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RAPID SCREENING AND DIAGNOSIS OF TUBERCULOSIS: A REAL CHALLENGE FOR THE MYCOBACTERIOLOGIST

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Abstract

Tuberculosis (TB) is an infectious, devastating and contagious disease, which infects third of the global population worldwide with high rates of incidence in the developing countries, where the health care providers face a serious problem and a real challenge during their clinical practice for controlling and preventing the transmission of this illness. Indeed the first step of control is the correct diagnosis and the initiation of the drug treatment regimen at the early stage of infection, which mandate the rapidity of screening and the accuracy of laboratory testing. In this paper we aim to highlight the different actual techniques, regarding the rapid screening and diagnosis of tuberculosis.

Key words: *Mycobacterim tuberculosis* MTB, Bacteriological techniques, Molecular techniques, Diagnosis, Identification.

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INTRODUCTION

Tuberculosis (TB) is an infectious, devastating and contagious disease caused by a *Mycobacterium*, called Koch s' bacillus (KB).

This disease is an ancient infection which affected the human being since millennia and that had been documented and detected in Egyptian mummies in several studies, either by direct conventional microscopic examination (111) or by molecular techniques (67,112).

Furthermore, tuberculosis is still a major threatening of humanity especially in developing countries and according to the last reports of The World Health Organization (WHO), it is estimated that one-third of the global population is infected with tuberculosis and that approximately 8.9- 9.9 million new cases of tuberculosis arise annually and over 1.3 million deaths among HIV-negative people and 0.38 million deaths among HIV-positive people (105). In almost cases; the causal agent of human tuberculosis is *Mycobacterium tuberculosis* (MTB). However TB can be caused by other Mycobacteria in some cases with special conditions (10), these Mycobacteria are belonging to the members of *Mycobacterium tuberculosis* complex (MTBc), (15, 85, 86) which include:

- *Mycobacterium africanum*: That can provoke human tuberculosis, principally in the west of Africa (18).
- *Mycobacterium bovis*: which infects Badgers, cattle, deer, elephants, goats, lions, seals, etc (15) and can spread to humans through inhalation of infectious droplet and by ingestion of raw milk. Furthermore, *M. bovis* has been associated with extrapulmonary tuberculosis in infants and children, generally occurring due to the consumption of milk, which had not been pasteurized or boiled, from infected cattle (93), especially in the HIV infected infants (37). Therefore the zoonotic risk for human represents a serious problem, mostly for those living at the animal-human interface (61).
- *Mycobacterium bovis,* Bacille Calmette–Guérin (*BCG)*: this has been largely used as a vaccine against human tuberculosis (6, 19) and can be obtained by the attenuation of *M.bovis* and deletion of the RD1 region (15).
- *Mycobacterium microti*: which infects voles, Cats, dogs (15, 82) and human as reported recently in immunocompetent patients in France by Panteix et al, 2010 (71).
- *Mycobacterium canetti*: which infects human in limited geographical location, in the horn of Africa (24).
- *Mycobacterium pinnipedii*: that infects seals, sea lions and marine mammals (8, 45).
- *Mycobacterium caprae*: which can infect domestic animals such as goats, sheep, ewes (60), camels, horses, pigs, dogs, cats (16), wild animal species (80) and human being.

Furthermore, it has been demonstrated that these members are sharing a common ancestor in their evolutionary events (34, 110) and has been settled that *M. tuberculosis* arose from *M. bovis* and *M. tuberculosis* emerged approximately 10–15,000 years ago, when the bovine tubercle bacillus was transmitted to the mankind (15, 34).

It is worth mentioning that there are other mycobacteria called atypical mycobacteria or non tuberculosis mycobacteria (NTM).

These mycobacteria are ubiquitous in the environment and in extreme circumstances (immunosuppression, HIV infection, underlying diseases……etc), some of them become pathogenic for humans and can induce opportunistic infections or mycobacterioses (30). These mycobacteria often are described based on their growth rate and pigmentation with and without exposure to light (5). The last group of mycobacteria is represented by *M leprae*, the causal agent of leprosy in humans and characterized by the inability to be cultured *in vitro* (42).

