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Exploring Different Methods for Performing Drug Susceptibility Testing of Fluoroquinolones in *M. tuberculosis.*

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ABSTRACT

Fluoroquinolone antibiotics are among the most potent second-line drugs used for treatment of multidrug-resistant tuberculosis and resistance to this class of antibiotics is one criterion for defining extensively drug resistant tuberculosis. Fluoroquinolones are also used for drug-susceptible tuberculosis in patients unable to tolerate first-line agents. Widespread Fluoroquinolone use in the community has resulted in Fluoroquinolone resistance in *Mycobacterium tuberculosis.* Despite this, Fluoroquinolone drug susceptibility testing is rarely performed in non- multidrug-resistant TB. The existing global evidence suggests that FQ resistance remains largely confined to MDR-TB strains. Currently there are many phenotypic and Genotpypic methods available for determining resistance to Fluoroquinolone. However, some of these techniques involve expensive equipment and infrastructure. Hence, in the current study, different methods were explored and the most reliable and feasible techniques in Mycobacterial laboratories have been discussed.

Keywords: Multi drug resistant tuberculosis, Fluoroquinolone Drug Susceptibility Testing

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INTRODUCTION

M. tuberculosis is the etiologic agent of tuberculosis (TB), a potentially fatal illness which results in approximately 2 million deaths worldwide each year [1]. Pulmonary tuberculosis is the commonest clinical presentation of tuberculosis, and sputum-positive cases are the most important sources of infection in the community. Since 2005 the global tuberculosis epidemic has been on the decline, yet the global incidence of tuberculosis has been growing slowly and much faster in sub-Saharan Africa, East Mediterranean and South-East Asia regions [2]. Multi drug resistant tuberculosis (MDR-TB), defined as in vitro resistance to at least isoniazid and rifampicin, is decreasing the effectiveness of standard treatments and leading to increased mortality. The first-line antituberculous drugs i.e isoniazid, rifampicin, pyrazinamide, ethambutol and Streptomycin are the most active agents with proven clinical efficacy that form the core of initial standardized treatment regimens. The WHO recommends second-line anti-tuberculous drugs for those with MDR-TB or people intolerant of first-line drugs [2]. WHO categorizes fluoroquinolones as bactericide drugs and recommends their usage in tuberculosis patients in which relapse has occurred because of therapy failure [3]. FQs are currently in use as second-line drugs in the treatment of MDR-TB. The WHO guidelines for the treatment of MDR-TB includes a second-line injectable agent from Group 2, a fluoroquinolone from Group 3 (Moxifloxacin (Mfx), levofloxacin (Lfx), ofloxacin (Ofx)), and then adding additional drugs from Groups 4 and 5 to create a treatment regimen with at least 4-5 active drugs. Fluoroquinolones are fluorine-containing nalidixic acid derivatives discovered as a by-product of the antimalarial chloroquine, characterized by broad-spectrum antimicrobial activity. They have been included in antituberculous regimens (particularly for MDR-TB) since the late 1980s, but the role of fluoroquinolones in tuberculosis treatment still remains controversial.

