Journal of Microbiological Methods 84 (2011) 155–160

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Review

Tuberculosis drug resistance testing by molecular methods: Opportunities and challenges in resource limited settings

Gemeda Abebe [a,e,*], Fabienne Paasch [b], Ludwig Apers [c], Leen Rigouts [d], Robert Colebunders [c,e]

[a] Department of Medical Laboratory Sciences and Pathology, Jimma University, Jimma, Ethiopia
[b] Department of Medical Microbiology, Vaccine and Infectious Disease Institute, University of Antwerp, Antwerp, Belgium
[c] Department of Clinical Sciences, Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium
[d] Mycobacteriology Unit, Department of Microbiology, Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium
[e] Department of Epidemiology and Social Medicine, University of Antwerp, Antwerp, Belgium

ARTICLE INFO

Article history:
Received 13 August 2010
Received in revised form 19 November 2010
Accepted 19 November 2010
Available online 1 December 2010

Keywords:
Tuberculosis
Drug-resistance
Resource-limited settings

ABSTRACT

One of the greatest threats to global tuberculosis (TB) control is the growing prevalence of drug-resistant bacilli. Correctly diagnosing drug-resistant TB patients is more problematic in resource-limited settings as there is no or limited infrastructure for drug susceptibility testing (DST) of TB bacilli. The conventional phenotypic DST method for TB takes weeks before declaring the results and initiating proper anti-TB treatment. Molecular DST offers advantages over the phenotypic methods mainly because of the short turnaround time. This review summarizes the different molecular DST methods for TB and discusses challenges and opportunities in implementing them in resource-limited settings.

Contents

1. Introduction

One of the greatest threats to the global control of TB is the growing prevalence of drug-resistant bacilli (Pablos-Mendez et al., 1998; WHO, 1999). It is well documented that early identification of multidrug-resistant (MDR)-TB patients and the administration of therapy based on *in-vitro* drug susceptibility testing (DST) reduce mortality, even among HIV-positive individuals (Turett et al., 1995; Park et al., 1996; O'Riordan et al., 2008). However, in most countries with limited resources, TB patients are treated with standard regimens including isoniazid (INH) and rifampin (RIF) without the knowledge whether the causative organism is susceptible to these drugs. The main reason behind is inadequate facility for DST.

Molecular DST offers advantages over the phenotypic methods mainly because of their short turnaround time. This review

* Corresponding author. Tel.: +251 911991285.
E-mail addresses: gemeda.abebe@ju.edu.et (G. Abebe), fpaasch@gmail.com (F. Paasch), lapers@itg.be (L. Apers), lrigouts@itg.be (L. Rigouts), bcoleb@itg.be (R. Colebunders).

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doi:10.1016/j.mimet.2010.11.014

summarizes the different molecular DST methods for TB, and discusses the challenges and opportunities in implementing them in resource-limited settings.

Culture-based DST methods will not be covered in this review.

2. Types and mechanisms of drug resistance in TB

Drug-resistant TB can be either primary or acquired. Primary resistance includes infections with *M. tuberculosis*-complex (MTBc) strains which are already resistant. This type of resistance may be the result of a previous undocumented or unreported treatment (Citron and Girling, 1987; Vareldzis et al., 1994). Acquired resistance refers to resistance that has developed due to improper exposure of the MTBc strain to anti-TB drugs and the consequent selection of resistant mutant bacilli. However, some of the drug-resistant isolates in previously-treated patients may actually represent primary resistance among patients who remain uncured (Frieden et al., 1993).

Mono-resistance is defined as resistance to one first-line drug only. Multi-drug resistance (MDR) is resistance to at least isoniazid and rifampin. Extensively drug-resistant (XDR) TB is MDRTB plus resistance to any fluoroquinolone and at least one of the three injectable second-line drugs (amikacin, capreomycin, or kanamycin) (WHO definitions available at: http://www.who.int/bulletin/volumes/85/5/06-035345/en/).

MTBc bacilli use several strategies to resist the action of antimicrobial agents. First, the mycobacterial cell is surrounded by a specific, highly hydrophobic cell wall that results in decreased permeability to many compounds. This cell wall is a strong barrier to a wide range of anti-mycobacterial agents (Jarlier and Nikaido, 1994; Lee et al., 1996). Second, resistance of MTB to anti-mycobacterial drugs is the consequence of spontaneous mutations in genes that encode either the target of the drug, or enzymes that are involved in drug activation. Third, active drug efflux systems and degrading or inactivating enzymes may play a role (Kwon et al., 1995; Cole et al., 1998).