Although the diagnosis of TB can be made by different techniques: radiological, clinical and immunological techniques, the identification of causative organism MTB in the clinical sample is the most accurate and reliable for the final decision of tuberculosis and for initiating the drug treatment regimen (17). In this paper we aim to outline the different actual techniques regarding the rapid screening and diagnosis of tuberculosis.

SCREENING AND DETECTION OF THE COMPLEX *Mycobacterium tuberculosis* (MTBc)

Diagnosis of the latent tuberculosis

In 2005, the CDC guidelines for controlling the tuberculosis and the reduction of the mortality and morbidity cases due to this horrible illness, one of the most important strategies for the achievement of this gaol is the identification of the persons with latent TB infection (LTBI), especially those who are at high risk for potentially active TB disease (3).

The diagnosis of LTBI has habitually been based upon results of tuberculin skin testing or Mantoux test (56). This test involves an intradermal injection of the Purified Protein Derivative (PPD) or the tuberculin (2). The response typically appears a day or two after the injection and consists of a raised, red, and indurated area in the skin, which then disappears as the antigen degraded (5, 10). This indicates the presence of antibodies or lymphocytes that are specific for that antigen (cellular immunity).

Moreover, the immunological tests (the QuantiFERON-TB Gold® and TSPOT®- TB tests) which are whole blood interferon gamma release assays (IGRAs), are now other options for detecting LTBI and are potential tools for diagnosing LTBI (49, 62). These tests are based on the principle that T cells from most persons that have been infected with MTB will release IFN-γ when re-exposed to the same mycobacterial antigens (*in vitro*).In fact these immunological tests on peripheral blood alone can not differentiate between active and latent TB (51).

Diagnosis of active TB

It is important to know that tuberculosis can be diagnosed accurately by identifying the causative organism, MTB in the clinical sample (17) and the suspicion of TB disease in a patient requires the completion more thorough medical examinations:

Radiological Examination: The radiological diagnosis can highlight the pulmonary form of TB and the chest X ray is the most traditional technique for the screening of TB (51). Although the presence of abnormality findings in the conventional radiography is not decisive for the TB infection (29, 39), the cavitation is still the most remarkable sign of the active TB or the post primary infection and can indicate the severity of disease (58).

Bacteriological examination: Generally, the investigation of the tubercle bacilli can be done on pulmonary samples after their decontamination, though it can also be performed on the other extrapulmonary specimens or other body fluids (Urine, cerebrospinal fluid, semen, gastric liquid……..etc) and the diagnosis of MTB can be achieved by:

- **Microscopic examination**

The smear microscopy is the first step and the key of bacteriological diagnosis of TB. However the smear microscopy can not distinguish between viable and dead microorganism and does not differentiate between the species of mycobacteria (35), Moreover it can only detect acid-fast bacilli in concentrations of 10,000 organisms per ml or more (109), but from an economical view the conventional microscopy is still the most useful tool or the sole tool in the low income countries (88), where the diagnosis of smear negative tuberculosis poses a serious problem for the health care providers, especially in the HIV co infected patients (9, 84). In fact there are different staining methods, but the widely used technique is the Ziehl-Neelsen method (13), which lack sensitivity and require the good expert technicians for the investigation about the bacillus in different fields on the smear (56). The problem of sensitivity can be improved by the concentration by centrifugation of the specimens after adding the disinfectant NaOCl as demonstrated by Gebre *et al*., in different developing countries (27). Nevertheless the sensitivity of the microscopical examination has been developed in the industrial countries by the utility of the Fluorescence microscopy (88), which provides more accurate results.

