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Review

Implementation of rapid colorimetric methods for drug resistance detection in *M. tuberculosis*: perspectives for low-resource settings

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ABSTRACT

It is estimated that one third of the world population is latently infected with *Mycobacterium tuberculosis* and drug resistance and multidrug resistance, defined as resistance to at least isoniazid (INH) and rifampicin (RIF), two critical drugs in the treatment of tuberculosis, is also of major concern for the control of the disease, stressing the need for early detection of drug resistant and multidrug resistant strains of *M. tuberculosis*. During the last decade several new approaches have been proposed for the early and rapid detection of drug resistant tuberculosis, many of them have relied on rapid molecular techniques that due to their cost and requirement for skilled personnel have not gained access yet to the clinical diagnostic laboratory, especially in developing countries where the situation of tuberculosis and multidrug resistance constitute a major problem. The present review will address several new colorimetric approaches recently proposed for the rapid detection of drug resistance in TB that due to their simplicity and low cost could constitute good alternatives for low-resource countries.

KEYWORDS: tuberculosis, drug resistance, colorimetric, nitratase, rapid methods

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INTRODUCTION

According to World Health Organisation (WHO) estimates, one third of the world population is latently infected with *Mycobacterium tuberculosis*. The last WHO report states that in 2003 there were 8.8 million new cases of tuberculosis (TB) and 1.7 million deaths were attributed to the disease [1]. This scenario is compounded by the human immunodeficiency virus (HIV) pandemic with almost 13 million people currently co-infected with HIV and TB [2]. Also, the emergence of multidrug resistant tuberculosis (MDRTB), defined as resistance to at least isoniazid (INH) and rifampicin (RIF), two critical drugs in the treatment of TB, is a major concern for the control of the disease. The last report of the WHO on drug resistance in the world, states that MDRTB reached levels of up to 14% amongst new patients and values as high as 50% in previously treated patients in some settings [3]. Thus, early detection of drug resistance represents an important component of the TB control programmes.

Although during the last decade several new approaches have been proposed for the early and rapid detection of drug resistant TB, many of them have relied on rapid molecular techniques that due to their cost and requirement for skilled personnel have not gained access yet to the clinical diagnostic laboratory, especially in developing countries where the situation of TB and MDRTB constitute a major problem. The present review will deal with several colorimetric approaches recently proposed for the rapid detection of drug resistance in TB that due to their simplicity and low cost could constitute good alternatives for low-resource countries.

Conventional methods for detecting drug resistance in TB

The so-called conventional methods for drug resistance detection in *Mycobacterium tuberculosis* are based on detection of morphological growth on solid culture media. These methods that include the proportion method, the resistance ratio method, and the critical concentration method are performed on Löwenstein-Jensen (LJ) medium or Middlebrook agar. Later on, a radiometric method based on the BACTEC TB-460 system was introduced and these four methods constitute the gold standard for diagnosis of drug resistance in clinical mycobacteriology laboratories around the world. However, they can be cumbersome and time-consuming, since they require at least 3 to 6 weeks for giving results [4, 5] or are faster, like the BACTEC TB-460 radiometric method, but require the use of heavy equipment and radioisotopes [6].

Newer developments for drug resistance detection in *M. tuberculosis*

In the last decade several new technologies have been proposed and applied for the rapid detection of drug resistance in TB. Among them, the Mycobacterial Growth Indicator Tube (MGIT), a commercial system, both in its manual version or automated format, the BACTEC MGIT960 (Becton Dickinson, Sparks, Maryland, USA) are reliable and relatively rapid methods for detecting drug resistance in TB [7, 8]. Also, commercially available, the E-test based on impregnated strips with antibiotics to be applied directly on solid medium [9] and the phage-based method commercially available as the *FastPlaque* test (Biotest, UK) [10] have been evaluated for rapid detection of drug resistance, especially to rifampicin, with good results.