Resistance-associated point mutations, deletions, or insertions have been described for all first-line drugs (isoniazid, rifampin, pyrazinamide, ethambutol, and streptomycin), and for several second-line and newer drugs (ethionamide, fluoroquinolones, macrolides, and nitroimidazopyrans) (Heym et al., 1994; Stover et al., 2000; Zhang and Telenti, 2000; Vester and Douthwaite, 2001). Horizontal transfer of resistance through mobile genetic elements such as plasmids and transposons, which are known to mediate drug resistance in various bacterial species, does not play a role in MTBc (Zhang and Yew, 2009). To date, no single genetic alteration has yet been found that is attributed to the MDR phenotype. Rather, MDR develops by sequential accumulation of mutations at different loci, usually because of inappropriate patient treatment. Mutations in the mycobacterial genome may confer resistance to anti-mycobacterial drugs or may not since some mutations could be silent causing polymorphic alterations without influencing the drug–target interaction (Kim et al., 1997). The most important drugs with their mode of action and related genes as well as the proportion of drug-resistant isolates showing resistance-conferring mutations in the respective genes are summarized in Table 1.

3. Molecular DST methods in MTB

There are different molecular methods to test for gene mutations associated with resistance to a particular anti-TB drug. Basically, they all include a DNA amplification step and can be categorized by large as real-time polymerase chain reaction (RT-PCR), solid phase hybridization assays and sequencing. Other less frequently applied methods include electrophoresis-based techniques, denaturing high performance liquid chromatography (HPLC) (Yip et al., 2006), multiplex-allele-specific PCR (MAS-PCR) (Evans and Segal, 2010) and mismatch analysis (Watterson et al., 1998).

Electrophoresis-based techniques depend on DNA characteristics such as fragment size and secondary structure or melting temperature that may be altered as a result of mutation. The altered characteristics in turn will affect the DNA mobility pattern during electrophoresis. These techniques include single strand conformation polymorphism (SSCP) (Bobadilla-del-Valle et al., 2001), PCR heteroduplex assay (Bidwell et al., 1994), double gradient denaturing gradient gel electrophoresis (DGGE) (Victor et al., 2002) and restriction fragment length polymorphism (RFLP) (Caws et al., 2007). These methods, however, attracted less attention compared with other molecular DST methods, mainly because they are technically demanding, labor intensive or not adequately sensitive (Nataraj et al., 1999; Johnson et al., 2006). Indeed, not all mutations may result in gain or loss of restriction sites (Victor et al., 2002) rendering these techniques ineffective. As a result, both their commercial success and field applications are limited. We will review the other methods which have attracted attention from the scientific community in the following sections.

3.1. Real-time PCR

Real-time PCR techniques monitor DNA amplification reaction directly while it is occurring. They are based on hybridization of

Table 1
Summary of most important drugs, their mode of action and related genes with the proportion of drug-resistant MTBc isolates showing resistance-conferring mutations in the respective genes.

Drug	Mode of action	Related gene	Mutation frequency among resistant MTBc isolates (%)	References
Rifampin	Inhibits RNA synthesis	*rpoB* gene encoding the β-subunit of the RNA polymerase	96–100	Telenti et al. (1993a), Hunt et al. (1994), Kapur et al. (1994), Caugant et al. (1995), Felmlee et al. (1995), Musser (1995), Whelen et al. (1995), Zhang and Telenti (2000)
Isoniazid	Inhibits biosynthesis of mycolic acids	*katG* gene encoding the catalase enzyme	42–58	Lavender et al. (2005)
		Promoter region of the *mabA* (*fabG1*)-*inhA*	21–34	
Streptomycin	Inhibits translation (ribosomal proteins)	*rpsL* gene encoding the ribosomal S12 protein	52–59	Nair et al. (1993), Musser (1995), Zhang and Telenti (2000)
		rrs gene encoding the 16s rRNA	18–21	
Ethambutol	Inhibits incorporation of arabinogalactan into cell wall	*embB* gene encoding for membrane indolylacetylinositol arabinosyltransferase	47–65	Musser (1995), Telenti et al. (1997), Zhang and Telenti (2000)
Pyrazinamide	Might inhibit membrane energetics	*pncA gene encoding the pyrazinamidase/nicotinamidase*	72–90	Musser (1995), Scorpio and Zhang (1996), Zhang and Telenti (2000)
Fluoroquinolones	Inhibit translation (DNA unwinding)	*gyrA gene encoding the gyrase A enzyme*	75–94	Takiff et al. (1994), Musser (1995), Zhang and Telenti (2000)