- **Culture**

The culture is the gold standard for the diagnosis and detection of TB (72). Most laboratories use solid medium for the isolation of MTB. After homogenizing and decontaminating the specimen, inoculation the media by means of a pipette delivering around 0.2ml as recommended by WHO (106). Two media are used for MTB growth, Middle brook's medium which is an agar based medium and Lowenstein-Jensen medium which is an egg based medium. Both types of media contain inhibitors to keep contaminants from out-growing MTB and the culture should be examined during 4-6 weeks to get visual colonies on either type of media. It is noteworthy to mention that there are also new liquid techniques that have been developed to shorten the time of culturing, comparing with traditional techniques of culturing on solid medium, such as: BAC-

Table 1. Some of the morphological and biochemical properties of tubercle bacilli.

Mycobacterial strain	Host	Colony Morphology	Consumption of Oxygen	Urease hydrolysis at 3 h	Urease hydrolysis at 18 h	PZA	TCH	Niacin accumulation	Nitrate reduction
M.tuberculosis	Humans	eugonic	aerophilic	-	+	S	R	+	+
M.bovis	Wild and domestic animals	dysgonic	Micro aerophilic	-	+	R	S	-	-
M. bovis BCG	----------	eugonic	Micro aerophilic	+	+	R	S	-	-
M. africanum	Humans in the West of Africa	dysgonic	Micro aerophilic	-	+	S	R/S	+/-	+/-
M. microti	Wild and domestic animals	eugonic	aerophilic	-	+	S	S	+	-
M.canetti	Humans in the horn of Africa	Very smooth, eugonic	aerophilic	+	+	R	R	+	+
M.caprae	Wild and domestic animals	eugonic	aerophilic	-	+	S	S	+	-
M.pinnipedii	Seals, sea lions	eugonic	aerophilic	-	+	S	S	+	-

TCH: thiophen-2-carboxylic hydrazide, PZA: pyrazinamide, R: resistant, S: sensitive

TEC 460 TB, BacT/ALERT 3D and BACTEC MGIT 960, ESP II culture system (1, 65, 73, 96). Although of the rapidity of liquid media in culturing mycobacteria, the solid medium still have the advantage of enabling the differentiation of mycobacterial growth and contamination, even more the colonial morphology, production of pigments, rates of growth and the optimal temperature of growth can help in the identification and the discrimination between rapid and slow growing mycobacteria (50). Table1 shows some of the colonial morphology, the production of pigments and oxygen consumption for the differentiation among the complex MTB. Therefore it is recommended to combine both of solid and liquid medium for the best recovery of mycobacteria (87).

- **Phenotypic and biochemical identification**

Even though their consumption of time and cumbersomeness, the biochemical identification is still useful in the developing countries for the differentiation among MTB complex and other mycobacteria, particularly the niacin test (26) and due to the large number of tests in this topic, we can not mention them in detail and briefly passing through the most important utilized phenotypic and biochemical tests, we can point out: Catalase, Niacin test, Nitrate reduction, Aryl sulphatase, Tween80 analysis, Urease Activity, Pyrazinamide (PZA), Iron Uptake, Growth inhibition by Thiophene 2 carboxylic acid Hydrazide (TCH), Growth on 5% sodium chloride, ß glucosidase, tellurite reduction (31, 52).

Levy-Frebault *et al.,* had evaluated 8 rapid biochemical and enzymatic tests for the identification of 18 species of mycobacteria (niacin, catalase, nitrate reductase, ß glucosidase, urease, penicillinase, trehalase and cephalosporinase) and they found that those tests were able in some cases to discriminate between closely related species (54).

Table 1 show also the most used biochemical tests for the phonotypical and biochemical differentiation among the complex MTB quoted from (15, 20).

- **Drug susceptibility testing DST**

After the detection and the identification of MTB, the DST is the main analysis to be performed for better management of TB, using different anti tuberculosis agents. The most used method of DST is the proportional method, adjusted from the method described by Canetti (12). This technique consists of observing the mycobacterial growth on the Löwenstein-Jensen medium containing different concentrations of tested antibiotics, this method is currently the method of choice for estimating drug resistance but it takes approximately four weeks for the final assessment. This period can be halved by the implementation of the DST on the BACTEC liquid media (33, 38, 98). There are also different commercially colorimetric methods for performing the DST; the Reszurin test is one of the low cost, rapid and accurate tests for the detection of drug resistance of MTB strains (53).

