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Commentary

Innovations in tuberculosis diagnostics: How far are we from reaching our goal?

Tuberculosis (TB) continues to be a major global health problem despite the fact that with timely diagnosis and appropriate treatment, most patients can be completely cured of the disease. In spite of the availability of an effective treatment regimen, 9.0-11.0 million individuals contracted active TB and 1.3 million died from the disease in 2017[1]. In certain countries, the disease burden has fallen considerably over the years, and these regions have only around 10 or fewer cases and less than one death per 100,000 population per year. However, for several other countries, victory in the fight against this disease is still a distant reality[1]. The WHO also stated that of the incident TB cases missed from the TB surveillance system, two-thirds were not reported and remaining one-third were not detected[1].

The diagnosis of TB by conventional method, namely direct smear examination by Ziehl-Neelsen staining for the presence of acid fast bacilli (AFB) is inexpensive, rapid and easy to perform but has low sensitivity (57-63%), especially in HIV-infected individuals[2,3]. Its inability to differentiate between the different mycobacterial species is also a major drawback[4]. Culture, 'the laboratory gold standard' is more sensitive than smear microscopy. However, this is a time-consuming process with culture on Lowenstein-Jensen medium taking at least 3-6 wk. Although the use of liquid culture such as in mycobacteria growth indicator tube (MGIT) hastens the growth of *Mycobacterium tuberculosis* (MTB), yet the time to culture positivity takes at least 8-14 days. Moreover, culture is cumbersome and requires well-trained laboratory staff. Thus, the delay in diagnosis with conventional microbiological techniques leads to a delay in the treatment of patients; during which time, they not only suffer but also remain infectious. This has led to the development of several rapid diagnostic assays. There has been a rapid evolution of molecular tools for diagnosis of TB with the availability of several nucleic acid amplification techniques, including real-time polymerase chain reaction (PCR) and loop-mediated isothermal amplification assays. However, successful diagnosis and treatment of drug-resistant TB depend on not only rapid identification of MTB complex (MTBC) but also universal access to accurate drug susceptibility testing (DST). Conventionally, the diagnosis of drug resistance in MTB isolates has relied on culture and DST in liquid or solid medium. Results are obtained after weeks to months of incubation, and culture-based methods require stringent laboratory biosafety practices, which is a challenge for several laboratories[1]. Moreover, the emergence and spread of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB and the challenges associated with performing phenotypic DST made it imperative to develop rapid assays that could not only detect MTBC but also drug resistance.

Drug resistance in MTBC is mainly conferred through point mutations in specific gene targets in the bacterial genome[5]. Therefore, it was possible to develop molecular tests for rapid testing and thus earlier initiation of appropriate treatment for drug-resistant TB. Rapid molecular tests such as line probe assays (LPAs) allow for the detection of a set of common resistance mutations in a few genomic regions[6]. Most of the assays target rifampicin (RIF) resistance, since it is considered to be a surrogate marker of MDR-TB, and along with isoniazid (INH), comprises the backbone of anti-TB therapy. RIF resistance can be detected by targeting a limited number of loci at the RIF resistance determining region (RRDR) of the *rpoB* gene[7]. In contrast, although the most important cause of INH resistance is mutations in *katG* and *inhA* genes, mutations in several other genes may also be involved[7]. The TB Drug Resistance Mutation Database has reported 22 mutations associated with INH resistance such as *katG*, *ahpC*, *inhA*, *kasA* and *ndh*[7]. The high number of mutations associated with INH resistance

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makes the creation of a minimal predictive mutation set difficult. Similarly, for many other drugs used in the treatment of drug-resistant TB, the mutations are spread over multiple genes and regions. Moreover, all mutations conferring resistance have not yet been identified[1]. Furthermore, limited information is available on the 'high-confidence mutations' that would accurately predict treatment outcomes[8]. In spite of these difficulties, molecular assays have been developed to detect mutations to INH, ethambutol (EMB), aminoglycosides (AG) and fluoroquinolones (FQ) in addition to RIF. The WHO endorsed rapid methods such as GeneXpert and Hain's LPA[9] changed the landscape of diagnostic mycobacteriology. However, these are limited in their inability to identify all mutations responsible for drug resistance as these target only the resistance-determining regions of the genome[3,6]. Thus, mutations outside this region are missed. Moreover, these methods cannot differentiate between silent mutations and mutations associated with drug resistance[10,11]. The ability to detect these mutations in patients of TB is important to guide appropriate therapy.

