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MONITORING AND SURVEILLANCE OF MULTI-DRUG- AND EXTENSIVELY DRUG-RESISTANT TUBERCULOSIS

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ABSTRACT

Multi-drug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB) area major problem in Kazakhstan and, hence,new research initiatives and treatment protocols are urgently required.World-wide, diagnosis of TB is largely reliant on smear microscopy; however,this method has low sensitivity, which can also be further compromised in an endemic HIV setting. Moreover, smear microscopydoes not provide information on drug susceptibility or the genotype of the causative agent. At present, drug susceptibility is mainly tested using culture-based methods.However, the methodology is time-consuming and requires specialized infrastructure, and thus,in many countries,is restricted to large centers and does not always include testing for second-line drug resistance.An alternative approach is the TB-SPRINT assay, which reflects the idea of an "all-in-one" assay that identifies the causative agentand also identifieswith high confidence any mutations conferring resistance. The TB-SPRINT assaycan act as a surveillance tool to monitor the epidemiology of disease in communities and countries. An improved tuberculosis diagnostic assay and improved understanding of risk factors for spread of drug-resistant TB will benefit local populations and will indirectly be very beneficial to the economy of Kazakhstan.

Key words: Tuberculosis, MDR-TB, XDR-TB, strains, genotyping, SNP, mutation, TB-SPRINT

INTRODUCTION

THE SITUATION IN THE WORLD AND IN KAZAKHSTAN

Tuberculosis (TB) is one of the top 10 causes of death worldwide.In 2015, 10.4 million people fell ill with TB and 1.8 million died from the disease (including 0.4 million among people with HIV). Over 95% of TB deaths occur in low- and middle-income countries [1].Globally in 2015, an estimated 480 000 people developed multidrug-resistant TB (MDR).and additional 100 000 people with rifampicin-resistant TB also required second line treatment in 2015 [1].MDR and extensively drug-resistant tuberculosis(XDR) tuberculosis is a major problem in Kazakhstan.In average, one out of 10 MDR-TB cases is an XDR-TB cases. Kazakhstan is among the 27 high MDR TB burden countries in the world (fig.1).

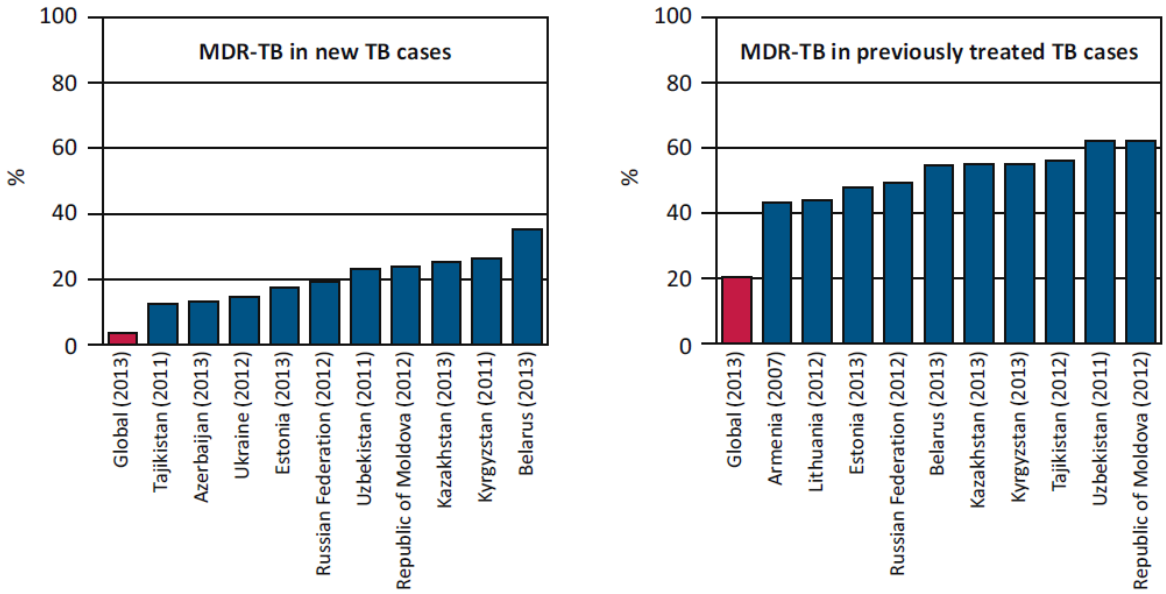


Fig. 1. The situation with MDR tuberculosis in Kazakhstan [2]

