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Archana Bharti
Department of Veterinary Pathology, College of Veterinary Science & A.H., Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh, India

Yamini Verma
Department of Veterinary Pathology, College of Veterinary Science & A.H., Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh, India

Amita Dubey
Department of Veterinary Pathology, College of Veterinary Science & A.H., Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh, India

Correspondence
Archana Bharti
Department of Veterinary Pathology, College of Veterinary Science & A.H., Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh, India

An update on diagnosis of tuberculosis: A review

Archana Bharti, Yamini Verma and Amita Dubey

Abstract
Tuberculosis (TB) is an infectious and contagious disease caused by various strains of *Mycobacterium* worldwide. In an estimate, 2 billion persons or approximately one third of the world's population are infected with contagious tuberculus bacillus among which 95 percent of cases occur in people of developing countries. However, it is estimated that only 10–12% of infections result in overt disease. The data gathered by World Organization of Animal Health stated that among 179 countries of world, more than half were reported with the presence of zoonotic TB in livestock and/or wildlife, demonstrating its wide geographical spread. In India bovine tuberculosis was found to be 14.31 per cent to 34.42 per cent. Researchers have proved that most of the active TB infections are curable with early diagnosis followed by appropriate treatment. The available conventional diagnostic techniques are useful but they neither rapid nor cost effective for early accurate and affordable diagnosis, represents a cornerstone to eradicate TB worldwide by 2030. However, current ongoing researches have confirm that TB detection via targeting disease biomarker and the development of new and cheaper tools for biomolecular recognition may circumvent the current limitations of diagnostic methods used in the global fight against TB.

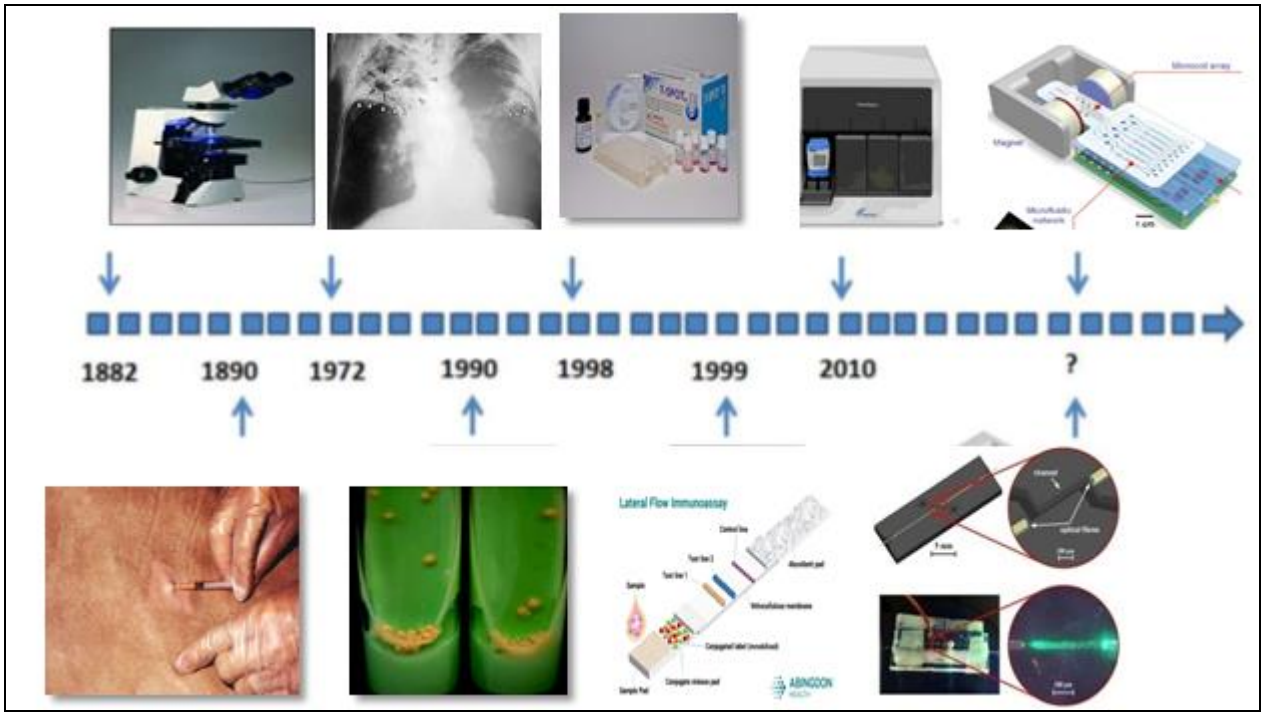
Keywords: Diagnosis, disease, *Mycobacterium*, tuberculosis