Other techniques like the INNO-LIPA-Rif.TB (Innogenetics, Ghent, Belgium) and GenoType MTBDR (Hain Lifescience, Nehren, Germany), both based on solid-phase hybridization assays, have also been introduced with good results especially for rifampicin and isoniazid resistance detection [11, 12]. On the other hand, PCR-sequencing, including new designs involving real-time PCR, continues to be the reference method for molecular assignment of drug resistance [13]. However, these methods, especially sequencing, are not readily available and require sophisticated, expensive equipment and/or highly skilled personnel. These disadvantages have precluded their implementation on a routine basis in most diagnostic laboratories in high-burden countries.

The colorimetric methods for detecting drug resistance in TB

Colorimetric methods for detecting drug resistance in *M. tuberculosis* were introduced a few years ago. They are based on the reduction of a coloured redox indicator added to the culture medium after *M. tuberculosis* has been exposed *in vitro* to different antibiotics. Resistance is detected by a change in colour of the indicator, which is directly proportional to the number of viable mycobacteria in the medium. Among the main reagents that have been used, tetrazolium salts or redox indicators like Alamar blue (Trek Diagnostics, Ohio, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and resazurin have been proposed for the rapid detection of resistance to various antituberculosis drugs and as rapid methods for drug susceptibility testing (DST) [14, 15, 16]. More recently another simple format of colorimetric method, the nitrate reductase assay has been proposed as a rapid method to detect drug resistance in TB [17, 18]. In the following sections we will briefly review each of the available formats and the studies performed to detect drug resistance in TB.

Rapid detection of *M. tuberculosis* drug resistance with Alamar blue

In 1995 Yajko *et al.* proposed for the first time a simple colorimetric method for quantitative measurement of *M. tuberculosis* susceptibility to antimicrobial drugs making use of the oxidation-reduction dye Alamar blue as an indicator of growth [14]. Alamar blue is a proprietary reagent that had been used before to study metabolism and viability in other micro-organisms [19, 20], and to measure toxicity in eukaryotic and prokaryotic cells [21]. The study showed that minimal inhibitory concentrations (MICs) of isoniazid (INH), rifampicin (RIF), streptomycin (SM), and ethambutol (EMB) could be determined within two weeks of incubation in 50 strains of *M. tuberculosis.* When compared to results obtained by the agar proportion method, a higher resistance was associated with higher MIC levels obtained by the Alamar blue colorimetric method. Overall, there was an agreement of 97% between the two methods.

The Alamar blue colorimetric method was also used by Collins & Franzblau in a microplate format for high-throughput screening of compounds against *M. tuberculosis* and *M. avium* [22] and by Shawar *et al.* for rapid screening of natural products for antimycobacterial activity [23]. Both studies confirmed the usefulness of the Alamar blue colorimetric method as a rapid and simple technique to screen for activity of antimycobacterial compounds. Franzblau *et al.* further applied this method to determine MICs of INH, RIF, SM and EMB in clinical isolates of *M. tuberculosis* [24]. A further simplification of the method was done by Palomino & Portaels who introduced one critical concentration of the first-line anti-tuberculosis drugs in a microtiter format performed with clinical isolates [25]. Additional studies using Alamar blue for detecting drug resistance in *M. tuberculosis* have been performed since then with similar good results [26-28].

The use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for rapid drug resistance detection in TB

Another compound commonly used now for detecting drug resistance in TB is the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). This compound was initially proposed by Abate and Mshana in a colorimetric assay for rapid detection of rifampicin resistance [29, 30]. They showed that a linear relationship existed between the number of viable bacteria and the ability to reduce the MTT compound. The study was performed in tubes and the reduction of MTT to blue formazan was quantified after six days of incubation with a spectrophotometer and also interpreted by the naked eye. The results obtained with the MTT assay matched completely those obtained with the BACTEC radiometric method used as the gold standard.

More recently the MTT assay was slightly modified to allow interpretation of INH resistance, not obtained before by Abate *et al.* In this modification the MTT assay was completed in four days for RIF and within 7 days for INH giving a specificity and sensitivity of 99.5 and 89.2% respectively when compared to the proportion method [31]. In an effort to implement a rapid and inexpensive method for determining MICs of several drugs for multidrug-resistant *M. tuberculosis*, the MTT assay has been also set up as a microplate-based assay (M-MTT) and evaluated with clinical isolates of *M. tuberculosis.* There was a good correlation as compared to the proportion method in 7H11 agar when testing for kanamycin, capreomycin, ethionamide, para-aminosalicylic acid, clarithromycin, cycloserine, clofazimine, levofloxacin and rifabutin [32].