METHODS TO DETECT RESISTANCE AND/OR PERFORM MOLECULAR EPIDEMIOLOGY

Progress towardstargets for diagnosis and treatment of multidrug-resistant TB (MDR-TB) is far off-track.World-wide, diagnosis of TB remains largely reliant on smear microscopy which has a low sensitivity that is further compromised in endemic HIV setting and does not provide information of drug susceptible.

Methodscanbedividedintotwo range.Methods to detectresistance in *M. tuberculosis*are basedeither on assessment of specificSNPs, sequencing of drugtargets or phenotype. The scheme of these methods is shown in the figure 2.

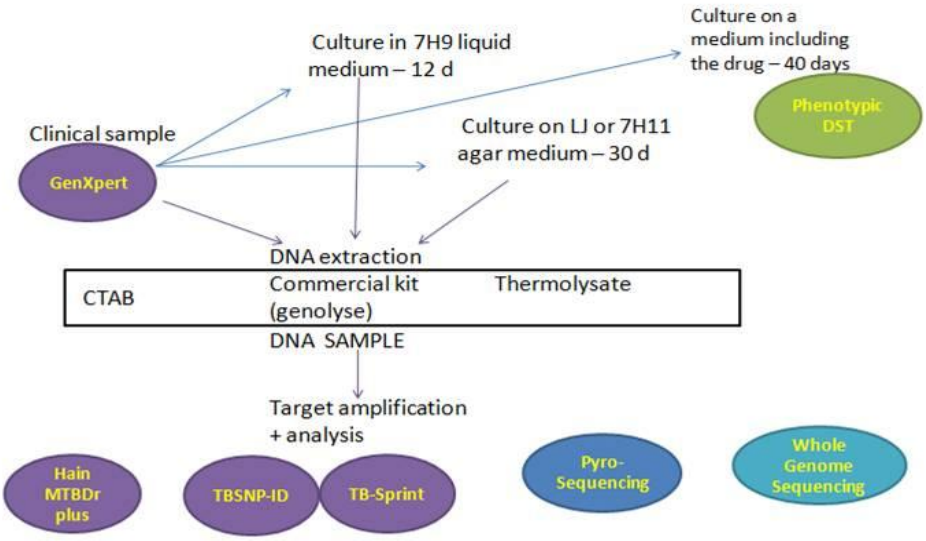


Fig. 2. The scheme of the methods todetectresistance in *M. tuberculosis*

Culture-based drug susceptibility testing remains the primary diagnostic tool to determine drug susceptibility. However, this methodology is time consuming and requires specialized infrastructure, and for this reason is restricted in many countries to large Centers and does not always include testing from second-line drug resistance.

Drug susceptibility testing (DST)

Drug susceptibility testing is the determination of a strain's susceptibility to drugs used in therapy: a "sensitive" result means that the patient with that strain has a high probability of treatment success, while a "resistant" result means that there is a high possibility of treatment failure and that the therapy should be changed. Thus, standardized and reliable DST of M. tuberculosis provides guidance for the treatment of the patient.DST methods currently in use are divided into two main groups – direct method and indirect methods [3].