The multiplex allele specific PCR (MAS-PCR) was developed as a simple and rapid assay and reported initially for *emb306* and *katG315* mutational analysis[12]. Sinha *et al*[13] also performed a study with the principle objective to use MAS-PCR as a rapid and cost-effective technique to detect drug-resistant MTB directly in clinical specimens, although the authors have not compared the cost of different techniques. The authors detected mutations in *katG315* codon and *rpoB516* by a nested MAS-PCR (NMAS-PCR) and mutations in *rpoB526* and *rpoB531* by nested allele specific PCR. Mutations in *inhA* promoter were detected by MAS-PCR[13]. The assay was performed on pulmonary and extrapulmonary specimens including urine, pus, fine needle aspirates, cerebrospinal fluid and pleural fluid. The sensitivity and specificity of the assays for INH resistance, when compared with conventional DST, were 98.6 and 97.8 per cent, respectively, while the sensitivity and the specificity for detection of RIF resistance were 97.5 and 97.9 per cent, respectively. Detection of multidrug resistance showed a sensitivity of 98.9 per cent and a specificity of 100 per cent[13]. In another study, Vadwai *et al*[14] evaluated four individual MAS-PCR assays on sputum samples and targeted *katG*315, *rpoB*531, *gyrA*94 and *rrs*1401 to determine resistance to INH, RIF, FQ and AG, respectively. MAS-PCR correctly identified MTBC in 97.2 per cent culture-positive specimens. Phenotypic DST correlated most with MAS-PCR for RIF resistance [94.9%; 95% confidence interval (CI): 91-97], followed by AG resistance 92.3 per cent (95% CI: 75-99), INH resistance 89.2 per cent (95% CI: 84-93) and FQ resistance 72.5 per cent (95% CI: 65-79). The authors recommended the use of MAS-PCR for rapid detection of drug-resistant TB. Another study from India used MAS-PCR for detection of RIF resistance in clinical isolates of MTB[15]. Distinct PCR bands were observed for different mutations in the RRDR and the region outside the RRDR. Mutations in regions other than the 81 bp RRDR were observed at codons 413 (11.1%), 511 (12.2%) and 521 (15.6%) of the *rpoB* gene. The concordance with phenotypic DST was 96.7 per cent[15]. Mistri *et al*[16] developed a modified NMAS-PCR assay enabling detection of MDR-TB directly from sputum samples and compared it with phenotypic DST and sequencing. The sensitivity and specificity of the MAS-PCR in comparison with phenotypic DST was 92.9 and 100 per cent for RIF resistance, respectively[16]. However, one of the limitations of allele-specific PCR, as in most other molecular technologies, is that only the most common mutations are targeted. Furthermore, it is worth considering if this technology will score over other existing technologies, in terms of ease of performing the assay and the expertise needed to run the assays.

Use of whole genome sequencing (WGS) can overcome these problems and can provide clinically relevant data rapidly. WGS can simultaneously identify all known resistance associated loci with high concordance to conventional drug susceptibility assays, with 96 per cent concordance reported between WGS and culture-based DST methods[8]. In addition, it can also characterize novel loci associated with drug resistance[3]. Moreover, the improvement in next generation sequencing workflow has shown the potential of WGS in identification of MTBC and drug-resistant mutants directly from clinical samples[6,17]. However, the high cost of these assays limits their use in resource-limited settings. In addition, most of the molecular techniques need expensive instrumentation and infrastructural facilities. In this context, there is a need for a rapid, consumer-friendly and cost-effective assay that can easily be used and sustained in resource-poor regions. It may be noted that it is just not the assay that needs to be affordable but also the running cost of the instruments. The assay needs to have increased accuracy, reduced turnaround time, reduced time to diagnosis and be highly

sensitive and specific[9]. It should also be able to detect drug-resistant TB and should be able to provide all these features at a highly economical cost so that it can be used easily in high-burden and resource-limited regions.

To meet the WHO End TB Strategy target set for 2030[18], research needs to be intensified. Experimental research needs to be supported and encouraged so that more innovative tools can be developed. There are several new assays in the global pipeline for diagnosis of TB, either under development or under evaluation[1]. The fact that no new assay for TB diagnosis has emerged in the last two years[1] underscores the lack of sufficient investment toward this goal. Increased and sustained funding along with stronger commitment is the need of the hour to fight this disease that has ailed mankind for thousands of years.

***Conflicts of Interest*:** None.

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