1. Introduction
Tuberculosis (TB) is an infectious and contagious disease caused by various strains of *Mycobacterium* worldwide viz. *Mycobacterium bovis* (bovine TB), *Mycobacterium avium* (avian TB) and *Mycobacterium tuberculosis* (human TB). It is the foremost leading cause of death of animal and human population [3]. In an estimate, 2 billion persons or approximately one third of the world's population are infected with contagious tuberculus bacillus among which 95 percent of cases occur in people of developing countries. The deadly disease is responsible for the death of 1.5 to 2 million people per year [65]. According to the World Health Organization (WHO), approx 147,000 new cases and 12,500 deaths are because of zoonotic TB related disease in human [46]. In 2017, 87% of new TB cases occurred in the 30 high TB burden countries. Eight countries accounted for two thirds of the new TB cases: India, China, Indonesia, the Philippines, Pakistan, Nigeria, Bangladesh and South Africa [70].
The data gathered by World Organization of Animal Health stated that among 179 countries of world, more than half were reported with the presence of zoonotic TB in livestock and/or wildlife, demonstrating its wide geographical spread [14]. An overall prevalence of tuberculosis was found in bovine 8 per cent, camels 11 per cent, shoats 2 per cent, pigs 15 per cent and wildlife 5 per cent [24]. This has also been noted by scientists that the 40-60 per cent cattle of herds are found infected in developing countries [54]. In India bovine tuberculosis was found to be 14.31 per cent to 34.42 per cent [53]. Srivasan *et al.* (2018) reported 7.3 per cent prevalence of bovine tuberculosis in India after systematic review and meta-analysis of 44 publications from 1942 to 2016 [58]. It is estimated that the seroprevalence of tuberculosis infection among captive elephants in southern India is nearly 15 per cent [72]. However, the prevalence of mycobacterial infection causing TB is far higher; it is estimated that only 10–12% of infections result in overt disease [17, 36]. In India over 70 per cent of the milk is sold unpasteurized, this raises concerns regarding the potential for zoonotic transmission of TB. India has the world's largest burden of human TB [61]. Due to chronic persistence of *Mycobacterium* infection farmers, dairy workers, zoo keepers and veterinarian are at greater risk.
Effort to stop TB since long time duration has resulted in a cure with increased survival rate. Eventually in April 1993 TB was declared a global emergency by the WHO. Most recently in developing countries especially in India, rare and very dangerous forms of TB have emerged.

A collaborative road map has been developed by the WHO, the Office International des Epizooties (OIE), the Food and Agricultural Organization (FAO) of the United Nations (UN) and International Union against Tuberculosis and Lung diseases to address the major health and economic impact of this disease [20].

Researchers have proved that most of the active TB infections are curable with early diagnosis followed by appropriate treatment. Beside that in animals, immune-deficient and immune-compromised individual TB treatment is much difficult as most of the times it gets undiagnosed since conventional diagnostic tools are unable to diagnose. However, currently available modern diagnostic techniques are useful but they neither rapid nor cost effective for early accurate and affordable diagnosis, represents a cornerstone to eradicate TB worldwide by 2030.

To overcome this awful situation innovative strategy for TB diagnosis has been needed. Current ongoing researches have proved that TB detection via targeting disease biomarker [19] and the development of new and cheaper approaches for biomolecular recognition may circumvent the current limitations of diagnostic methods used in the global fight against TB. The present review discuss about TB diagnostic, drawbacks and limitation of conventionally used diagnostic techniques.



Diagnostic evolution of tuberculosis

2. Clinical Diagnosis

Tuberculosis has usually prolonged course and takes months or years to appear clinically. TB can entail disease in many organ systems of body. Clinically tuberculosis can diagnose by progressive emaciation, lethargy, weakness, anorexia, and a low-grade, fluctuating fever. In chronic condition, moist cough with later signs of dyspnoea and tachypnea may occur, that indicating condition like bronchopneumonia; on auscultation and percussion the destructive granulomatous bronchopneumonia can be detected which is suggestive to TB [51, 60].

Superficial lymph node enlargement is a useful clinical sign in diagnosis of TB when present. But affected deeper lymph nodes cannot be palpated, in severe condition they may obstruct the major airways, pharynx, and gut, leading to dyspnoea and ruminal tympany. When the digestive tract is involved, intermittent diarrhea and constipation can be seen [18, 64]. Tuberculous mastitis in livestock is important, because of threat to public health by its ability to spread infection to young ones, via raw milk consumption. However, clinically diagnosed tuberculous mastitis in animals is rare, and mostly undiagnosed in subclinical condition [22].

3. Microscopic Diagnosis

3.1 Direct Smear Microscopy

3.1.1 Ziehl–Neelsen (ZN) stain

Microscopic examination of direct smear with Ziehl–Neelsen (ZN) staining is commonly used to detect acid fast bacteria (*Mycobacterium)*, especially in low-income countries, owing to its rapidity, low cost, and high predictive value [8]. However, sensitivity and specificity of ZN stained detection of mycobacteria is very low (a minimum of $1x10^4$ bacterium per ml/gm of sample), and TB remain undiagnosed in most of the subclinical condition where bacterial shedding is intermittent [73].