Direct drug susceptibility method*:* in the direct DST method, a smear-positive sputum (at least 2+) is inoculated directly onto drug-containing and drug-free media. The inoculum size is adjusted according to the number of AFB (Acid Fast Bacilli) observed on smear microscopy of treated and concentrated sputum; one control tube is inoculated with a dilution (10^{-2}) of the sample.This method has certain advantages compared with indirect methods. First, the bacillary population used for performing DST is more representative of those existing in vivo, since all the bacilli in the sputum are represented in the inoculated sample. Second, the direct method takes 3-4 weeks less than indirect testing and there is no need for subculturing.There are also some disadvantages. The direct method can be used for highly smear-positive cases only (>or=2+). This method has a high contamination rate and is more difficult to calibrate because both live and dead bacilli are seen on the smear. The failure rate of the method is 10-15%.

It is mandatory to perform identification at the species level before reporting DST results*:* for indirect DST methods, organisms from clinical materials are isolated in culture; either a homogeneous suspension of their growth or a broth culture is inoculated onto control and drug-containing egg-based media.The three main methods are:absolute concentration method;resistance ratio method;proportion method [3].

In Kazakhstan, rifampicinand isoniazid susceptibility tests are carried out on LJmedium(Lowenstein Jensen Medium) containing 40 mg/L rifampicin, 0.2 mg/L, isoniazid, or 1 mg/L, isoniazid using the absolute concentration method according to the World Health Organization(WHO) recommendations [4].

MTBDRplusXpert MTB/RIF methods

In order to decrease the time for diagnostics and to expand theavailability of diagnostics, the WHO has endorsed threemolecular-based TB diagnostics: Genotype MTBDR plus and MTBDRllineprobe assays and the Xpert MTB/RIF assay. However, each of these assays has distinct limitations which restrict their use ascomprehensive diagnostic tools.WHO guidelines, in 2012 the NTP

(National tuberculosis program) of Kazakhstan decided to implement Xpert with the aim to improve case finding and management of MDR-TB patients by reducing time to diagnosis and allow earlier initiation of appropriate treatment. The procurement and installation of the first Xpert instruments in Kazakhstan was supported by USAID as part of a larger global health and development initiative [5].In particular, Genotype MTBDR*plus* does not allow newly described RIF (Rifampin) and INH (Isoniazid) mutations to be detected. The Xpert MTB/RIF does not provide any information relative to INH and second-lane drug mutations and remains expensive. For these reasons, other systems, that are already used as Research Use Only. Methods to performmolecularepidemiology for *M. tuberculosis*are shown on the figure 3.

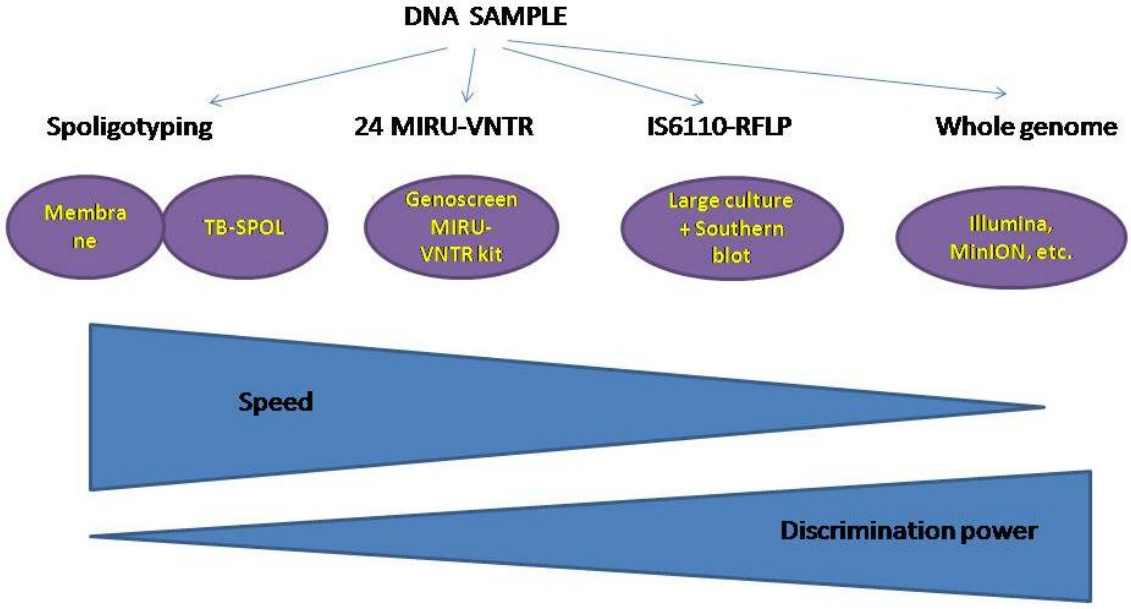


Fig. 3. The scheme of the methods to performmolecularepidemiologyfor *M. tuberculosis*

