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Chronicles of Biomedical Sciences

journal homepage: https://cbsciences.us/index.php/cbs

Advances in diagnosis of *Mycobacterium tuberculosis complex*: Journey from conventional to Genotyping techniques and their importance

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ARTICLE INFO

Article Type:
Review Article

Keywords:
Genotyping
Mycobacterium tuberculosis
MTB complex
Epidemiology
Diagnosis

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Received on: March 19, 2024.
Revised on: April 22, 2024
Accepted on: April 23, 2024

ABSTRACT

Tuberculosis rests a foremost global health concern being categorized as a rampant by World Health Organization. Although an ancient disease but tuberculosis had no diagnostics for long. Actual tubercle was said to be first discovered by Sylvius in 1679, Benjamin Marten during 1720 came up with the idea for first time that minute living organisms may be the reason for TB and presented possible insights of human to human transmission of disease through direct contact. Robert Koch finally discovered MTB during 1882 using a special staining method enabling conjuring up of disease causing organism. Primary diagnosis of tuberculosis is based on observation of acid fast bacilli by smear microscopy and culturing the clinical specimens to isolate Mycobacterium tuberculosis. In cases of extra-pulmonary tuberculosis, therapeutic and clinical decisions are to be taken afore availability of definite diagnosis. Sensitivity of smear microscopy is very poor while gold standard culture takes long time of 6-8 weeks. Use of polymerase chain reaction (PCR) through amplification of mycobacterial DNA provided promising outcomes. Present discussion concludes the success in development of various molecular techniques for identification and fingerprinting of MTB complex

Introduction

Tuberculosis (TB) rests a foremost global health concern and categorized as a rampant by World Health Organization (WHO). Basically TB affects the lungs though it can affect every tissue and body organs in circulatory system, lymphatic system, central nervous system, alimentary system and hence can affect almost any part of the body. [1] A one third of global population is estimated to be infected with MTB and at risk of developing the disease. The estimated global incidence rate of TB remained 127 cases per 100,000 population and 1.5 million people died of TB in 2020 including 214000 Human Immunodefficient viruses (HIV) positive cases. [2]

Substantial development Goals (STG's) are set by WHO to End TB strategy consist of various milestones and global targets for reduction of the incidence of TB and related deaths. According to SDG-3, global epidemic of TB must be ended by 2030 and reduction in 35% deaths and 20% rate of incidence was expected till 2020 which remained below half by 2020 and Global reduction in death toll remained to be 14% and incidence as 9%. Pakistan is facing

a high incidence of 263/100,000 cases of all types i.e. pulmonary and extra pulmonary TB cases every year till 2019, moreover death rate due to TB remained 19.9/100,000 population in Pakistan became lower than the neighbor country India that comprises 32.6/100,000. [3]

Considerable influence of TB is posed on socioeconomic status of Pakistan as country bears 5.8% burden of TB alone from total national disease burden where country is ranked as 6th highest TB cases containing nation. [2] Multi drug resistant (MDR) TB is the disease caused by the strain of *Mycobacterium tuberculosis* (MTB) complex which is resistant to at least isoniazid and rifampicin with or without resistance to any other first line anti TB drugs.

Basic diagnostic readily available laboratory tests to diagnose TB are observation of acid fast bacilli (AFB) by smear microscopy and MTB culture on Lowenstein Jensen (LJ) medium in clinically suspected cases. [4] Smear microscopy with Ziehl Neelsen (ZN) stain, theough cheap and rapid thus lacks sensitivity among variety of clinical specimens. Culture for MTB on LJ medium also takes a long duration of 6-8 weeks for incubation to reach definite diagnosis. On the other hand clinical and therapeutic decisions are to be established afore laboratory diagnosis for the sake of patients and at risk populations. [5] Following primary TB infection, it may reactivate in any part of body at any time [6] excluding the lungs to be called extra-pulmonary TB. Sites of extra-pulmonary TB may vary and are expected to be influenced by ethnicity and geographical location further gained attention due to HIV epidemics during previous two decades. [7] An up-surging trend of extra-pulmonary TB as 7.8% during 1960's to 20% during 2000's has been proposed in literature.[8] A high proportion of extra-pulmonary TB patients have co-infection with pulmonary TB. Prevalence of extra-pulmonary TB ranges 15%-20% in Indo-Pak region. Its incidence has been published to be much higher in china; additionally higher rates of peritoneal and lymphatic TB are also reported as compared to other Asian populations. [9]