Ofloxacin, levofloxacin, and moxifloxacin are fluorinated quinolones, a synthetic class of antibiotics [4]. The fluoroquinolones are generally well-absorbed after oral administration, with bioavailabilities of 85-95% for ofloxacin, ≥99% for levofloxacin, and 86 - 91.2% for moxifloxacin in healthy adults. Newer fluoroquinolones, including moxifloxacin and gatifloxacin, exhibit potent activity against *M. tuberculosis*, and show potential to shorten the duration for TB treatment [5]. The favorable combination of pharmacodynamic and pharmacokinetic characteristics of fluoroquinolones may be enhanced when added to antituberculous regimens. It adds to the bactericidal and sterilizing effect of the combination therapy by inhibiting DNA-gyrase and increasing penetration into the infection loci. It also improves adherence to treatment due to the potentially better safety profile as compared with the first-line drugs and by allowing shorter courses of antituberculous treatment. Inspite of having such advantages, fluoroquinolones also have the potential to do harm. They are known to cause, Increase liver and central nervous system (CNS) toxicity of antituberculous drugs and cause clinically significant drug interactions with antituberculous, anti-HIV and other drugs, resulting in reduced efficacy and potential toxicity. They also cause additional adverse drug reactions, such as musculoskeletal damage, gastrointestinal problems (pseudo- membranous colitis), cardiac arrhythmias, infections from fungi or bacteria, psychosis, and convulsions. Further, it is also known to induce resistance in *M. tuberculosis*, which may become cross-resistant to all the representatives of the fluoroquinolone class. The problem of resistance to fluoroquinolones is further complicated by the broad indications of this class of antimicrobials in treatment of various lower respiratory tract and other infections. This may at least be partially responsible for the rising resistance rates among *M. tuberculosis* strains to fluoroquinolones [2]. Levofloxacin is the *l*-isomer and more active component of the racemate ofloxacin and therefore has approximately twice the activity of ofloxacin against most bacterial pathogens. Moxifloxacin has an added methoxy-group, which increases its affinity for DNA gyrase and Topoisomerase IV. Ofloxacin is generally considered to be a second-generation FLQ, levofloxacin a second or third-generation, and moxifloxacin a fourth-generation FLQ [4]. Bacterial DNA gyrase is an important target of antibacterial agents, including fluoroquinolones. DNA gyrase is an ATP-dependent enzyme that acts by creating a transient double-stranded DNA break. It is unique in catalyzing the negative supercoiling of DNA and is essential for efficient DNA replication, transcription, and recombination. DNA gyrase is a tetrameric A2B2 protein. The A subunit carries the breakage-reunion active site, whereas the B subunit promotes ATP hydrolysis [6]. Fluoroquinolones cause inhibition of topoisomerase II or DNA gyrase and topoisomerase IV of bacterium [3]. In *M. tuberculosis*, only type II topoisomerase (DNA gyrase) is present and, thus, is the only target of fluoroquinolone activity [6]. The *M. tuberculosis* genome analysis has identified a gyrB-gyrA contig in which gyrA and gyrB encode the A and B subunits, respectively. *M. tuberculosis* DNA gyrase is thus a validated target for anti-tubercular drug discovery [7]. The main mechanism of development of fluoroquinolone resistance in *M. tuberculosis* is by chromosomal mutations in the quinolone resistance-determining region (QRDR) of *gyrA* or *gyrB*. The most frequent mutations found are at position 90 and 94 of *gyrA* but mutations at position 74, 88 and 91 have also been

reported [6]. One interesting finding reported that the simultaneous occurrence of mutations T80A and A90G in *gyrA* led to hyper susceptibility to several quinolones. This finding could point out that the problem of fluoroquinolone resistance in *M. tuberculosis* might be more complex than was thought initially. Cross-resistance is assumed to occur between fluoroquinolones although isolated reports have acknowledged the presence of strains resistant to gatifloxacin and moxifloxacin that were still susceptible to ofloxacin. Also, the involvement of efflux mechanisms has been suggested as a possible cause for fluoroquinolone resistance in *M. tuberculosis* [6]. The development of drug resistance in association with low $CD4^+$lymphocyte levels may be associated with the rapid proliferation of *M. tuberculosis* for longer periods of time in patients with decreased cell-mediated immunity, which allows accelerated acquisition of clinically significant resistance [8].

MATERIALS AND METHODS

In vitro methods used for Fluroquinolone susceptibility testing was searched using the internet. Key words such as "Fluroquinolone", "Fluroquinolone susceptibility testing" and "antitubercular drug susceptibility testing" were used for the search. Research articles which described novel methods of testing Fluroquinolone susceptibility testing were reviewed. Definitive diagnosis of drug-resistant TB requires that *M. tuberculosis* be detected and resistance to anti-TB drugs determined. This can be done by isolating the bacteria by culture, identifying it as belonging to the *M. tuberculosis* complex (MTBc), and conducting drug susceptibility testing (DST) using solid or liquid media or by performing a WHO endorsed molecular test to detect TB DNA and mutations associated with resistance [9].