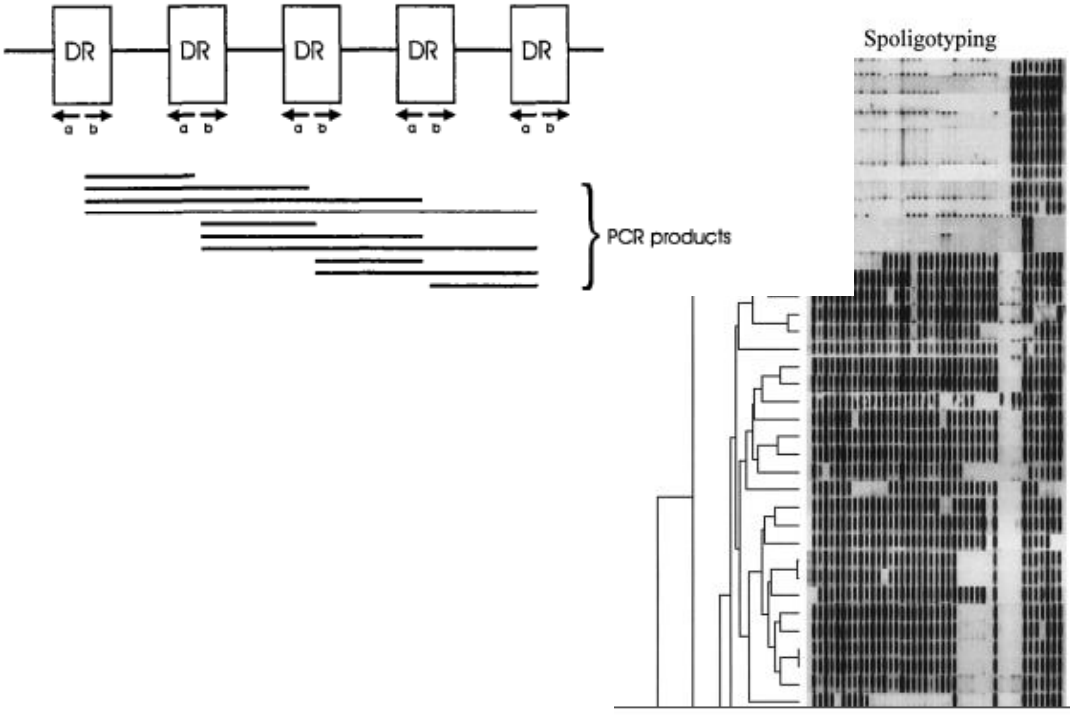
IS6110restriction length fragment polymorphism

IS6110restriction length fragment polymorphism (IS6110-RFLP) is a traditional *M. tuberculosis*typing method based on comparing patterns of fragments generated by digestion of isolated genomic DNA with restriction enzymes. The standardized and most widely applied molecular typing method for *M. tuberculosis*complex isolates is IS6110RFLP typing. This method is based on differences in the IS6110copy numbers per strain, ranging from 0 to about 25, and variability in the chromosomal positions of these IS6110insertion sequences. To visualize IS6110RFLP patterns, DNA is extracted and purified from a bacterial culture. Thereafter, the DNA is digested with the restriction enzyme *PvuII* and the restriction fragments are separated on an agarose gel. The separated restriction fragments are transferred to a DNA membrane. In order to visualize the IS6110-containing restriction fragments, a peroxidase-labelled probe with a DNA sequence complementary to the IS6110DNA sequence is added to a hybridization buffer which is poured onto the membrane. The *PvuII* restriction fragments the IS6110probe hybridizes to, are highlighted by a chemiluminescence reaction initiated by adding two substrates. The membrane is packed in plastic and the RFLP patterns are detected by putting a light-sensitive film on the packed membrane in a light-blocked cassette. An example of the heterogeneous IS6110RFLP patterns of*M. tuberculosis*complex isolates from the Netherlands is depicted in fig. 4[6].