Also, with the purpose of speeding up the detection of drug resistance in clinical samples, the MTT assay has recently been applied directly in sputum samples for rifampicin resistance detection. The sensitivity and specificity of this direct MTT assay matched those of the standard indirect DST on 7H10 agar with 98.5% of the samples giving interpretable results within 2 weeks [33].

The introduction of resazurin for detection of drug resistance in TB

The third and most recent introduction for colorimetric determination of drug resistance in *M. tuberculosis* is the use of resazurin as the indicator of growth. Resazurin is an oxidation- reduction indicator that has been used before to assess viability and bacterial contamination and to test for antimicrobial activity [34-36]. Since Alamar blue was eventually identified as resazurin in cell cytotoxicity studies [37], Palomino *et al.* introduced the use of resazurin for detection of multidrug resistant TB in a simple microtiter assay, the REMA plate, which showed a very good agreement with results obtained with the gold standard proportion method in LJ [38]. The REMA plate was later adapted for rapid detection of resistance to second-line anti-tuberculosis drugs and ofloxacin [39, 40]. Also, very recently the REMA plate has been used in a modified assay using nicotinamide for detection of resistance to pyrazinamide, an anti-tuberculosis drug not easy to test *in vitro* since it is only active at an acid pH [41]. The main advantage in using resazurin as an indicator of growth is that it is a non-proprietary reagent, very easy to obtain and of very low cost that could be very important for the introduction of this type of techniques in low-resource countries.

A further simplification of this test has been recently proposed by Coban *et al.* by performing the test in screw-cap tubes with good results [42]. Recent publications have also reported the implementation of the REMA plate in high-burden low-resource countries with good results [43, 44].

The nitrate reductase assay (NRA) for rapid detection of drug resistance

A new colorimetric approach for the rapid detection of drug resistance in TB is by the nitrate reductase assay (NRA) based on the capacity of *M. tuberculosis* to reduce nitrate to nitrite. The NRA also known as the Griess method is an old technique used in the differentiation of *M. tuberculosis* from other mycobacterial species [5]. A recent re-evaluation of this technique allowed its introduction as a rapid method for detecting drug resistance in TB [17, 18]. Reduction of the nitrate could be detected using specific reagents, which produce a colour change. When testing a panel of *M. tuberculosis* strains with various resistance patterns, the sensitivity and specificity of the NRA as compared to the BACTEC 460 method were 100 and 100% for RIF, 97 and 96% for INH, 95 and 83% for SM, and 75 and 98% for EMB. These results were mostly available in 7 days. The NRA could correctly identify most resistant and sensitive *M. tuberculosis* strains. In an attempt to simplify and speed up results, performance of the NRA in liquid medium has also been proposed but only for INH and RIF [45]. Also, a microplate nitrate reductase assay has been evaluated with clinical isolates and compared to the proportion method and Alamar blue assay [46].

A practical approach for an easier introduction of these colorimetric methods would be its application directly on clinical samples. In this direction are two recent studies reporting the application of the NRA directly in sputum samples. Musa *et al.* evaluated the NRA for DST of *M. tuberculosis* directly from smear-positive sputum samples containing more than ten acid-fast bacilli per microscopy field [47]. Sensitivity and specificity as compared to the direct proportion method were 100 and 100%, 93 and 100%, 76 and 100%, and 55 and 99% for RIF, INH, SM, and EMB respectively, with results in most cases available in 14 days. A similar study has been performed by Solis *et al.* comparing the sensitivity and specificity of the direct NRA with the proportion method in LJ for determining resistance to INH and RIF among clinical specimens. In this case the sensitivity and specificity of the NRA for INH resistance was 99.1% and 100% and for RIF resistance 93.5% and 100% respectively [48]. Both studies have shown the feasibility of implementing the NRA as a direct method in sputum samples.