Molecular Examination: Nowadays, there are many molecular techniques are commercially available which have decreased the time for the diagnosis, detection, identification of MTB and drug susceptibility pattern (44). However, they have an excellent sensitivity and specificity when used from a culture, these techniques have currently insufficient sensitivity when applied directly to respiratory and other samples (46), these tests are numerous and we can list the most important tests:

- **DNA Amplification by PCR, including Real Time PCR**

The utilisation of PCR for the detection of MTB in clinical samples has been reported and commercially available amplification and detection kits as well as manual in-house methods are also applied in routine diagnostic laboratories (28, 40, 55, 63, 75-77, 83, 104, 107) and its reliability has been questioned (68). Moreover, the paucibacilary nature of specimens (sputum, biopsies, pus and body fluids) was a challenge for the detection of this microorganism.

The insertion sequence IS 6110 (7, 22, 23, 91) 16S rRNA (14, 48, 64) and hsp 65 (47) are the most common targets used for the MTB diagnosis in the clinical specimens. Additionally there are different types of PCR-based assays have been developed and modified for this purpose, the Real-time PCR is one of the promising tools with high sensitivity for the rapid identification of different mycobacterial species, with the advantage of possible identification and detection of a given target sequence directly from clinical specimens (95).

The Multiplex PCR is also a potential tool for distinguishing between the pathogenic species of mycobacteria and it is supposed to be simple, rapid, cost effective and superior to the traditional methods (30). Recently, Warren *et al.,* have developed a new multiplex PCR for the differentiation the members of MTBC by the amplification of genomic regions of difference (RD1, RD1^{mic}, RD2^{seal}, RD4, RD9, RD12) (103). The PCR-restriction endonuclease analysis (PRA) had been used and evaluated for the differentiating between 39 pathogenic rapid growing mycobacteria (RGM) by Wang *et al.,* and they found good results (102) , in addition of the fast identification comparing with conventional method and it could be used into the clinical laboratory setting, particularly for patients who are suffering of infection due to pathogenic RGM and according to Varma-Basil *et al.,* the PCR-RFLP was recently used for the direct identification of MTB in the clinical specimens and could offer a considerable benefit for clinician for their chemotherapy choice (101).

- **Nucleic acid Probe**

The DNA probe is one of the efficient methods for the identification of MTB members from pure culture (66) and the most used DNA probes in the routine laboratories are the AccuProbe for the rapid identification of MTB.

Badak *et al.,* demonstrated that the AccuProbe allows the accurate and rapid identification of MTB when applied directly on the positive MB/BacT broth (4). Furthermore it can also be used for the identification of other non tuberculosis mycobacteria (97)

- ***In situ* Hybridization**

The fluorescence hybridization *in situ* by using Peptide Nucleic Acid Probes has been recently demonstrated a good results for the identification of the members of the MTBC and the differentiation tuberculosis and non tuberculosis mycobacteria, especially in the liquid culture (21, 36, 70).

- **Automated Sequencing**

Genetic sequencing techniques have progressively become useful tools for mycobacterial differentiation (74) and sequencing of a fragment of conserved genes such as 16S rRNA (32, 81, 108) or hsp65 (78, 79, 90) are the most sensitive methods for identification of a large number of mycobacterial species. Recently a single step sequencing technique has been applied for the identification of the eight closely related members of the complex MTB, called the Exact Tandem Repeat D or the ETR-D sequencing (20). Importantly, sequencing is also used for DST to characterize the genetic mutations associated with resistance to antituberculosis drugs.