Spoligotyping

Spoligotyping is based on the presence or absence of the 43 distinct “spacers” separating insertion elements in direct repeat (DR) regions of the *M. tuberculosis*genome. The DR locus contains multiple 36-bp DRs separated by 30- to 40-bp unique spacer sequences.43 different synthetic oligonucleotides, each corresponding to one of the unique spacer DNA sequences within the DR locus, are covalently linked to a membrane in parallel. The next step is to amplify and label all DNA sequences within the DR region of a given strain by PCR. The primers used are based on the sequence of the DR, and allow the amplification of the spacer(s) between the target DRs (fig. 4). The resulting PCR products differ in length because of

two reasons: the DR primers can initiate polymerization at any DR, irrespective of the number of DVRs (direct variant repeats)between the primers, and PCR products can act as a primers and become elongated with one or more DVRs. Spoligotyping is traditionally performed by reverse hybridization of biotinylated PCR products to spacers applied on a nitrocellulose membrane.



The use of the two primers, DRa and DRb, for in vitro amplification, will lead to the amplification of individual spacers or blocks of neighboring spacers and DVRs Primer DRb is labeled with biotin, allowing nonradioacitve detection of PCR products after hybridization

Fig. 4. Principle of the in vitro amplification of DNA within the DR region of *M. tuberculosis* complex bacteria [7]

MIRU-VNTR

Molecular genotyping of *M.tuberculosis*is an important laboratory tool in the context of emerging drug resistant TB. MIRU-VNTR is based on the analysis of 24 loci containing variable numbers of tandem repeats (VNTRs) of genetic elements called mycobacterial interspersed repetitive units (MIRUs).The standard 24-loci MIRU-VNTR typing includes PCR amplification followed by the detection and sizing of PCR fragments using capillary electrophoresis on automated sequencers or using agarose gels [8].

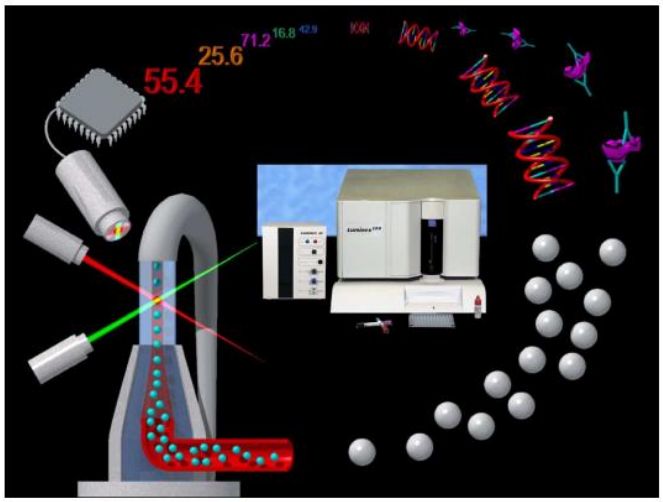
Next generation Sequencing (NGS)

Other competing technologies are Next generation Sequencing (NGS). However, NGS information flows remains extremely sophisticated and hazardous. NGS technology itself can be done using a wide-range of technologies that are in competitions one against another. Moreover, the price per assay, even if it is decreasing fast, remains too high for public health systems, and there is a clear demand of simple, post-NGS projects that will try to detect the maximum of relevant information using the minimal technical requirements; as such, many public health systems (even the richest) will be reluctant to sequence all clinical isolates/samples if the clinical added value is not evident. This is quite obvious that information systems and flow require optimization and after fashion trends, it is evident that systems that will provide economical gain will be preferentially chosen that systems whose cost/efficiency ratios are low. For these reasons, cheap array systems have a role to play in the future. Miniaturization will also allow to decrease costs [9,10]. Thefull genome of M. tuberculosis is studied in Kazakhstan. Kairov and others presented a reportof the draft genome sequences of two clinical isolates of *Mycobacterium tuberculosis* (MTB-476 and MTB-489) isolated from sputum of Kazakh patients [11].