amplified nucleic acids with fluorescent-labeled probes spanning DNA regions of interest. The increase in fluorescence signal is monitored on-line and directly proportional to the amount of amplified product. Different types of probes have been used including the TaqMan probe (Espasa et al., 2005), fluorescence resonance energy transfer (FRET) probes (Saribas et al., 2005), molecular beacons (Varma-Basil et al., 2004) and bioprobes (Edwards et al., 2001). Although, real-time PCR was initially developed for MTBc strains, more recently it has been successfully applied directly on clinical samples. In a study conducted to determine RIF resistance by molecular beacons among 148 clinical MTBc isolates, RIF-associated mutations were detected in 63 of 65 RIF-resistant isolates, and no mutations were found in any of the 83 RIF-susceptible isolates (El-Hajj et al., 2001). In the same study, mutations were detected in all sputum specimens of 11 patients infected with RIF-resistant TB. In a separate study conducted in two different TB-high-incidence populations the sensitivity and specificity of real-time PCR using molecular beacons to correctly identify RIF-resistant TB were 89% and 99% respectively (Varma-Basil et al., 2004). However, the requirement to design individual probes for each SNP increases the cost. Beacon and TaqMan probes may be best suited for detecting commonly encountered SNPs, such as those in codon 315 of *katG* or codon 531 of the *rpoB* gene. On the other hand, although FRET probes require two rounds of PCR amplification, they are ideal for initial screening of multiple SNPs and they are more cost efficient for high-throughput screening.

Real-time PCR does not only permit detection of specific DNA genes or mutations, it also allows for their quantification. Different probes were evaluated for their efficiency in detecting antibiotic resistance conferring single nucleotide polymorphisms (SNPs) in mixed MTBc DNA extracts (Yesilkaya et al., 2006). They allowed detecting heteroresistance by discriminating different subpopulations in the clinical sample, thus helping to detect the possible emergence of resistance in an early stage.

The main advantages of real-time PCR techniques are the speed of the test (results can be available 1.5–2.0 h after DNA extraction) and a lower risk of contamination due to the closed single-tube system. The main disadvantages are the requirement for expensive equipment and reagents, and the need for skilled technical personnel.

Cepheid (Sunyvale, CA) has recently introduced a real-time PCR based approach, Xpert MTB/RIF, for RIF drug resistance testing. In this approach the detection of MTBc and resistance to RIF can be performed in an integrated hands-free sputum-processing and real-time PCR system. It utilizes a single-use-processing cartridge and the result can be available in 2 h. The first evaluation study on clinical specimens reported an overall sensitivity of 98–100% with 72% sensitivity in smear-negative samples. The method proved to be 100% specific (Helb et al., 2010). In another study on the analytic sensitivity and specificity of the test method, 79/79 *M. tuberculosis* isolates were correctly identified and 89 non-tuberculosis isolates correctly excluded. The assay proved capable to detect resistance in the presence of different mixtures of RIF-resistant and RIF-susceptible DNA, requiring 65–100% of the mutant DNA to be present in the sample for 95% certainty of resistance detection, depending on the type of mutation (Blakemore et al., 2010). Even though it's potential in solving the problems related to specimen processing and handling looks promising, its feasibility and affordability in resource-limited settings remain to be determined.

3.2. Solid phase hybridization assays

These assays are based on interactions between two complementary strands of double-helical DNA molecules. It involves the hybridization of labeled PCR amplicons with probes bound to a solid support. First, the targeted resistance region of the clinical strain is amplified using labeled primers. Second, the resulting amplicon is hybridized with the immobilized probe and visualized by an enzymatic color reaction or fluorescence signal. If a mutation is present in the target region of interest, the amplicon will not hybridize with the probe representing the wild type sequence, but only with probes representing the complementary strand of that specific mutation.