Early diagnosis with definite microbiological evidence is essential effective treatment and good patient care thus to control further spread of TB. Major draw-back also creating the difficulty in diagnosis of extra-pulmonary TB is low yield of AFB in clinical specimens, subsequently linked with lower sensitivity of smear and culture as well. [10] This paucibacillary nature of specimens is the main reason to search a faster, specifically sensitive and microbiological definite test for clinical samples taken from pleura, meninges and lymph nodes.[11] Another drawback with most of these extra-pulmonary sites is the requirement of invasive techniques to draw a sample for diagnosis resulting many deficiencies ultimately causing delay in definitive treatment.

The distribution of TB in various geographic areas is categorized by prevalence of many strains along diverse virulence and pattern of resistance. [12] Various host and environmental factors are thought to be the reason for transmission of various strains of MTB. [13] An essential phase of TB control program must be its capacity to demonstrate the mode and site of transmission for prevention from further spread of infection and active TB by finding recently infected individuals and providing treatment further observing treatment completion leading to cure. Such practice has aided to find the transmission connections among individuals and to determine instances in which associated cases were infected through distinct strains.[14] Various molecular techniques can be used for genotyping of MTB which serve for studying molecular epidemiology of different species. Moreover these techniques can be applied to pursue various tenacities like differentiation or identification of MTB and non-tubercular mycobacteria (NTM) at species level further to find the patterns of drug susceptibility in various isolates of MTB. Aim of this article is to review, discuss and compare different conventional and modern methods available for early diagnosis of pulmonary and extra-pulmonary tuberculosis. Further to see major techniques being used for genotyping and their application in various aspects of research on mycobacteria and MDR-MTB complex are reviewed.

History of Diagnosis of Tuberculosis

TB is historical disease and as old as mankind. It has been present in humans since ancient times. The oldest definite diagnosis of MTB is

evidenced in remnants of bison, expected to be present 18000 years afore the time today. Skeletal structures founded from Neolithic Settlement in Eastern Mediterranean revealed prehistoric (7000 BC) humanoids were infected with TB since tubercular decay was observed in the spines of cadavers from 3000-2400 BC. Different terms were used to refer TB in the history include phthisis, consumption, Pott's disease, white plague and scrofula etc. It was also referred as Koch's disease after scientist Robert Koch. [15]

Although TB was existing from old days but its diagnosis was not present. Hippocrates during 460 BC had also present it as invariably fatal, actual tubercle was said to be first discovered by Sylvius in 1679, Benjamin Marten during 1720 came up with the idea for first time that minute living organisms may be the reason for TB and presented possible insights of human to human transmission of disease through direct contact. Robert Koch finally discovered MTB during 1882 using a special staining method enabling conjuring up of disease causing organism. [16] Scientists including Ziehl. Ehrlich, Neelsen and Rindfleisch were trying to upgrade the Koch's technique and proposed few alterations in procedure and reagents. Franz Ziehl was the first scientist who used phenol (Carbolic acid) as mordant. Ziehl's mordant was retained by Neelsen who however replaced the primary stain with basic fuchsin which was primarily used by Ehrlich in 1882. Therefore this method was named as Ziehl-Neelsen (ZN) during 1890's. [17]

Currently Available Conventional Techniques

Smear microscopy and LJ culture are the mainstays in diagnosis MTB infection. Different faster techniques consisting liquid medium and observation of radiometric growth by BACTEC system are also used to overcome the issue of delayed diagnosis are overpriced, requires more trained staff with higher responsibilities even then requires >18 days for definite results. Non-conventional serological techniques bearing immune complexes of antigen and antibodies use blood samples could not convince the clinicians and consultants. Other serological approaches used secretory and excretory proteins are also failed to attain courtesy. In-vitro amplification of MTB DNA through PCR is succeeded greatly. [18]