Validation of colorimetric methods for drug resistance detection in *M. tuberculosis*

After the introduction of colorimetric methods using redox indicators or the nitrate reductase assay, there have been a few studies with the purpose of validating these techniques in different settings or comparing them to other techniques used in different laboratories. A first attempt to do this was performed by Lemus *et al.* who studied the performance of the colorimetric methods using MTT, resazurin and nitrate reduction for the detection of RIF resistance in a panel of 20 coded *M. tuberculosis* strains tested blindly [49]. The results obtained were compared with those of the gold standard methods: the proportion method on LJ medium and the BACTEC TB 460 system. Additionally, the results were validated with two commercial tests: the MGIT system and the INNO LiPA Rif.TB kit. A complete agreement was obtained among all methods. Another study performed by Montoro *et al.* validated the same three techniques in one hundred *M. tuberculosis* clinical isolates comparing the results with those obtained with the gold standard proportion method on LJ medium [50]. A sensitivity and specificity between 94% and 100% was obtained for the detection of RIF and INH resistance. There was also a high level of agreement of these three colorimetric methods compared with the gold standard proportion method.

One important step in the introduction of new techniques in general, and of these colorimetric methods in particular, will be to assess their reproducibility and robustness; this could be better assessed through simultaneous studies performed in different settings. In connection with this, there have been two multicentre evaluations recently carried out to assess the performance of these methods. Martin *et al.* performed a multicentre study to assess the colorimetric methods using MTT and resazurin for rapid DST of first-line anti-tuberculosis antibiotics [51]. The study was performed in seven laboratories located in six countries in Latin America. Each site received thirty coded *M. tuberculosis* strains and performed the colorimetric assays using MTT and resazurin blindly with the first-line drugs RIF, INH, EMB and SM. MICs obtained were compared to the conventional proportion method on LJ medium. Excellent results were obtained for RIF, INH and EMB, with levels of specificity and sensitivity between 96% and 99%. The second study, also from the same group, performed a multicentre evaluation of the NRA [52]. The study was performed in five laboratories in different countries and the results were compared with those obtained with the gold standard proportion method on LJ medium. The NRA was highly accurate in detecting resistance to RIF, INH and EMB with an accuracy of 98%, 96.6% and 97.9%, respectively. The assay proved easy to implement in countries with limited laboratory facilities.

Table 1 shows the major studies performed during the introduction, evaluation and validation of colorimetric methods using redox indicators or the NRA as presented in the text.

Implementation of colorimetric methods for drug resistance detection in *M. tuberculosis*

Most of the studies described above have been mainly performed and evaluated with *M. tuberculosis* isolates while just a few have been applied directly in clinical specimens. Furthermore, for the proper implementation of these new colorimetric methods for rapid drug resistance detection in TB, they should be preferentially evaluated in well-designed and controlled clinical trials and tested in high-endemic, low-resource settings where the implementation and use of these methods are most needed.

