MOLECULAR LINE PROBE ASSAYS FOR RAPID SCREENING OF PATIENTS AT RISK OF MULTIDRUG-RESISTANT TUBERCULOSIS (MDR-TB)

POLICY STATEMENT

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1. Background

Multidrug-resistant tuberculosis (MDR-TB) poses a formidable challenge to TB control due to its complex diagnostic and treatment challenges. The annual global MDR-TB burden is estimated at around 490 000 cases, or 5% of the global TB burden; however, less than 5% of existing MDR-TB patients are currently being diagnosed as a result of serious laboratory capacity constraints. Alarming increases in MDR-TB, the emergence of extensively drug-resistant TB (XDR-TB), potential institutional transmission, and rapid mortality of MDR-TB and XDR-TB patient with HIV co-infection, have highlighted the urgency for rapid screening methods.

Conventional methods for mycobacteriological culture and drug susceptibility testing (DST) are slow and cumbersome, requiring sequential procedures for isolation of mycobacteria from clinical specimens, identification of *Mycobacterium tuberculosis* complex, and *in vitro* testing of strain susceptibility to anti-TB drugs. During this time patients may be inappropriately treated, drug resistant strains may continue to spread, and amplification of resistance may occur. Novel technologies for rapid detection of anti-TB drug resistance have therefore become a priority in TB research and development, and molecular line probe assays focused on rapid detection of rifampicin resistance (alone or in combination with isoniazid) are most advanced.

Line probe assay technology involves the following steps: First, DNA is extracted from *M. tuberculosis* isolates or directly from clinical specimens. Next, polymerase chain reaction (PCR) amplification of the resistance-determining region of the gene under question is performed using biotinylated primers. Following amplification, labeled PCR products are hybridized with specific oligonucleotide probes immobilized on a strip. Captured labeled hybrids are detected by colorimetric development, enabling detection of the presence of *M. tuberculosis* complex, as well as the presence of wild-type and mutation probes for resistance. If a mutation is present in one of the target regions, the amplicon will not hybridize with the relevant probe. Mutations are therefore detected by lack of binding to wild-type probes, as well as by binding to specific probes for the most commonly occurring mutations. The post-hybridization reaction leads to the development of coloured bands on the strip at the site of probe binding and is observed by eye.

2. Evidence base

2.1 Process

An Expert Group was convened by the World Health Organization (WHO) and the UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) in March 2008, to assess available data on line probe assays with a view towards policy recommendations on their use. Data from published literature, laboratory validation studies, and investigator-driven laboratory and field demonstration studies were used to assess assay performance and feasibility of programmatic implementation. The associated laboratory infrastructure, human resource requirements and research gaps were also defined.

An extensive literature search resulted in published information on proven efficacy from two commercial line probe assays. To the best of knowledge, these are currently the only

products available. Although the specific commercial assays are under patent, the underlying line probe assay technology is in the public domain.

Tests used to inform patient care can only be ethically justifiable if performed with a product that has met pre-defined performance targets in carefully controlled evaluation studies, and which have been registered for a given indication. Both commercial assays evaluated are manufactured under ISO 13485:2003 certification, offering the advantage of quality-assured reagents and test kits, and labeled for use under defined conditions. The tests are also approved by the Regulatory Authority in Europe (CE-Marked) and elsewhere.

In-house line probe assays, developed in academic research settings, have not been adequately validated or evaluated outside of such settings, and their use for clinical care of patients is therefore not recommended.

While it is likely that additional assays may become available in the future, these will need to be subjected to the same level of validation and expert review before their implementation can be recommended by WHO.

2.2 Results

Data from systematic reviews and meta-analyses to evaluate assay performance results against conventional DST methods showed that line probe assays are highly sensitive (>=97%) and specific (>=99%) for the detection of rifampicin resistance, alone or in combination with isoniazid (sensitivity >=90%; specificity >=99%), on isolates of *M. tuberculosis* and on smear-positive sputum specimens. Overall accuracy for detection of MDR was equally high at 99%, and retained when rifampicin resistance alone was used as a marker for MDR. These results were confirmed by laboratory validation and field demonstration data in several countries, most notably in the large-scale demonstration project in South Africa, executed by the Foundation for Innovative New Diagnostics (FIND), the SA Medical Research Council (SAMRC) and the SA National Health Laboratory Service (NHLS).