In vitro susceptibility testing utilizing solid media

Conventional drug susceptibility testing (DST) with solid media such as Löwenstein-Jensen (LJ) or Middlebrook 7H10 agar requires 3 or more weeks to be completed. Indirect proportion method is recommended by WHO (Table 1). This interpretation gives susceptibility results at the 1% proportion basis i.e. proportions of the number of colonies in the study-drug media relative to the number of colonies in the drug-free medium are determined; M. tuberculosis isolates with proportions of >1% determined were considered to be resistant [10]. Ginsburg AS et al (2003) studied Fluoroquinolone resistance in 55 patients with newly diagnosed tuberculosis by PM method and reported high incidence of *M. tuberculosis* fluoroquinolone resistance with newly diagnosed tuberculosis, particularly among patients with prior fluoroquinolone exposure [8]. Considering the potential use of gatifloxacin and moxifloxacin in the treatment of tuberculosis, Somasundaram S in 2006 studied resistance to both these drugs in Fifty *M. tuberculosis* isolates using absolute concentration method on Lowenstein-Jensen medium (LJ), the proportion susceptibility testing method (PST) on LJ and 7H11 agar media, and the BACTEC radiometric method. The results showed acceptable concordance between the tested methods and proved that the Minimal Inhibitory Concentration (MIC) of gatifloxacin and moxifloxacin were much lower than the MICs of other quinolones like ofloxacin and ciprofloxacin. Additionally, these two drugs have shown a low mean MIC and low concentration as a definition of resistance, which might help in treating the patients with low levels of quinolone resistance [11]. In another investigation by Sulochana S et al (1999), the conventional drug susceptibility testing procedures using the 1% proportion method on Löwenstein-Jensen (LJ) and Middlebrook 7H10/7H11 agar media were compared with a recent radiometric method. There was a 100 per cent agreement with the conventional MIC method by the proportion tests on L-J as well as on 7H11 media. The BACTEC radiometric method, at the same concentration, yielded 98 per cent agreement. Thus, any of these methods could be used depending upon the infrastructure available [12].

In vitro susceptibility testing utilizing liquid media

Broth-based DST methods provide significantly rapid and reliable results. In recent times, many automated liquid culture systems have been developed and their performance has been evaluated against the traditional "gold standard" for TB culture work which is LJ medium [13]. The radiometric BACTEC method (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.), introduced in 1980, is considered the standard method for DST for both first and second line anti-tuberculosis drugs. The procedures for DST using the broth-based Bactec 460 TB system are well established; however, there are increasing concerns about the disposal of the radioactive material with the use of this system. The recently introduced non radiometric Bactec Mycobacterial Growth Indicator Tube (MGIT) 960 System (Becton Dickinson Diagnostic Systems) has been reported to be equivalent to the radiometric system in performance. A study by Rodrigues C et al., (2008)

helped to establish a rapid and reliable DST method by using the Bactec MGIT 960 test procedures and the critical test concentrations for the second-line anti-tuberculosis drugs that are being used to treat drug-resistant cases in their area [14]. The current status of DST methodology and critical concentrations for second-line DST recommended by WHO [15] [25] is given in Table No: 1.

Table 1: Current status of DST methodology and critical concentrations for second-line DST

Drug group[a]	Drug	DST method available	DST critical concentrations (µg/ml)			
			Löwenstein Jensen[b]	Middle brook 7H10[b]	Middle brook 7H11[b]	MGIT960
Group 3 Fluoroquinolones	Ofloxacin	Solid, liquid	4.0	2.0	2.0	2.0
	Levofloxacin	Solid, liquid	-	1.0	-	1.5
	Moxifloxacin	Solid, Liquid	-	0.5/2.0	-	0.5/2.0
	Gatifloxacin	Solid	-	1.0	-	-

[a] WHO Guidelines for the programmatic management of drug-resistant tuberculosis.
[b] Indirect proportion method recommended. Other solid media methods (resistance ratio) have not been adequately validated for second-line drugs. Concentrations for the absolute concentration method were not evaluated.

Calorimetric methods to detect FQ resistance

Colorimetric assays employing oxidation-reduction indicators for DST have been previously used with Mycobacteria. Resistance is detected as change of color in the medium. Different indicators have been evaluated, such as MTT-(3- 4,5- dimethylthiazol- 2-yl), XTT (2,3-bis2-methoxy-4-nitro-5-sulfophenyl- 2H-tetrazolium-5-carboxanilide), Alamar blue, Malachite green and Resazurin [16,17] The resazurin microtiter assay (REMA) plate method is well described by Palomino et.al(2002). In 2005, Martin.A demonstrated REMA plate method to be an alternative low-cost and rapid method to detect resistance to OFX. The results obtained in this study are in complete agreement with those obtained by the PM. The microplate format offers many advantages: many DSTs can be performed at the same time, and the method is cost-effective and rapid and gives quantitative (MIC) results. One disadvantage of resazurin assays is the use of liquid media in a microplate format, which might be a biohazard, since aerosols could be generated [18, 19].