Importance of early and confirmed diagnosis of TB, especially in challenging extra-pulmonary TB patients has positive impact in starting timely treatment thus to control further transmission. International Standards of Tuberculosis Care (ISTC) recommends for suspected patients considering of pulmonary or extra-pulmonary TB require appropriately respective samples from suspicious site of infection must be obtained for smear microscopy, LJ culture in routine and histology if desired. In addition sputum should be examined in extra-pulmonary TB suspects because a considerable proportion of associated pulmonary TB. [19] Three samples for sputum smear microscopy are preferred among suspects of pulmonary TB [20], thus it is utmost impossible to duplicate the samples of extra-pulmonary TB for examination through smear microscopy among cases could not present AFB in first smears. Cultures may also get contaminated in first samples at times hurdle to delay further. Poor outcomes of conventional microbiological methods due to above mentioned reasons have enthused the utilization of PCR test in laboratory diagnosis of TB. [18]

Modern Molecular Techniques

Nucleic acid amplification (NAT) notably PCR technique has shown a great potential in revolutionizing the microbiological investigation and already shown it effect on clinical diagnosis of TB. [18] According to previous reports diagnosis of MTB through NAT in clinical suspects was lacking its validity among under developed nations which turned to be useful and game changer in recent years with variety of amplification techniques. [21]

Line Probe Assay; Line probe assays being used for diagnosis of MTB complex (Genotype MTBC), identification of multidrug resistant TB (Genotype MTBDR*plus*) and detection of MTB resistant to fluoroquinolones, ethambutol and second line injectables (Genotype MTBDR*sl*) have been developed by the Hain Life Sciences are reputed to have good quality products. [22] These tests are frequently utilized to find resistance among MTB isolates but LPA based GenID RIF/isoniazid, fluoroquinolones

and second line injectable are not espoused far and wide yet. Even a study from high TB incident region demonstrated the performance of LPA to only an acceptable tool with compromised sensitivity and specificity in comparison to the WHO suggested profiles. [23]

GeneXpert

Provision of GeneXpert MTB/RIF assay to the various reference and tertiary care TB laboratories by National TB Control Program has influenced the diagnosis to a certain level. This technique produce the final results in just two hours, differentiates MTB from *Mycobacteria* other than TB (MOTT) further provides rifampicin susceptibility concurrently. This test has been strongly recommended by WHO for TB diagnostics and supported well throughout developing countries [24] Oligonucleotide sequences having a probe sequence pop in between two arms of the sequences are said to be molecular beacons. This molecular beacon technology is used in GeneXpert MTB RIF assay to find amplified DNA sequences in a hemi-nested rt-PCR technology. Five different forms of acid hybridizing probes [25] are used to react in a multiplex which is presented in figure 2.3. Each probe is paired to specific target sequence within 81 bp core region of *rpo*B gene in rifampicin susceptible MTB and are labeled with different tinted fluorophore.

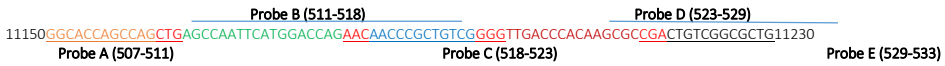

Figure 1: RDRR of rpoB gene and overlapping probes

The two complementary arm sequences hybridize to form a stem and loop structure under assay conditions where probe is situated inside loop structure. A covalent linkage of fluorophore takes place at one end and non-fluorescent quencher at the other end of an arm. Fluorescence is suppressed by the fluorophore and quencher in presence of free non-hybridized probe. Whereas, in case of complementary binding of probe sequence on DNA target, conformational change takes place in molecular beacon which leads to the separation of two arms, thus a bright fluorescent is produce by fluorophore and quencher. [26] A further higher diagnostic sensitivity has been achieved by introduction of MTB/RIF Ultra. [27]