Data from the validation and field demonstration studies in South Africa also indicated the feasibility of introducing line probe assays in high-volume public health laboratories. Detailed costing data from South Africa showed that the reduction in cost of line probe assays under routine diagnostic algorithms amounted to between 30% and 50% when compared to conventional DST methods. As expected, the cost was lowest when the line probe assay was directly applied to smear-positive specimens and highest when the assay was used on isolates from liquid primary culture.

Cost-effectiveness and cost-benefit of line probe assays remain to be assessed, and will be dependent on screening and diagnostic algorithms in different epidemiological settings. Detailed cost-effectiveness and patient impact data will only be available once large-scale field demonstration projects have been completed.

Apart from the impact on morbidity, mortality and transmission of MDR-TB, introduction of these assays in screening and diagnostic algorithms could significantly reduce the need for sophisticated and costly conventional laboratory infrastructure, still vastly inadequate in most high-burden countries. The Expert Group concluded that there was sufficient generalisable evidence to justify a recommendation on the use of line probe assays for rapid detection of MDR-TB, at country level, and with further operational research to address country-specific implementation needs.

Line probe assays are not a complete replacement for conventional culture and DST, as mycobacteriological culture is still required for smear-negative specimens while conventional DST is still necessary to confirm XDR-TB. Nevertheless; the implementation if line probe assays in MDR-TB screening algorithms may significantly reduce the demand on conventional culture and DST laboratory capacity.

3. Implementation considerations

As with any new technology, a range of implementation issues was identified, without which line probe assays would not be useful. These include:

3.1 Specimen collection, storage and transport

The quality of sputum specimens submitted to the laboratory is critical in obtaining reliable results with line probe assays, as with other tests. Although contamination of specimens due to inappropriate storage and long transport times of specimens to the laboratory is less of a concern than with conventional culture-based approaches, a reliable specimen transport system will ensure that the full benefit is gained from use of a rapid assay, by reducing diagnostic delay times.

Current WHO recommendations call for MDR strains to be screened for XDR, both during surveys and in clinical settings where XDR-TB patients are suspected or confirmed. Refrigerated transport of specimens and rapid delivery systems are essential for conventional culture and DST procedures; therefore, strict adherence to standard operating procedures for specimen collection, storage and transport will be necessary if laboratories wish to implement second-line culture-based DST on specimens found to be MDR-TB by line probe assay.

3.2 Biosafety

Line probe assays require the digestion, decontamination and concentration of clinical specimens prior to DNA extraction. These processes involve aerosol-producing methods such as homogenization and centrifugation which pose a considerable risk of infection as well as cross-contamination of specimens. The processing of specimens for line probe assays should therefore be performed in a laboratory with adequate and appropriate biosafety level precautions.

Current WHO recommendations specify that specimen processing for mycobacterial culture be performed in a biological safety cabinet (BSC) under at least biosafety level 2 (BSL2) conditions. Procedures involving manipulation of *M. tuberculosis* cultures (identification, sub-culturing and DST) must be performed in laboratories complying with BSL3 standards. Applying these recommendations to line probe assays, processing of smear-positive specimens for direct testing should be performed in a BSL2 level laboratory, whereas performing the assay on positive cultures would require BSL3 facilities.

Conceivably, sputum specimens could be rendered non-infectious before shipping to the referral laboratory, obviating the need for BSL2 facilities; however, while line probe assays are likely to perform well on specimens inactivated/disinfected after collection, there are currently no sufficient data to recommend this practice. It should also be kept in mind that inactivation/disinfection of specimens result in loss of viability of organisms and that subsequent culture (eg. for smear-negative specimens) and DST (eg. for second-line anti-TB drugs to detect XDR) will not be possible.

Once the decontaminated specimens have been denatured (by heating), organisms present in the specimen are rendered non-viable. Subsequent steps may therefore be performed outside of the BSC; however, due consideration needs to be given to preventing amplicon contamination through stringent cleaning and working practices.