- **Strain typing and DNA fingerprinting**

Tuberculosis epidemiology has been clarified significantly by the development of molecular biological techniques which allow the relatively unambiguous identification of a particular clinical isolate (5). The restriction fragment length polymorphism (RFLP) using the insertion sequence IS6110 has become the standard international method for fingerprinting isolates of *M. tuberculosis* (62, 100). The IS6110 is a transposable element present in the members of MTB complex in multiple copies (up to 25 copies) (92, 94) except *M. bovis* BCG which harbours a single copy (68) and absent in other mycobacteria. Furthermore, due to its high numerical and positional polymorphism, has become a widely used marker in the epidemiological studies (57, 59, 69), identification (99) and phylogenetic analysis (94). In the last decade many methods for typing of clinical strains of MTB were developed, the most commonly used methods; the spoligotyping and MIRU-VNTR.

The spoligotyping relies on the analysis of a unique chromosomal region of the genome with high polymorphism, called DR or direct repeat and contains of 36 bp that are separated by non repetitive DNA spacers (41), the order of spacers is identical in all mycobacterial strains, but their presence or absence varies from one isolate to another (Figure 1).

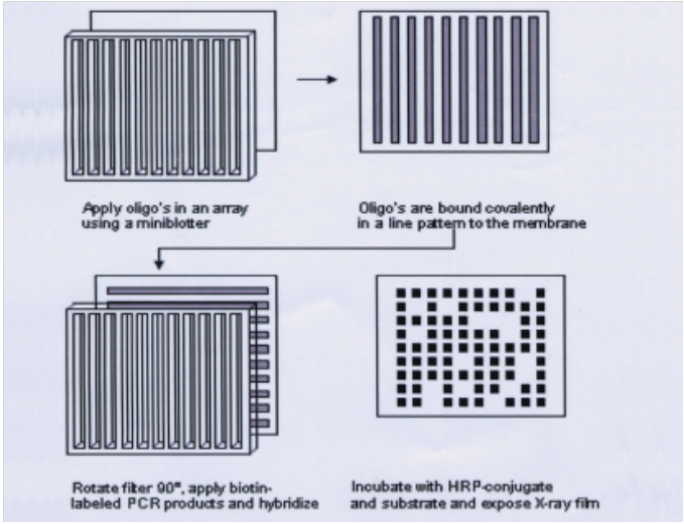

Figure 1. Steps of Spoligotyping method.

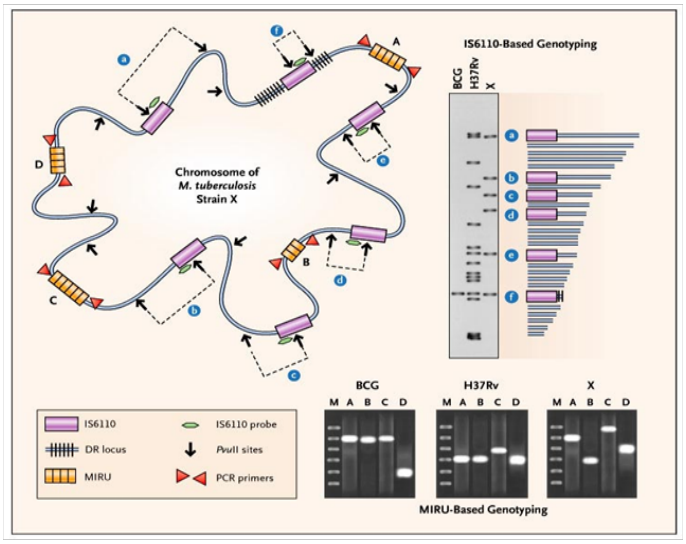

Figure2. Chromosome of MTB (X) strain, genotyping of *M. bovis* BCG, H37Rv MTB strain on the basis of repeated units MIRUs. On the top: a chromosome of MTB strain, the three lower panels show the results of genotyping based on MIRUs. The MIRUS contain repeated units; the analysis of MIRUS involves a PCR amplification followed by electrophoresis to look for the number and size of the elements repeated in 12 independent loci, each with one repeated sequence.The sizes of molecular weight marker (M) and PCR products (A, B, CD) BCG, H37Rv and X strains are given. The specific sizes of the different MIRUS in each strain are the result of a distinct fingerprint of the strain.