LuminexxMAP

Multiplexing represent a future of molecular diagnostics. Microsphere-based suspension array technologies, such as the LuminexRxMAPi system, offer a new platform for high-throughput nucleic acid detection which is being used in a variety of applications.This technology has revolutionizing the clinical diagnostics and life science industries by enabling companies and laboratories to perform bioassays far more quickly and cost-effectively than with other systems, while ensuring optimum accuracy. It is applicable to antibody/antigen or DNA/RNA/probes detection in multiplexed (parallel) assays [12,13].

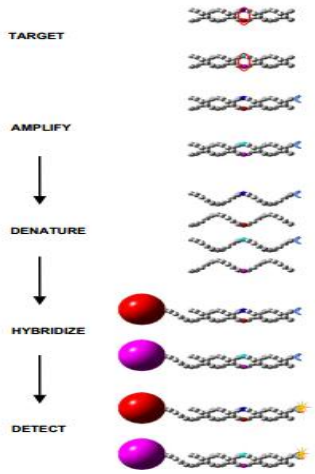
The LuminexxMAP system incorporates 5.6 Am polystyrene microspheres that are internally dyed with two spectrally distinct fluorochromes. Using precise amounts of each of these fluorochromes, an array is created consisting of 100 different microsphere sets with specific spectral addresses. Each microsphere set can possess a different reactant on its surface. Because microsphere sets can be distinguished by their spectral addresses, they can be combined, allowing up to 100 different analytes to be measured simultaneously in a single reaction vessel (fig.5) [13].



The four main components of the xMAP system are shown, clockwise from top-right: biomolecular reactants; fluorescently color-coded microspheres; fluidics and optics; and high-speed digital signal processing. The Luminex 100 analyzer with XY platform and sheath delivery system is pictured in the center

Fig. 5. LuminexxMAP system and components [13]

Several assay chemistries have been used for nucleic acid detection on the xMAP system. One approach is to use direct hybridization of a labeled polymerase chain reaction (PCR)-amplified target DNA to microsphere sets bearing oligonucleotide capture probes specific for each sequence.



Target DNA is PCR-amplified. One of the primers is biotinylated. The amplified products are denatured, hybridized to allele-specific probecoupled microsphere sets and labeled for detection with streptavidin-Rphycoerythrin.

Fig.6. Diagram of the microsphere-based direct hybridization assay forma [13]

Multiplexing allows a number of experiments to be simultaneously performed in a single tube, at relatively low additional cost,producing a deluge of data. Multiplexed diagnostic data are invaluable to control and monitor disease at the population level as well as guide individual therapy [14,15,16].

Teams in the Netherlands and in France have become experts in applying these multiplexed technologies to public health and are continuously developing new assays with improved cost-effectiveness. This was acknowledged by the publication of many papers in clinical microbiology, epidemiology and public health [17-21].

TB-SPRINT technology (Tuberculosis-spoligo-rifampin-isoniazid typing)

A classical genotyping technology, «spoligotyping» was transferred onto microbrad-based hybridization format in 2004 [22].The 43 spacer oligonucleotide probes used in study of Cowan were the same as those used in the traditional spoligotyping assay [23].The probes were synthesized with a 5′-terminal amino group with a six-carbon spacer to allow covalent attachment to membranes or microspheres.primers that hybridize to the 36-bp DR were used to amplify the DR region, simultaneously amplifying all of the spacers present. The average size of the amplicons produced was 75 bp. One primer was biotinylated at the 5′ terminus for subsequent detection using a streptavidin conjugate.