Line probe assays (LPAs) and DNA microarrays (DNA biochips) are the most widely known techniques using immobilized probes on a solid support. In LPAs the probes are attached to a nitrocellulose strip. Currently there are two commercially available tests that were endorsed by WHO: GenoType® MTBDR and MTBDRplus (Hain Lifescience, Nehren, Germany) and INNO-LiPA Rif.TB (LiPA) (Inogenetics, Ghent, Belgium). They can detect MTBc-specific DNA and genetic mutations associated with drug resistance from smear-positive sputum specimens or culture isolates, after DNA extraction and PCR amplification.

In a systematic review and meta-analysis including 15 studies using LiPA, 14 studies reported a sensitivity between 82% and 100% for correctly identifying RIF resistance among MTBc isolates, with a specificity ranging from 92% to 100% (Morgan et al., 2005). Twelve of these studies reported a sensitivity of 95% or more, and 5 of them even reached 100% sensitivity. Of the 4 studies testing LiPA directly on clinical specimens, reported sensitivity estimates were consistently high (80% to 100%), with a specificity of 100% (Morgan et al., 2005).

In a separate systematic meta analysis of studies on direct DST, the pooled sensitivities for Genotype® MTBDR were 99% and 98%, and for the new Genotype® MTBDRplus 99% and 99% (Bwanga et al., 2009). GenoType MTBDR assay detects the resistance to both INH and RIF simultaneously, and thus can detect MDR-TB (Hillemann et al., 2005; Hillemann et al., 2006; Somoskovi et al., 2006; Ahmad et al., 2009; Huyen et al., 2010).

The application of the new GenoType MTBDR*sl* (Hain Lifescience, Nehren, Germany) for detection of MTBc resistant to second-line drugs was reported recently (Hillemann et al., 2009). The method has shown a sensitivity of 90.6% to correctly detect fluoroquinolone resistance among MTBc isolates, 84.8% for amikacin and 86.7% for capreomycin. The sensitivity for detecting ethambutol resistance was low (69.2%). On clinical specimens the sensitivity was lower with 88.9%, 75%, 87.5% and 35% for fluoroquinolone, amikacin, capreomycin and ethambutol respectively. The specificity for all drugs both in strains and clinical specimens was 100%.

Another type of solid phase hybridization assays are DNA microarrays. They analyze several genetic markers in a single hybridization step due to the immobilization of a large set of oligonucleotides at a precisely defined spot on a polyacrylamide gel or glass carrier (Cherkasova et al., 2003). The fluorescence-labeled DNA amplicon can hybridize to the microarray oligonucleotides. The resulting fluorescence intensity between the different positions in the microarray defines the pattern of mutations of the clinical strain. Such an approach has previously been used for the successful detection of *rpoB* mutations conferring RIF resistance (Gingeras et al., 1998; Troesch et al., 1999; Gryadunov et al., 2005; Caoili et al., 2006) and *pncA* mutations for PZA resistance (Denkin et al., 2005). A recent report of DNA microarray analyzed culture isolates and clinical samples for mutations in seven genes related to five anti-TB drugs (INH, RIF, SM, kanamycin and EMBl). The report indicated a high sensitivity (>90%) for all five drugs (Shimizu et al., 2008). In this study specificities for RIF and EMB were nearly 90%, but the specificity for INH (60%) and kanamycin (67%) was not satisfactory. More recently, further attempts were started for rapid detection of resistance to fluoroquinolones by microarrays (Antonova et al., 2008).

3.3. Sequencing

DNA sequencing is the reference molecular method as it visualizes the complete nucleotide sequence of the target DNA. It is the most direct and reliable method for studying mutations and allows for

detection of both previously recognized and unrecognized mutations. The mutations detected by sequencing, however, could be related to drug resistance or not as some mutations could be silent, or just represent DNA polymorphism not related to drug resistance. Unfortunately, sequencing is not as easily applicable for routine identification of drug resistance mutations as it is for identification of mycobacterial species: many different genes may be involved for a single drug, as is the case in INH resistance, or the mutations may be scattered in a large segment of the gene. This implies that, in order to obtain complete drug-resistance data for a single MTBc isolate, a range of sequencing reactions needs to be performed or the whole genome should be sequenced. However, for targeted use such as *rpoB* analysis, where mutations associated with RIF resistance are concentrated in a very short segment of the gene, PCR-based sequencing is a useful technique (Pai et al., 1997).