Table 2. Culture and Molecular based techniques (Principles, advantages and limitations)

Culture based techniques			
Technique	**Principle**	**Advantages**	**Limitations**
Culture based techniques (MTB detection, identification and DST)	Detection of bacterial growth after inoculation of clinical specimens on media culture	Gold standard Characteristics, morphology and pigmentation of colonies can help in the detection, identification of MTB and DST testing	Cumbersome and takes a long time (up to 8 weeks) especially on solid media. The presence of viable bacteria is necessary and it is not always possible, especially in treated people.
Molecular based Techniques			
Technique	**Principle**	**Advantages**	**Limitations**
PCR and various PCR techniques: Real time PCR Multiplex PCR…etc	Detection of the presence of MTB genomic DNA in clinical samples after amplification	High specificity. Fast results. Allows identification and investigation of genetic resistance patterns	Higher cost and limited availability. Variable sensitivity. Inferior sensitivity for non respiratory specimens. Does not allow ruling out tuberculosis
Nucleic acid Probe	Detection of a complementary target sequence in a nucleic acid	Rapid identification of MTBC members from pure culture. Differentiation between MTB and NTM	The sensitivity of MTB detection in clinical specimen has been questioned.
In situ Hybridization	Fluorescence hybridization of the Bacterial DNA with Peptide Nucleic Acid Probes	Identification of MTBC members Differentiation between MTB and NTM especially in liquid culture.	High cost and necessitate a good expert skills.
Automated Sequencing	The determination of the nucleotides order in the genes	Rapid Identification of a large number of mycobacterial species	Higher cost is the main limitation and good expertise in the analysis of sequences
Strain typing and DNA fingerprinting Techniques (RFLP Spoligotyping and MIRU	The discrimination between mycobacterial strains based on polymorphism of the insertion sequence IS*6110* and repetitive DNA elements such as the polymorphic GC-rich sequence (PGRS) and the direct-repeat (DR) region.	The IS 6110 is the standard international method. The strain typing and DNA fingerprinting help in the epidemiological studies	The IS 6110 is the standard international method, but this technique is difficult to perform. The spoligotyping and MIRU-VNTR must be combined for better discrimination and differentiation between strains.

MIRUs (Mycobacterial Interspersed Repetitive Units) are loci in the MTB genome that contain variable numbers of tandem repeats (VNTRs) (25). MIRU-VNTR typing based on PCR amplification targeted areas and determining the number of repetitions of the unit. The end result is a numeric code corresponding to the 12-digit number of repeats observed each chromosomal locus (66) (Figure 2). Recently a standardized method based on analysis of 15 MIRU loci instead of 12, with a discriminatory power has been proposed as a new standard for epidemiological studies and 24 loci was anticipated as a method of high-resolution for phylogenetic studies (89).

- **Microarray analysis**

The microarray analysis or the DNA chips are a collection of labeled DNA probes fixed on a solid surface, after the hybridization with the DNA in question, the intensity can be computed and fluorescent image can be visualised (66).

The microarray of the mycobacterial genom provided a new insight of understanding and measuring the genes expression on multiple regions on the genome, which can be exploited for improving drugs, vaccines, and diagnostics tools for controlling mycobacterial diseases (11, 43).

CONCLUDING REMARKS

The screening of TB can be performed by different techniques: radiologic, clinical, immunological, bacteriological and molecular techniques. However the final decision of the infection of TB can be merely completed by the identification of MTB.

The conventional techniques of MTB identification are based on the bacteriological examinations which are time consuming. However in the over last decades, different molecular techniques have been developed and provided excellent, rapid and accurate results.

The main limitation of their utilization is the high cost of their application in routine analysis and in conclusion, the culture for MTB is irreplaceable but molecular techniques are optional to save the life of patients in the high suspected cases of tuberculosis and briefly the principles, main advantages and limitations of culture and molecular based techniques have been summarized in Table 2.

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