In Europe, the IGEPE team introduced the method in 2009 [24]. In this study, they describe the implementation of and improvements to the spoligotyping technique in different formats, including suggestions for an increased discrimination format or simplifications to create a reduced cost-effective form suitable for a wide range of settings. They found the multiplex microbead-based spoligotyping technique to be versatile and easily implemented and it can be adapted (classical 43 spacers format for Europe, improved 68 spacers format for Asia, simplified 10 spacers format for first-screen genotyping in resource-limited settings).

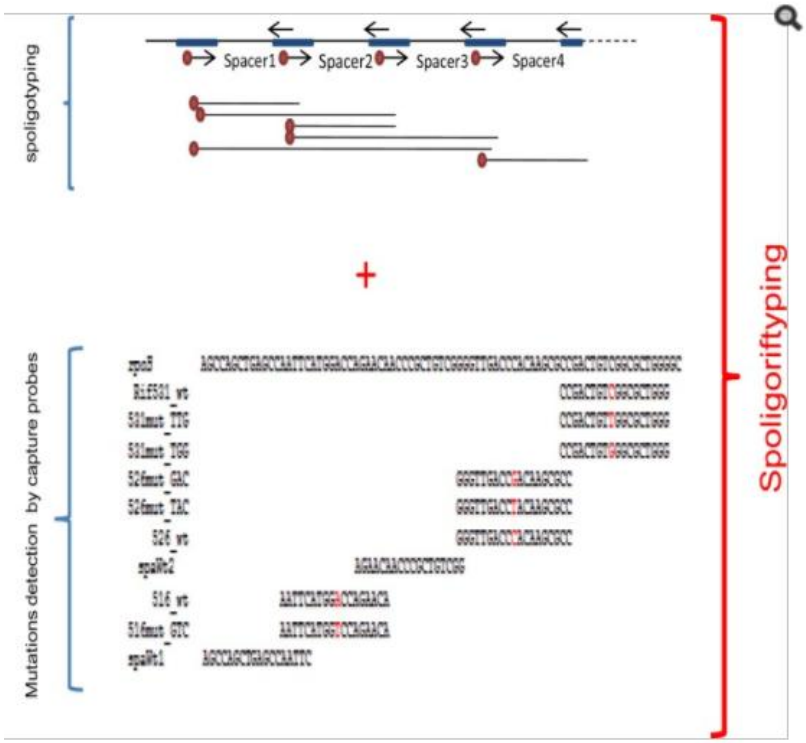
Later a new technique showed that clinical isolates DNA could be simultaneously used to detect high confidence mutations conferring resistance to isoniazid and rifampicin and to obtain a genotyping profile.

Sola and his colleagues developed “spoligoriftyping,” a 53-plex assay based on two preexisting methods, the spoligotyping and “rifoligotyping” assays, by combining them into a single assay. Spoligoriftyping allows simultaneous spoligotyping (i.e., clustered regularly interspaced short palindromic repeat [CRISPR]-based genotyping) and characterization of the main rifampin drug resistance mutations on the *rpoB*hot spot region in a few hours. This test partly uses the dual-priming-oligonucleotide (DPO) principle, which allows simultaneous efficient amplifications of *rpoB*and the CRISPR locus in the same sample [25].

Spoligoriftyping is based on a simultaneous analysis of the polymorphism in the clustered regularly interspersed short palindromic region (CRISPR) and those in the *rpoB*gene hot spot region, as shown in fig. 7. Specifically, spacers 1 to 43 are amplified, as well as the most frequent SNPs, i.e., TTG at codon 531, TGG at codon 531, GAC at codon 526, TAC at codon 526, and GTC at codon 516, with specific capture probes used for detection (fig.7).