Likewise, GeneXpert MTB/XDR also based on nucleic acid amplification test being used for diagnosis of other drugs resistances in one test following rifampicin resistance diagnosed by former GeneXpert tests. This is suitable technique and preferred in intermediate and peripheral level laboratories. It finds resistance against isoniazid, fluoroquinolones, capreomycin, ethionamid and amikacin. The main advantage of GeneXpert MEB/XDT over LPA is its one step closed automated system providing higher accuracy with lower chances of contamination and human error. [28]

Comparisons of Different Methods of TB Diagnosis

Various studies presented an extensive range of AFB smear positivity for diagnosis of extra-pulmonary samples extending from the lowest 0% to the highest 75%[29], few however presented sensitivity of AFB smears as 0-6% and 20% for culture on LJ medium.[23] Miyazaki et al., on the other hand presented sensitivity of nucleic acid amplification as low as 10 fg which is approximated to be two organisms of MTB.[30] Kesarwani et al., also presented a sensitivity of PCR as 97.87%, specificity as 100%, positive predictive value 100% and negative predictive value as 97.36%.[5] Tiwari et al., also showed a significantly higher positivity rate of 62.16% by

PCR diagnostic technique as compared to only 12.8% with AFB smear for extra-pulmonary TB samples.[31] Negi et al., compared the ZN smear with culture on LJ medium and BACTEC system presented sensitivities of 33.79%, 48.9% and 55.86% while a sensitivity for PCR was presented to be 97.29%.[32]

A study lately, based on line probe assay provided with variety of pulmonary and extra-pulmonary specimen has shown sensitivity, specificity, PPV and NPV of 92%, 100%, 100% and 83% respectively, moreover also elaborated the additional diagnostic value of this technique over TB culture. [33] Another study under same settings also concluded that Genotype MTBDR*plus* assay is highly sensitive technique for detection of MDR TB in both smear positive and smear negative subjects. [34]

Detection and confirmation of MTB is possible within few hours by using molecular amplification techniques further these technique may also be used to diagnose MTB from formalin fixed or even paraffin embedded. Benefit arises in this way is to investigate the specimens previously suspicion to other pathologies. [35]

Genotyping of mycobacteria for epidemiological purposes

Knowledge and control of TB has been greatly enhanced through molecular typing of MTB as it allows to find the unsuspected transmission, detection of false positive isolates and distinguish the relapse and re-infection within a single case. Molecular indicators offer vital techniques for detection and transmission of TB that helps to find fast evolution involved in rapid active diseases. Dynamics of TB transmission and its understanding have been greatly enhanced by finding different methods of DNA typing where whole genome sequencing is being set as standard for genotyping of MTB. [36] Genotyping is a reliable tool for evaluation of TB outbreak or other diseases. Epidemiological data suggesting occurrence of an outbreak can also be verified by genotyping of isolates hence may help to find actual occurrence of outbreak or coincidental pseudo manifestation of high number of cases [37] This approach may evidence the severity of outbreak and provide an action plan for public health to control the further transmission of disease.

Genetic variety of MTB should have imperative clinical magnitudes are the growing evidence at present. This is not a novel thought as about 55-60 years before Mitchison et al. compared outcomes of guinea pigs followed by infecting them with MTB isolates obtained either from Indian or British patients suffering from pulmonary TB. Report suggested higher virulence of British isolates and produced widespread and sever infection compared to Indian isolates resulted to kill higher proportion of animals. However scientists were unable to illustrate the diversity of infecting organisms at genetic level further association of virulence among humanoids and guinea pigs could not be defined. The advent of mycobacterial genotyping leads to better learning of association among MTB clinical phenotypes and genotypes. [38]

Genotyping is very important and useful in variety of situations; results could be used to confirm cross contamination of specimen in the laboratory. Epidemiologically unrelated TB patients represent vast variability in genotypes of MTB isolates, while the patients infected by a common source show identical genotypes may called clustered cases of TB and suspected to have recent infection in contrast genotypically distinctive isolates generally thought to have reactivation of infection. [39]