3.3 Laboratory design

Precautions to reduce the risk of cross-contamination of DNA molecular procedures are critical. This is achieved by using different rooms for DNA extraction, preparation of reagents for PCR (pre-amplification), PCR amplification and hybridization, and interpretation of results (post-amplification). Restricted access and uni-directional workflow through the rooms further

reduce the likelihood of amplicon contamination. Careful cleaning of all rooms after each use is also critical.

Due to space constraints it may not be possible to provide separate rooms for each step of the process in all settings. As a minimum requirement, three separate rooms for the different molecular steps should be established – one for DNA extraction, one for pre-amplification procedures, and one for amplification and post-amplification processes. As large equipment is not required for running the assays, the rooms can be fairly small in size. Restricted access, attention to the direction of workflow, and meticulously followed procedures for cleaning, are critical in attaining satisfactory results.

In most settings, renovations may be required to provide adequate facilities for performing line probe assays and the time, space and resources required for such renovations should be carefully considered.

3.4 Electrical supply and back-up power

Reagents used in line probe assays must be refrigerated or frozen, while amplification and hybridization procedures must be conducted under closely monitored temperature conditions. Uninterrupted power supply (UPS) connection is required during PCR amplification and use of the automated hybridization systems to avoid interruption of the procedure and subsequent loss of results.

Use of line probe assays therefore poses challenges in settings where interruption of the electrical power supply is common. Connection of laboratory power supply to a back-up generator and UPS is strongly recommended in such settings.

3.5 Reagent quality and shelf-life

Molecular grade water and Taq polymerase are required for PCR amplification. The quality of these reagents may critically affect the performance of line probe assays, and locally available reagents should be validated prior to use.

Short expiration dates of reagents are a concern for laboratories, especially in relatively inaccessible areas with complex customs clearance procedures. Management of inventory based on usage, shelf-life and lead time for deliver of orders is therefore needed.

3.6 Equipment

In addition to the equipment required for initial digestion-decontamination of sputum specimens (such as BSCs and safety centrifuges), line probe assays require specific equipment for molecular procedures such as a thermal cycler, shaking platform and water bath, heating block, sonicator, micro centrifuge and tubes, hybridization instrument, fridge, freezer, micropipettes and pipette tips, and PCR tubes. These are available from various commercial suppliers.

Correct specifications of equipment should be confirmed with line probe assay manufacturers and adequate lead time for procurement of such items must be allowed when implementing these assays.

Certain equipment such as incubators and automated hybridization systems are product-specific. Both commercial line probe assays can be used in automated, product-specific hybridization systems.

Manual line probe assay systems are appropriate for use in laboratories processing small numbers of specimens. Automated systems require a much larger initial capital outlay but can process up to 48 samples per run, taking between 2 and 3.5 hours, and are therefore an option to consider for high-throughput laboratories.

3.7 Human resources and training

Successful implementation and interpretation of line probe assays is highly dependent on the skill of laboratory staff performing the testing and the quality of supervision. The FIND/SAMRC/NHLS Demonstration Project has shown that these assays can be successfully implemented in high-volume laboratories; however, this is heavily reliant on the quality and training of personnel and their adherence to strict working practices, including cleaning and uni-directional workflow.

Since skilled and highly trained personnel are required for performing line probe assays, the human resource requirements need to be carefully assessed prior to implementation. It should be noted that supervision in most of the published studies was performed by scientists with postgraduate training in molecular technology.

Interpretation of results of line probe assays must be done with care due to the complexity of interpreting the banding patterns. A high level of skill is required to interpret banding patterns in cases of unusual mutations or mixed mycobacterial populations. These issues must be covered in initial training, with access to ongoing access to technical support when unusual results are obtained. Post-training supervision and monitoring (*ad hoc* or remote) of staff by a senior person with expertise in molecular assays is therefore strongly recommended.

3.8 Technical support

Coordination between commercial suppliers and customers with regard to ordering, shipping and customs clearance is critical to ensure smooth delivery of reagents and equipment and to avoid customs delays which may cause product deterioration due to inadequate storage conditions in transit.