A)

B)



(A) The CRISPR region is amplified by a single pair of primers that hybridize within the repeat sequences and overlap the spacers; (B) The full span of the *rpoB*gene hot spot region is determined**.**

Fig. 7. Schematic representation of the spoligoriftyping test principle [25]

Molecular mechanisms of *Mycobacterium tuberculosis*complex (MTC) drug resistance are well known and are reported to involve mutations in specific genes. Rifampin (RIF) resistance involves mutations in the *rpoB*gene (the 81-bp rifampin resistance-determining region [RRDR]), and isoniazid (INH) resistance is associated with mutations in *katG* (codon 315), *inhA* (positions −15 and −8 in the *inhA*promoter sequence)The method developed by the IGM team, “TB-SPRINT” is easily adapted to allow for the inclusion of additional resistance markers [26,27,28].

As a follow-up of the “spoligoriftyping” development, Sola and his colleague present an extension of this technique which includes the detection of isoniazid resistance-associated mutations in a new 59-plex assay, i.e., tuberculosis-spoligo-rifampin-isoniazid typing (TB-SPRINT), running on microbead-based multiplexed systems.

This assay improves the synergy between clinical microbiology and epidemiology by providing mutation-based prediction of drug resistance profiles for patient treatment and genotyping data for tuberculosis (TB) surveillance. Spoligotyping patterns obtained by the TB-SPRINT method were 100% concordant with those obtained by microbead-based and membrane-based spoligotyping. Genetic drug susceptibility typing provided by the TB-SPRINT method was 100% concordant with resistance locus sequencing. Considering phenotypic drug susceptibility testing (DST) as the reference method, the sensitivity and specificity of TB-SPRINT regarding *Mycobacterium tuberculosis*complexrifampin resistance were both 100%, and those for isoniazid resistance were 90.4% (95% confidence interval, 85 to 95%) and 100%, respectively [25].

The method of TB-SPRINT was tested in the genotyping of mycobacterium in Brazil [29], Kazakhstan (not published).

Acknowledgements

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КЕҢ ЖӘНЕ КӨП ДӘРІГЕ ТӨЗІМДІЛІГІ БАР ТУБЕРКУЛЕЗДІ БАҚЫЛАУ ЖӘНЕ ҚАДАЛАУҒА АРНАЛҒАН ЗАМАНАУИ ӘДІС

Жолдыбаева Е.В.
Қазақстан Республикасының Білім және ғылым министрлігі Ғылым комитетінің «*Ұлттық* биотехнология *орталығы*» *республикалық мемлекеттік кәсіпорны*
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Туберкулез (ТБ) Қазақстандағы қоғамдық денсаулық сақтаудың маңызды мәселесі болып қалуда, өйткені жыл сайын көп дәріге төзімділігі (MDR) бар, сонымен қатар дәріге төзімділігі кең (ДТК-ТБ) ТБ жағдайлары тіркелуде, сәйкесінше бұл ауру ғылыми зерттелуді талап етеді.

Әлемдік практикада ТБ диагностикасы көп жағдайда қақырық мазогын микроскоптауы болып табылады. Бірақ бұл әдістің сезімталдылығы төмен, дәрілік препараттарға сезімталдылығына немесе туберкулез микобактерияларының генотипіне қатысты ақпарат бермейді.

Бірақ, қазіргі кезде бұл әдіс көп еңбекті қажет етеді, сонымен қатар арнайы мамандандырылған инфрақұрылымды талап етеді, сондықтан көптеген елдерде және үлкен орталықтарда оны қолдану шектелген, және екінші қатарлы препараттарға тұрақтылықты тестілеуді үнемі қоспайды.

TB-SPRINT әдістері қоздырғышты анықтауға, тұрақтылыққа жауап беретін дәлділігі жоғары мутацияларды анықтауға қабілетті «барлығы біреуінде» талдауының идеясын айқындайды; бұл әдістер қоғам мен елдерде ауру эпидемиологиясы мониторингінің құралы ретінде жұмыс істейді.

Туберкулездің жақсартылған диагностикасы, сонымен қатар дәріге тұрақты туберкулездің таралу қаупінің факторларын білу жергілікті тұрғындарға тиімді, сонымен қатар Қазақстан экономикасына едәуір пайда әкеледі.

Негізгі сөздер: туберкулез, MDR-TB, XDR-TB, штамдар, генотиптеу, SNP, мутация, TB-SPRINT