Epidemiologic studies on the basis of molecular genotyping have presented the dynamics of TB transmission and communication differ significantly according to geographic regions. Importantly homelessness, crowded shelters, health care facilities and other public areas are often considered foci of TB transmission. It is observed that DNA polymorphism has been exploited to distinguish the irrefutable MTB cultures for research on epidemiological bases and assumed that DNA fingerprints of identical strains are linked. [39], [40]

Genotyping Methods

Various repeat sequences are studied through the genome of MTB complex consisting transposable elements, tri-nucleotide repeats, mutable digital tendon repeats, MTB intermingled repeats and regions with direct repeats (DR). [41] Any region with DR is most

abundantly used loci which consist of 36 base pair (bp) direct repeat sequences scattered with unique spacer sequences of 34-41 bp, which in combination are said to be direct variable repeat (DVR) probes. [42] The deletion of DVR region brings evolution to DR sequences and process involves homologous recombination of single nucleotide mutations and the addition of IS*6110* elements. Such proceedings are thought to be unidirectional and make the DR region as a useful locus for learning the evolution and epidemiology of MTB complex. [43]

Restriction fragment length polymorphism (RFLP)

The standard approach for genotyping of MTB isolates is analysis of RFLP for dispersal of IS*6110* in various strains and sufficient database is also available. This technique is based on the difference in IS*6110* copy number per strain approximately in the range of 0-25 with variations in chromosomal targets of these insertion sequences. [44] IS*6110* base genotyping can only be performed on culture positive isolates and require sub-culturing to get moderate amount of DNA. From cultures DNA is first extracted and then purified followed by treatment with restriction enzyme *puv* II which recognizes specific 6 nucleotide palindromic arrangement of DNA which releases it on every repeat of sequence. Restriction of fragments is achieved by separating through electrophoretically transfer on nylon membrane arranged according to their size with IS*6110* probe hybridization. This probe precisely scripts repetitive elements IS*6110* containing fragments thus presence of specific bands denotes the molecular weight of IS*6110* contained copies of fragments. [45] Perfect method to apply IS*6110* RFLP typing does not exists yet while other molecular techniques even then these tools contributed significantly to understand MTB transmission and epidemiology. [46]

Restriction Enzyme Analysis (REA)

One of the main targets in DNA fingerprinting is *hsp*65 probes which are definitely conserved for specific species and therefore may be utilized in taxonomic research studies. This tool has been widely used as it works with only two restriction enzymes for diverse recognition of MTB species. [47] Various studies used this technique with variety of modifications for identification and prevalence of MTB complex as well as non tubercule mycobacteria. [47], [48]

Spoligotyping

It is a molecular typing tool which targets DR locus of mycobacterial chromosome based on reverse hybridization, the technique being used for documentation of possible source of infection and epidemiological inquiries of TB. This technique is useful where there is absence or low copy number of IS*6110* elements and developed to transfer information through DR region of alone MTB strains or among various members of MTB complex. [49] Advantages of spoligotyping over RFLP are; it requires less amount of DNA therefore could be performed directly on the clinical specimens and secondly qualitative as well as quantitative results can be expressed. However this technique has low power to discriminate MTB complex than does IS*6110* based genotyping. [49]

Mycobacterial inter-spread repetitive unit variable number tandem repeats (MIRU-VNTR)

The utmost dependable molecular techniques rely on PCR replication of multiple loci consisting MIRU-VNTR that provides a possible solution over different drawbacks heavily existed in other techniques further the method is reproducible, delivers convenient findings and much time saving as compared to IS*6110*-RFLP typing which is considered gold standard. MIRU-VNTR technique could be directly used in crude extracts obtained from mature or early culture plates [12] This MIRU analysis may be automatic and can be used to assess high number of strains thus provision of digital findings may be catalogued in ease on softwares of computer.