A detailed plan for training, based on country-specific human resource needs, must be developed. Training may be provided directly by the manufacturer, by a nominated local distributor, or by an accredited third-party, depending on the location and circumstances. Agreements as to responsibilities for training should be made in advance of supply of equipment and reagents.

In addition, ongoing technical support and continuous supply of consumables and reagents is essential, best provided for in a formal service contract between the supplier and customer. Such a contract should cover the following aspects:

- Maintenance of equipment and provision of a servicing contract, including the repair or replacement of faulty equipment at short notice;
- Supply of consumables and reagents with at least six months expiry after arrival at the laboratory;
- A detailed plan for provision of ongoing technical support and the channels through which this will be provided, eg. a local distributor, via helpline, or internet-based support;

3.9 Quality assurance

The exquisite sensitivity of nucleic acid amplification assays such as PCR is also a draw-back, since even the smallest amount of DNA can be amplified. Target amplification methodologies such as line probe assays therefore require strict adherence to a number of procedures to minimize the risk of contamination leading to false-positive results.

Sources of contamination occurs when unwanted DNA is introduced to the assay through water, reagents, laboratory disposables and equipment, or through the environment, such as sample carry-over between tests or introduction of nonspecific amplification products through unrelated activities in neighbouring laboratories.

Providing each room with separate sets of equipment and supplies substantially reduces the risk of carry-over contamination. Working areas, equipment and everything that is routinely touched by hand (including doorknobs, telephones, handles of fridges and freezers, etc.) must be cleaned on a regular basis using appropriate cleaning agents and strategies. Additionally, the risk of contamination can be reduced by careful waste disposal.

From a clinical perspective, prevention of false-negative results is equally important. In nucleic acid amplification assays false-negative results are mostly due to the presence of inhibitors (often arising from laboratory surfaces), sub-optimal assay conditions or omission of key steps, or the absence of positive controls and internal process controls.

Aside from strict adherence to process and cleaning protocols and appropriate use of positive- and negative controls, monitoring of results based on expected outcomes is very useful to detect false-positive and false-negative trends. Knowledge of the underlying prevalence of MDR-TB in the populations from which the specimens are obtained is particularly useful.

Although data are limited, multi-centre PCR quality assurance studies have shown alarmingly high false-positive rates, but also indicating that procedural problems rather than specific assays were responsible. While internal quality control should be executed continuously by laboratory staff, external quality assurance through blinded rechecking of subsets of specimens or proficiency testing by an independent external organization is strongly recommended.

Standardized external quality assurance programmes for line probe assays are not yet available. Development of such systems is therefore an urgent priority.

3.10 Recording and reporting

In order to gain full benefit from implementation of line probe assays, systems must be implemented to ensure that results are reported rapidly to clinicians and patients to ensure that appropriate treatment is initiated. Furthermore, where conventional DST is used to confirm rapid assay results, the possibility of discrepant results must be considered, and a mechanism for explanation of implications of discrepancies to clinicians should be established.

4. Research needs

While these should not prevent or delay the implementation of line probe assays, priorities for research include:

- The evaluation of line probe assays in screening and diagnostic algorithms in different epidemiological settings;
- The cost-effectiveness and cost-benefit of line probe assays in different programmatic settings;
- The role of line probe assays in combination with conventional culture in smear-negative specimens;
- The impact of specimen inactivation/disinfection procedures on line probe assay performance;
- Methods to optimize DNA extraction, especially from specimens with low numbers of organisms.

5. Policy recommendations

The use of line probe assays is recommended by WHO, with the following guiding principles:

5.1 Adoption of line probe assays for rapid detection of MDR-TB should be decided by Ministries of Health within the context of country plans for appropriate management of MDR-TB patients, including the development of country-specific screening algorithms and timely access to quality-assured second-line anti-tuberculosis drugs;

5.2 Line probe assay performance characteristics have been adequately validated in direct testing of sputum smear-positive specimens and on isolates of *M. tuberculosis* complex grown from smear-negative and smear-positive specimens. Direct use of line probe assays on smear-negative clinical specimens is not recommended;