Nitrate reduction assay

The nitrate reduction assay which is routinely used for biochemical identification is based on the ability of *M. tuberculosis* to reduce nitrate to nitrite, [20] which is easily detectable with specific reagents, is another very interesting low-cost, rapid method. When used as a drug susceptibility test, the nitrate reduction assay uses the detection of nitrite as an indication of growth. In a study, Martin.A(2005) for the first time evaluated testing of the susceptibility of *M. tuberculosis* to OFX by the nitrate reductase assay and found that all results were concordant with those of the PM and the other methods tested.. They proposed a concentration of 2 µg of OFX/ml for performing DST by the nitrate reductase assay; this is the same concentration used by the other methods. Since the critical drug concentrations for second-line drugs have not been completely established, this study helps to optimize the cutoff value and the ideal OFX concentration for DST. Biosafety problems are limited as the medium used is solid [19].

MODS Assay

The Microscopic observation drug susceptibility assay (MODS) uses liquid media and is fast to perform. The method is based on the principle that *M. tuberculosis* grows faster in liquid medium and its characteristic growth 'cord formation' can be observed through an inverted light microscope. A largest population-based study to examine MGIT 960, MODS and NRA for fluoroquinolone susceptibility testing in *M. tuberculosis* was performed by Devasia RA et.al in 2009. Using ofloxacin, all three methods were highly sensitive and specific compared with the gold standard PM. MODS assay was found to be equally sensitive and specific with MGIT 960 and NRA, and faster for fluoroquinolone susceptibility testing than the PM. Advancing

and standardizing the use of MODS assay for fluoroquinolone susceptibility testing in *M. tuberculosis* are necessary and will be an important tool in the treatment of tuberculosis [21].

Genotypic DST

PCR-based techniques provide new possibilities for the rapid diagnosis of second line drug resistance. The MTBDR*sl* test is a molecular line probe assay for the rapid detection of mutations associated with resistance to fluoroquinolone and aminoglycoside antibiotics and cycloserine. These assays detect mutations in the *gyrA* gene (fluoroquinolone resistance), *rrs* gene (KM, AMK, and CM resistance), and *embB* gene (ethambutol resistance). Published studies have shown that the assay performs well when using culture isolates [22]. The test involves DNA extraction from culture isolates or AFB smear-positive respiratory specimens, followed by PCR amplification and reverse hybridization. It provides a visual reading of test results on nitrocellulose strips. Hillemann D(2009) studied sensitivity and specificity of the new MTBDR*sl* assay for the detection of FLQ, AM, CM, and EMB resistance-associated mutations in 64 culture and direct clinical specimens. The MTBDR*sl* assay produced interpretable results for all of the isolates, and the MTBC-specific control band appeared accurately [23]. Barnard et al (2012) assessed the diagnostic performance of the GenoType MTBDR*sl* LPA on 657 paient specimens and concluded that this assay significantly improved diagnostic yield ($P < 0.001$) while simultaneously decreasing diagnostic delay for reporting second-line DST [22].

The emerging new Polysequencing (PSQ) assay involves three essential parts, namely, amplification of gene segments by PCR, capture of biotinylated single-stranded DNA on streptavidin-Sepharose beads, and sequencing with the PSQ technology and the entire procedure. PSQ assay is a rapid effective method for identifying MTBC and detecting drug resistance mutations from clinical specimens and isolates. Once sufficient data associating specific mutations with MIC distributions have been accumulated, PSQ may become a useful tool for predicting the level of drug resistance [24].

CONCLUSION

In conclusion, it is evident that flouroquinolone DST is gaining importance in the diagnosis of drug resistant TB. The key to control TB, in the community is to rapidly diagnose and initiate drug resistant TB treatment so that the chain of disease transmission is cut. Although there are many techniques available for detection of Flouroquinolone drug resistance, Molecular diagnostic methods based on the detection of mutations conferring drug resistance are promising technologies for rapidly detecting multidrug or extensively drug-resistant tuberculosis. The GenoType MTBDR*sl* LPA seems to be the best and most reliable technique to be used in a public health laboratory for rapid detection of Flouroquinolone resistance.

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