Genotyping for investigation of drug resistance

The drug sensitivity pattern of MTB strain is highly unbalanced characteristic as most of the strains usually acquire drug resistance during treatment or individuals may be directly infected with already resistant strain. In most of areas one strain predominates amongst MTB isolates. [50] Genotyping allows to evaluate the various drug susceptibility patterns in identical isolates which can help in diagnosing weather primary organism developed drug resistance

during/after anti TB treatment or re-infected with a different MTB strain.

Genotyping for clinical identification

Mycobacteria can be distinguished into two groups; MTB complex and NTM. TB due to one of the MTB complex strains is most common among under developed however NTM have clinical relevance also especially for immune-compromised patients. Such NTM infections are mainly found in advanced countries bearing the low incidence of TB. [51] Although higher morbidity and mortality being linked to NTM even then far less work on genotyping of mycobacteria has been reported from developing countries including Pakistan and no national prevalence of NTM is available yet.

MTB complex includes mainly MTB, *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium microti*, *Mycobacterium canattii*, *Mycobacterium caprae* and *Mycobacterium pinnipedii*. Although they all are differ on the bases of host tropism, phenotypes and pathogenicities but are closely related genetically. The commonest human pathogen is MTB species which may further be separated among genetic clusters which also present variations in levels of their virulence, geographical distributions and immunogenecities. [52] It is necessary to distinguish the species of MTB complex among obligatory human specific or zoonotic TB so that proper treatment can be initiated. These species have been studied in various studies in different parts of the world and showing variety of results as presented in the table I.

Table I: Distribution of various MTB complex species isolated in different studies.

Sr. No	Mycobacterium Species	Richter *et al.* [53] N=78 n (%)	Bouakaze *et al.* [54] N=59 n (%)	Bouakaze *et al.* [55] N=56 n (%)
1.	*Mycobacterium tuberculosis*	29 (37.1)	48 (81.4)	36 (64.2)
2.	*Mycobacterium africanum*	6 (7.8)	6 (10.2)	4 (7.2)
3.	*Mycobacterium bovis*	17 (21.8)	5 (8.4)	12 (21.4)
4.	*Mycobacterium caprae*	17 (21.8)	-	1 (1.8)
5.	*Mycobacterium microti*	9 (11.5)	-	1 (1.8)
6.	*Mycobacterium canattii*	-	-	1 (1.8)
7.	*Mycobacterium pinnipedii*	-	-	1 (1.8)

Distinction of MTB species and *Mycobacterium bovis* is essential because the latter is certainly resistant to pyrazinamide. Genotyping of MTB isolates is not only useful in addressing evolutionary questions but also helpful in finding transmission dynamics of this deadly pathogen to create future outbreaks. [55] Recognition of MTB complex at the level of species can be performed phenotypically and biochemical physiognomies of mycobacteria following culture that takes much time. With the advancement of knowledge and development of modern techniques it is necessary for the mankind to be a step ahead from such deadly pathogens and make every effort to stop transmission hence saving lives. Journey of diagnosis may be better explained by the figure 2.

Present discussion concludes that conventional methods especially ZN smear however provide support in many cases to minimize economic loses and prevents waste of resources in many

cases. Culture on LJ medium is although gold standard but takes longer time even with liquid culture may take more than ten days. The success in development of various molecular techniques for identification and fingerprinting of MTB complex. However emphasize should be given to high TB prevalent nations to work in different angels using these techniques and help National TB control programs to modify their strategy as required. Exact prevalence of MTB complex species is still unknown in Pakistan further drug resistance patterns of each species, most prevalent MDR species and most frequently mutating species are few areas required to be studied.