There are some directions to better accomplish this as has been proposed in two

Table 1. Major studies performed with colorimetric methods

Reference	Method	Type of study	Performed on	Antibiotics
Yajko *et al.* 1995	Alamar blue	Proof of principle	Clinical isolates	RIF, INH, SM, EMB
Collins *et al.* 1997	Alamar blue	Proof of principle	*M. tuberculosis* *M. avium*	Thirty antimicrobials
Shawar *et al.* 1997	Alamar blue	Application	*M. bovis* *M. intracellulare*	Natural products
Franzblau *et al.* 1998	Alamar blue	Application	Clinical isolates	RIF, INH, SM, EMB
Palomino *et al.* 1999	Alamar blue	Application	Clinical isolates	RIF, INH, SM, EMB
Bastian *et al.* 2001	Alamar blue	Application	Clinical isolates	KM
Pracharktam *et al.* 2001	Alamar blue	Application	Clinical isolates	OFX, LVX
Sungkanuparph *et al.* 2002	Alamar blue	Application	Clinical isolates	INH, RIF, EMB
Mshana *et al.* 1998	MTT	Proof of principle	Clinical isolates	RIF
Abate *et al.* 1998	MTT	Proof of principle	Clinical isolates	RIF
Foonglada *et al.* 2002	MTT	Proof of principle	Clinical isolates	RIF, INH
Morcillo *et al.* 2004	MTT	Application	Clinical isolates	Nine antibiotics
Abate *et al.* 2004	MTT	Proof of principle	Sputum samples	RIF
Lemus *et al.* 2004	MTT	Validation	Clinical isolates	RIF
Montoro *et al.* 2005	MTT	Validation	Clinical isolates	INH, RIF, SM, EMB
Martin *et al.* 2005	MTT	Multicentre study	Clinical isolates	INH, RIF, SM, EMB
Palomino *et al.* 2002	Resazurin	Proof of principle	Clinical isolates	RIF, INH
Martin *et al.* 2003	Resazurin	Application	Clinical isolates	ETH, KM, CAP, OFLX, PAS
Lemus *et al.*2004	Resazurin	Validation	Clinical isolates	RIF
Montoro *et al.* 2005	Resazurin	Validation	Clinical isolates	INH, RIF, SM, EMB
Martin *et al.* 2005	Resazurin	Application	Clinical isolates	OFLX
Martin *et al.* 2005	Resazurin	Multicentre study	Clinical isolates	INH, RIF, SM, EMB
Martin *et al.* 2006	Resazurin	Proof of principle	Clinical isolates	PZA
Coban *et al.* 2006	Resazurin	Application	Clinical isolates	INH, RIF
Nateche *et al.* 2006	Resazurin	Evaluation	Clinical isolates	INH, RIF
Umubyeyi *et al.* 2006	Resazurin	Evaluation	Clinical isolates	OFLX
Ängeby *et al.* 2002	NRA	Proof of principle	Clinical isolates	INH, RIF, SM, EMB
Panaiotov *et al.* 2002	NRA	Proof of principle	Clinical isolates	INH, RIF, SM, EMB
Syre *et al.* 2003	NRA	Proof of principle	Clinical isolates	INH, RIF
Lemus *et al.* 2004	NRA	Validation	Clinical isolates	RIF
Montoto *et al.* 2005	NRA	Validation	Clinical isolates	INH, RIF, SM, EMB
Martin *et al.* 2005	NRA	Multicentre study	Clinical isolates	INH, RIF, SM, EMB
Kumar *et al.* 2005	NRA	Proof of principle	Clinical isolates	INH, RIF, EMB
Musa *et al.* 2005	NRA	Proof of principle	Sputum samples	INH, RIF, SM, EMB
Solis *et al.* 2005	NRA	Proof of principle	Sputum samples	INH, RIF

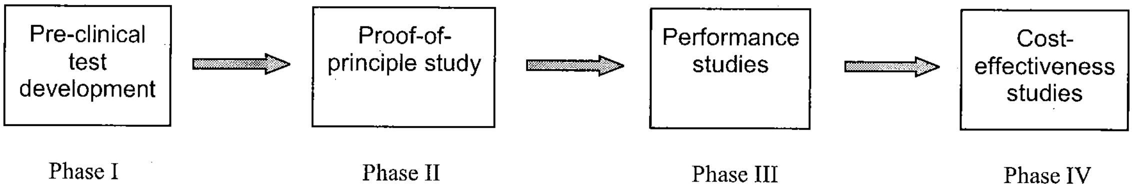

Fig. 1. Multi-phase development proposed for new diagnostic methods.

previous publications [53, 54]. Some common errors in the design or reporting of trials of TB diagnostics have included: not describing the selection criteria for sampling or an inadequate sample size, giving positive and negative predictive values without taking into account the population for which the test was intended, the use of inadequate gold standards and not stating when the test under study is indicated. Ideally, studies should be performed following a multi-phase sequence as is done for drug and vaccine development. A phase I study would involve the pre-clinical test development, phase II will carry out proof-of-principle studies that can distinguish a positive from a negative sample in easily accessible populations, phase III should demonstrate the performance of the test in the target populations, and phase IV will study the cost-effectiveness and impact of the new tests in comparison with existing tools. Figure 1 shows a diagram summarizing the different phases for testing new methods. Furthermore, as has been stressed recently, efforts should be made to implement good laboratory practice (GLP) and good clinical practice (GCP) rules in well-designed diagnostic trials for evaluation of these new tools [55]. As long as all new tests, like the colorimetric methods here described, follow this path they will be closer for a smooth implementation in TB diagnostic laboratories especially in high-burden countries where they are more necessary.

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