by BACTEC methodology failed to provide a result with the LiPA test, although the internal control and IS*6110* target DNA amplification were successful with this specimen. The lower sensitivity of the LiPA test could rest on the fact that the target for amplification is a single copy gene,[26] in contrast to the insertion sequence IS*6110*, which may be present in multiple copies in the *M. tuberculosis* genome.[27] Upon cultivation, this specimen yielded 1–10 colonies on LJ with MIC values of >8 μg/ml. Furthermore, patient records revealed that this patient had received 4–6 months of treatment and was responsive to anti-tuberculosis therapy, as determined by reversion of smears and cultures to negative that remained so for 6 months thereafter.

While we have demonstrated the utility of LiPA for rapid detection of RMP-resistant tuberculosis infection, the high cost and complexity of the techniques renders it unsuitable for routine use in low-income countries. We have demonstrated the use of MPRA as a rapid method of screening *M. tuberculosis* isolates grown on LJ slopes for susceptibility to RMP and streptomycin. Genotypic tests for resistance to streptomycin are not yet available, and the ability to rapidly test susceptibility to this first-line anti-tuberculosis drug will be of advantage in the assessment of MDR infection. The rapidity of this method compared to other currently available low-cost methods may enable improved management of suspected MDR patients in poor resource settings.

Although phages were used to demonstrate susceptibility of mycobacteria to anti-tuberculosis drugs 20 years ago,[9] their use for routine testing was not explored further until the development of luciferase reporter phages by Jacobs et al. in 1993.[28] A susceptibility test using D29 phages has previously been described in which the bactericidal effect of anti-tuberculosis drugs was assessed following a 3-day incubation period.[29] However, the application of proportional methodology for interpretation of the results requires quantification of both bacteria and pfu, which is technically difficult and labour intensive. The MPRA is simple to perform, and the 96-well format facilitates the processing of large numbers of strains; the result is obtained by a single visual readout and is available within 48 hours. The test does not utilise expensive or sophisticated equipment or reagents such as required by BACTEC, MGIT or LiPA, and stocks of phages and *M. smegmatis* indicator cells may be maintained in house, thus reducing dependence on imported reagents and further facilitating application of this technology in low-income countries. More extensive validation studies and the investigation of other anti-tuberculosis drugs are to be undertaken. We have previously demonstrated detection of *M. tuberculosis* bacilli in sputum using Mycobacteriophage D29,[11] and direct detection of drug resistance in clinical specimens may prove possible using this technology. However, assessment of susceptibility may require enumeration and speciation of the bacteria present within the specimen, providing an additional challenge for this technology which is currently under investigation.

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RÉSUMÉ

OBJECTIF : Investiguer la détection rapide d'une tuberculose résistante aux médicaments grâce à la technique génotypique inno-LiPA Rif TB associée à un nouveau test de susceptibilité peu coûteux basé sur les bactériophages.
SCHÉMA : On a comparé la performance du test de réplication des phages en microplaques (MPRA) sur 18 isolats de patients suspects de tuberculose à germes multirésistants (MDR) à celle du test LiPA pratiqué directement sur les échantillons d'expectoration. Les mutations du gène *rpoB* identifiées par LiPA et qui confèrent la résistance à la rifampicine ont été confirmées par le séquençage de l'ADN, alors que les sensibilités ont été confirmées par la méthode des proportions et le BACTEC. L'on a inclus pour comparaison 19 isolats supplémentaires subissant un dépistage de routine pour la sensibilité à la rifampicine et à la streptomycine.
RÉSULTATS : La sensibilité à la rifampicine a été déterminée dans 17 des 18 (94,4%) échantillons d'expectoration testés par le LiPA. La corrélation entre MPRA, les méthodes moléculaires et les méthodes conventionnelles a été de 100% pour la détection de la sensibilité à la rifampicine. Toutefois, un résultat discordant a été trouvé en ce qui concerne la sensibilité à la streptomycine : un isolat sensible à la streptomycine par la méthode des proportions s'est avéré résistant à 2 μg/ml de streptomycine par la méthode MPRA. De manière similaire, un isolat initialement résistant par la méthode MPRA a été trouvé sensible lors du test répétitif, en accord avec la méthode conventionnelle.
CONCLUSION : LiPA permet une détection rapide de l'infection à germes résistants aux médicaments, alors que MPRA fournit un test de sensibilité simple et n'exigeant pas une technologie avancée, ce qui peut favoriser son application dans les pays à faibles revenus.

RESUMEN

OBJETIVO : Investigar la detección rápida de la tuberculosis resistente a las drogas mediante el empleo del genotipo Inno-LiPA Rif TB y un nuevo test de sensibilidad de bajo costo, en base a bacteriófagos.

MÉTODO : Se comparó la eficacia del test de replicación de fagos en microplacas (MPRA) en 18 cepas de pacientes sospechosos de tener tuberculosis multirresistente (MDR) con el test LiPA efectuado directamente en muestras de esputos. Las mutaciones en el gen *rpoB* identificadas por LiPA y que confiere resistencia a la rifampicina fueron confirmadas por las secuencias del ADN, mientras que la sensibilidad fue confirmada por el método de las proporciones y el BACTEC. Otras 19 cepas sometidas a un catastro de rutina para sensibilidad a la rifampicina y a la estreptomicina fueron incluidas para la comparación.

RESULTADOS : La sensibilidad a la rifampicina fue determinada en 17/18 (94,4%) de las muestras de esputos controladas por LiPA. La correlación entre MPRA, métodos convencionales y moleculares fue del 100% para la detección de la sensibilidad a la rifampicina. Sin embargo, se halló un resultado discrepante para la sensibilidad a la estreptomicina : una cepa sensible a la estreptomicina por el método de las proporciones fue hallada resistente por MPRA a 2 μg/ml de estreptomicina. En forma similar, una cepa con resistencia inicial por MPRA, en forma repetida, fue hallada sensible con los métodos convencionales.

CONCLUSIÓN : LiPA permite una rápida detección de la infección con gérmenes resistentes, mientras que MPRA ofrece un test de la sensibilidad simple, de tecnología sencilla, que puede ser adecuado para su aplicación en los países de bajos recursos.