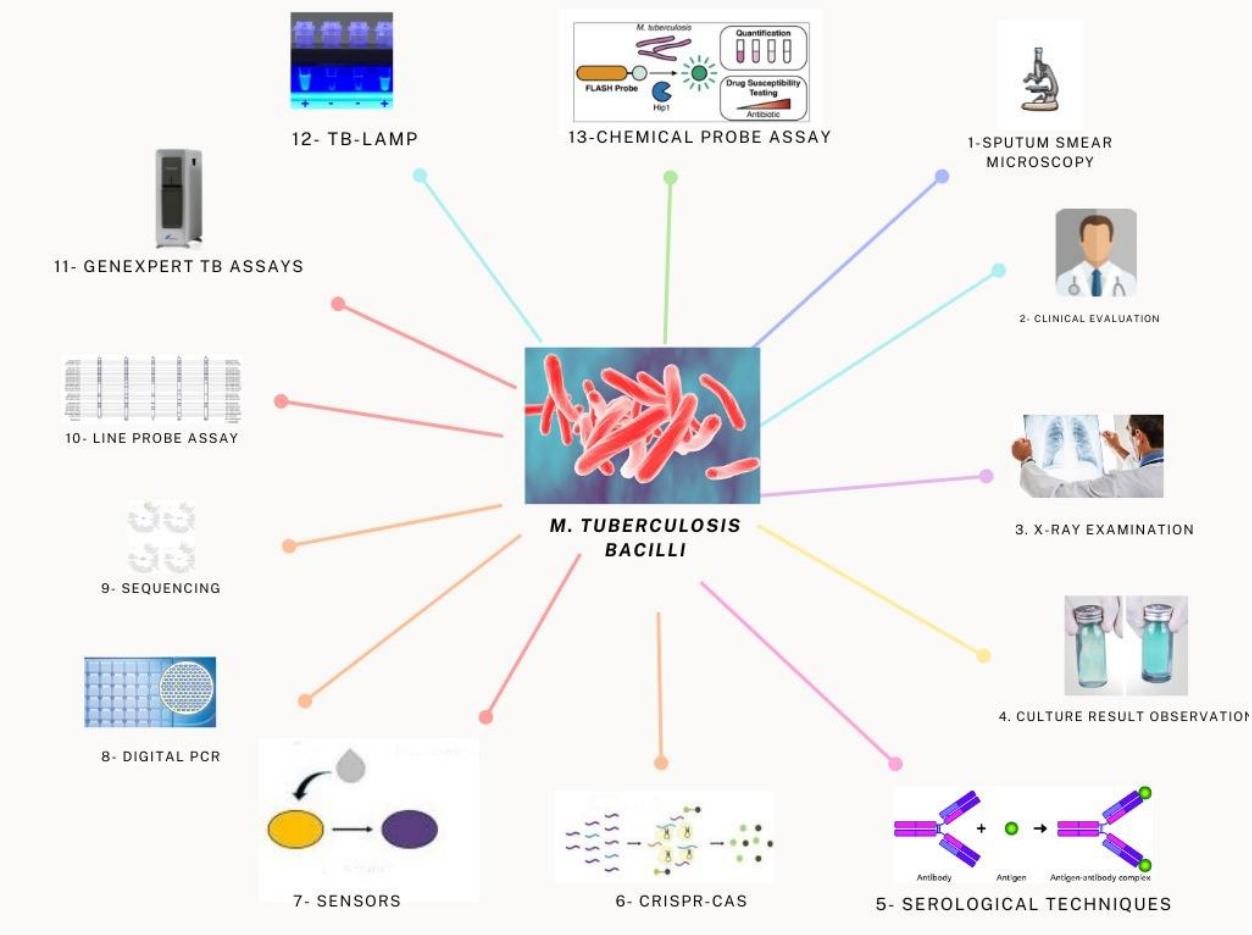

Figure 2: Summary of Journey in Diagnosis of TB

Acknowledgements: We express our deepest gratitude to Prof. Saqib Saeed, Head Department of Pulmonology, King Edward Medical University for his unanimous support and encouragement in preparing this manuscript.

Author Contribution: Conceptualization, MKM; Writing (Draft preparation, review, editing) SS, HA, and AA. Both authors read and approve the final manuscript. Revisions; MKM

Funding: This is a non-funded work.

Declarations

Consent for Publication: Both Authors are agreed to publish this version of manuscript

Competing Interest: Authors have no competing interest.

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