5.3 The use of commercial line probe assays rather than in-house assays is recommended to ensure reliability and reproducibility of results, as in-house assays have not been adequately validated or used outside limited research settings. Any new or generic line probe assays should be subject to adequate validation, ie. published laboratory validation studies, adequate data to allow systematic review and meta-analysis (including assessment of data quality), and results from field demonstration projects documenting feasibility and consistent performance equal to conventional methods and commercial line probe assays. New or generic line probe assays for MDR-TB should have the following characteristics:

5.3.1 A specific probe to identify *M. tuberculosis* complex;

5.3.2 Multiple probes to detect the most common mutations in *rpoB* (codons 531, 526 and 516);

5.3.3 Multiple overlapping wild-type (susceptible) probes covering the RRDR region of *rpoB;*

5.3.4 Preferably, multiple probes to detect both high-level (*catG* mutations) and low-level isoniazid resistance (*inhA* mutations);

5.3.5 Strip technology, with appropriate assay procedure controls, allowing visual detection of results;

5.3.6 Line probe test production under ISO 13485:2003 standards;

5.3.7 Performance characteristics equal to those of conventional DST methods;

5.3.8 Performance characteristics equal to those of current commercial line probe assays.

5.4 Adoption of line probe assays does not eliminate the need for conventional culture and DST capability; culture remains necessary for definitive diagnosis of TB in smear-negative patients, while conventional DST is required to diagnose XDR-TB. However, the demand for conventional culture and DST capacity may change, based on the local epidemiological situation and the use of line probe assays in country-specific screening algorithms;

5.5 As current line probe assays only detect resistance to rifampicin and/or isoniazid, countries with documented or suspected cases of XDR-TB should establish or expand conventional culture and DST capacity for quality-assured susceptibility testing of second-line drugs, based on current WHO policy guidance;

5.6 Adoption of line probe assays for rapid detection of MDR-TB should be phased in, starting at national/central reference laboratories or those with proven capability to conduct molecular testing. Once this has been accomplished, expansion could be considered, within the context of country laboratory strengthening plans, and considering availability of suitable personnel in peripheral centres, quality of specimen transport systems, and country capability to provide appropriate treatment and management of MDR-TB patients once diagnosed;

5.7 Adequate and appropriate laboratory infrastructure and equipment should be provided, ensuring that required precautions for biosafety and prevention of contamination are met:

5.7.1 Specimen processing for culture must be performed in biological safety cabinets (BSCs) in at least Biosafety Level (BSL) 2 facilities:

5.7.2 Procedures for manipulation of cultures (conventional identification, subculture for DNA extraction and conventional DST) must be performed in BSL3 facilities;

5.7.3 Laboratory facilities for line probe assays require at least three separate rooms - one each for DNA extraction, pre-amplification procedures, and amplification and post-amplification procedures. Restricted access to molecular facilities, uni-directional work flow, and stringent cleaning protocols must be established to avoid amplicon contamination leading to false-positive results;

5.7.4 Successful establishment, staffing, and maintenance of BSL2, BSL3 and molecular laboratories are demanding. Upgrading of facilities and establishment of the required infrastructure for molecular assays should be carefully planned and adequately financed;

5.8 Appropriate laboratory staff should be trained to conduct line probe assay procedures, especially those relating to amplification and interpretation of results. Supervision of staff by a senior individual with adequate training and experience in molecular assays is strongly recommended;

5.9 A detailed commercial sales contract and customer support plan should be negotiated with manufacturers, guaranteeing ample and continuous supply of materials, appropriate shipment conditions, customs clearance, equipment installation, maintenance, repair and replacement, and provision of training and ongoing technical support;

5.10 Stringent laboratory protocols, standard operating procedures for molecular line probe assays, and internal quality control mechanisms must be implemented and enforced. A programme for external quality assessment of laboratories involved in line probe assays should be developed as a matter of priority.

5.11 Mechanisms for rapid reporting of line probe assays results to clinicians must be established to provide patients with the benefit of an early diagnosis;

5.12 WHO and partners should assist countries with operational plans to introduce line probe assays within the appropriate epidemiological and resource availability context.

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