Sequencing can be performed by conventional Sanger's method or the Illumina automatic sequencer (Loman and Pallen, 2008). Both are based on the same principle of chain termination but the speed and length of readout are better in the latter, as it can produce a staggering 1 GB of nucleotide per run. Pyrosequencing, on the other hand, applies the DNA sequencing-by-synthesis principle, and is mostly used for short read sequencing and SNP analysis. It has been used for the detection of drug-resistance in MTB as well (Zhao et al., 2005; Jureen et al., 2006; Marttila et al., 2009). Compared with routine conventional DST testing, pyrosequencing was reported to have sensitivities of 97.4% and 66.7%, for correctly identifying rifampin and isoniazid resistance among MTBc isolates respectively (Marttila et al., 2009). In another study, it detected relevant mutations in the *rpoB* gene for 96.7% of rifampin-resistant isolates, in *katG* for 64% of isoniazid-resistant isolates, and in *gyrA* for 70% of ofloxacin-resistant isolates (Bravo et al., 2009). Pyrosequencing has gained attention over the conventional sequencing method mainly because of its improved turnaround time (Lindstrom et al., 2004). However, the inherent problems associated with pyrosequencing—mainly the length of DNA sequence that can be sequenced—made it impossible to replace the conventional method. Pyrosequencing allows to sequence a string of up to 20–50 nucleotides. If a longer DNA fragment is to be analyzed, the conventional Sanger's method or Illumina is preferable (Zhao et al., 2005).

4. Opportunities and challenges in implementing molecular DST methods for TB

The traditional methods for DST have a long turnaround time. Nevertheless, they are still standard practice in high-resource countries but not necessarily in resource-limited countries as culture-based methods are often not available yet (Ridderhof et al., 2007). WHO already approved line probe assays with current known developers GenoType® MTBDR and MTBDRplus (Hain Lifescience, Nehren, Germany in collaboration with FIND) and INNO-LiPA Rif.TB (Innogenetics, Ghent, Belgium) (WHO, 2008).

Molecular tests provide a way to centralize testing services without the need of "cold chain" transport systems as required for culture (Harries et al., 2004; Drobniewski et al., 2006). As the tests do not require viable mycobacteria, locally inactivated specimens can be transported to the central lab in an affordable, secure manner, decreasing the biosafety risk during transport and in the laboratory. Inactivation of TB bacilli can be achieved by heating or addition of ethanol (Bemer-Melchior and Drugeon, 1999; Doig et al., 2002; Elbir et al., 2008). On the other hand it is not possible to recover viable MTBc bacilli from inactivated samples for further phenotypic testing of additional drugs. The use of molecular tests can also directly reduce the cost associated with testing. On the basis of FIND-negotiated prices, the cost of GenoType MTBDRplus is less than 50% of that for conventional liquid culture and DST for INH and RIF (Barnard et al., 2008).

The last decade, WHO has encouraged the expansion of liquid cultures in resource-limited settings. This expansion is expected to create increased capacity to diagnose MDR-TB, but at the same time could constitute a support for the quality assurance of molecular assays. Combined efforts could improve management of MDR-TB patients at clinics through early and effective diagnosis of MDR-TB. Some countries like Ethiopia are going in this direction (Ministry of Health of Ethiopia, 2009). With the implementation of molecular DST tools, countries will not only be in a position to formulate realistic plans for MDR-TB diagnosis and treatment, but might also be able to provide more systematic data on the prevalence of (M)DR-TB, data that are still lacking in many resource-limited settings.

Issues related to the feasibility of molecular DSTs are presented in Table 2. The challenges to implement molecular DST in resource-

Table 2
Feasibility of molecular DST for TB.

	Drugs		Specimen		Infra-structure	Equip-ment cost[a]	Consumables cost[a]	Training[b]	Turnaround time[c]	References
	1st-line	2nd-line	Sputum	Isolates						
Real-time PCR										
Xpert® MTB/RIF	R	N	Y	Y	BSL2[d]	High	High	Minimal	2 h	WHO (2008), Helb et al. (2010)
Manual-in house	Y	Y	Y (1st-line)	Y	BSL2[d]	High	High	Moderate	3 h	Wada et al. (2004)
Solid phase hybridization assays										
Microarray	Y	Y	Y	Y	BSL2[d]	High	High	High	12 h	Gryadunov et al. (2005), Balabanova et al. (2009)
LPA					BSL2[d]	High	High	Moderate (3 days)	1–2 days	Morgan et al. (2005), WHO (2008), Morcillo et al. (2002)
GenotypeMTBD	R	N	Y	Y						
Rplus®	H	F	Y	Y						
Genotype MTBDRsl®	R	A	Y	Y						
INNO-LiPA.RIF.TB®	R	N								
Sequencing										
Conventional (illumina)	Y	Y	Y	Y	BSL2[d]	High	High	High	3.8 +/− 1.8 days	Djelouadji et al. (2009)
Pyrosequencing	Y	Y	Y	Y	BSL2[d]	High	High	High	5 h	Djelouadji et al. (2009)

Y = Yes, N = No, NA = no data, BSL1 = bio-safety level 1, BSL2 = bio-safety level 2; R = rifampicin, H = isoniazid, E = ethambutol; F = fluorquinolones; A = aminoglycosides.
[a] Product prices may vary depending on geographical location and terms of supply; ranges are indicative only: Low (minimum–2000 US$); Medium (2001–7000 US$); high (7001+ US$) (WHO, 2008).
[b] Assuming that basic TB lab procedures and the principles of PCR are known.
[c] From sample receipt to result.
[d] BSL1 is sufficient when handling only inactivated samples.

limited countries are multifaceted and include logistics, technical aspects, human resources and the inherent limitations of molecular techniques.

4.1. Logistics

(1) Most consumables used in the molecular testing are imported from overseas. The short expiry date of the reagents and the long procurement and complex custom clearance procedures are only some of the challenges faced. (2) After procurement some consumables may require strict storage and transport conditions, mostly they must be refrigerated or frozen. (3) The amplification procedures should be performed under regular and uninterrupted temperature conditions, and a continuous and regular power supply is a precondition before introducing any of these devices. (4) The equipments used in molecular DST need to be serviced and maintained on a regular basis. Domestic companies that can ensure these services on a regular and timely basis should be in place. These logistic problems are not strictly specific to molecular methods but are also faced for conventional DSTs, especially if commercial culture media are being used.

4.2. Technical aspects

The inherent vulnerability of molecular techniques for contamination is another challenge to face. Cross-contamination can result in false-resistant results. To avoid such problems separation of working areas, rigorous techniques, strict procedures and regular internal quality control are needed. Although not specific to resource-limited settings, setting up PCR-based facilities requires expertise and a basic infrastructure. On the other hand, molecular-based analyses offer the opportunity to share facilities for different (infectious) diseases. Finally, the absence of international standardized external quality assurance specifically for molecular DST for TB, poses problems in monitoring the laboratory qualities.

4.3. Human resources

If all technical and logistic challenges are met, one is left with perhaps the most important condition: adequate human resources. The training and retention of laboratory personnel may be a challenge as the retention scheme in most resource-limited settings is problematic. This is mainly evident in most African countries as they are suffering from a high staff turnover within the country or a worrying brain drain to other countries (Anyangwe and Mtonga, 2007).

4.4. Limitations of molecular techniques

Despite their advantages, molecular techniques for MTB drug resistance are not without shortfalls. The major drawback of most molecular techniques, except sequencing, is that they can only be used for detecting known mutations in a defined site or region, and are not sensitive enough to be used for detecting unknown mutations since their design emanates from known mutations. This can be substantiated by comparing the sensitivities and specificities of molecular tests for rifampin (which is associated with mutations concentrated in one region) and isoniazid (dispersed mutations). Today, the commercially available molecular tests which are endorsed by WHO can detect resistance to RIF and INH only. Consequently, they can be applied for MDR-TB only, not for diagnosis of XDR-TB.

The level of the health care system at which molecular tests could be applied should be determined on a case by case approach. The availability of quality control and the before mentioned conditions need always to be taken into account. For these tests to be cost effective and of good quality, only central or regional implementation, depending on the size of the country in question, shall be considered.

5. Conclusion

Molecular methods offer many advantages for drug-resistant TB case management, mainly for the following reasons:

- Most of the molecular methods tested so far have shown good specificity and sensitivity for rifampin which is the surrogate predictor of MDRTB.
- The implementation of molecular DST for TB can create the capacity for early treatment of MDRTB cases.
- The infrastructure that is required to implement molecular DST is less expensive compared to conventional phenotypic methods, and facilities can be shared for various (infectious) diseases.
- Tests can be easily centralized.
- The safety of technical personnel can be better ensured by using molecular methods than the conventional phenotypic methods.

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