3.1.2 Fluorescence Stain

Fluorescence staining using auramine O staining has approximately 10% greater sensitivity, equivalent specificity than ZN staining [38, 48]. The Fluorescent staining method with its relatively high sensitivity, low incidence of false negative results make this efficient for screening test. The Fluorescence stained microscopical method is nowadays been further improved by a more advanced, cost effective, and stronger light source using light-emitting diodes (LEDs) [13, 63, 65]. LED-FM has better sensitivity for the diagnosis of TB as compared to conventional ZN microscopy. Therefore, WHO

recommends LED-FM as an alternative for conventional ZN microscopy [63]. However, these diagnostic techniques are not much effective and cannot differentiate pathogenic mycobacterial species.

4. Microbiological Diagnosis

4.1 Culture-based Diagnosis

4.1.1 Conventionally used culture techniques

Mycobacterium culture is considered as a gold standard technique among the current conventional diagnostic techniques. Although, difficulty in TB diagnosis in this technique is due to slow growth of *Mycobacterium*, which subsequently takes much time in evaluating the susceptibility of *Mycobacterium* to antibiotics [2]. Using this current tool, a primary culture is obtained in two to four weeks on average, and antibiotic susceptibility is determined after an additional two to four weeks. Therefore, four to eight weeks are needed to obtain an isolate and determine its susceptibility to antibiotics [23, 33].

4.1.2 Automated liquid culture techniques

Most recently automated liquid culture systems such as Bactec MGIT 960 (BD Diagnostics, Franklin Lakes, NJ, USA) and BacT/Alert MB (bioMerieux, Craponne, France) with higher sensitivity, and a decreased time are introduced in conventional diagnosis [2, 62]. The two types of liquid culture system i.e. radiometric and non-radiometric are involved in this technique.

4.1.2.1 Radiometric

The radiometric detection test is based on metabolic activity of MTB by radiologic detection. MTB (and also some other mycobacteria) are known to produce CO_2 from carbon sources like glycerol or acetate. The important selective principle is that MTB cannot form CO_2 from glucose. This helps to differentiate MTB from other *Mycobacterium* sp. and bacteria. Utilizing this selective property, the capability of MTB in producing $^{14}CO_2$ from ^{14}C-U-glycerol or ^{14}C-U-acetate, but not from ^{14}C-U-glucose is measured. The radiometric semi-automated BACTEC 460 TB system (Becton Dickinson, Sparks, Md.), is the first system to permit the significantly earlier detection of mycobacteria [4]. This technique has several drawbacks, however: it involves the use of radioactive material, and reading of cultures is labor-intensive and is associated with a potential risk of cross-contamination and risk of stick injury [9, 57].

4.1.2.2 Non radiometric

BACTEC MGIT 960 system is a fully automated and non-radiometric instrument. The system exploits the fluorescence of oxygen sensor that detects growth of tuberculous mycobacteria in culture. However, contamination rate is found higher than that for the radiometric BACTEC 460 system and needs to be improved [42, 50, 71].

Various experiments prove that these automated liquid culture system mislead TB diagnosis resulting in large number of misdiagnosed cases, with cross-contamination events and the erroneous detection of multidrug-resistant outbreaks [1, 55].

5. Immunological Diagnosis

An immunological diagnosis measures how strong the affected animal's immune system reacts to pathogen. Tuberculin test and Interferon gamma release assay are most commonly used immunological assay that is based on the immunological response of individual and are widely applied for diagnosing latent TB infection (LTBI) and active TB.

5.2 Tuberculin test

The Tuberculin test is performed by injecting a small amount of *Mycobacterium* purified protein derivative (PPD) into the patient skin. If the skin area reacts to the antigen and is larger than or equal to 10 mm, it is considered a positive reaction [21]. However, researches prove that the tuberculin test gives high rate of false positive as well as false negative results. Tuberculin tests false-positive results may found in individual who have been vaccinated with Bacillus Calmette–Guérin (BCG), and false negative responses in immune-compromised individuals [52].

5.3 IFN-γ assay

White blood cells release interferon-γ (IFN-γ) when ever infected with *Mycobacterium*. Concentration of IFN-γ of specific antigens (ESAT-6 and CFP10) released in the plasma separated from blood can be determined by enzyme-linked immunosorbent assay (ELISA) [27, 39]. However, experiments by scientists get poor agreement between the whole blood IFN-γ assay and tuberculin test for the diagnosis of latent TB. They found specificity of interferon gamma assay was more than tuberculin test. But sensitivity of whole blood IFN-γ assay may have lower than the tuberculin test in diagnosing TB infection. Researches proved that a significant proportion of whole blood IFN-γ assays get fail when used as a screening assay in routine practice [11].

5.4. Latex agglutination assays

In immunological assays like latex agglutination, the MTB antigen binds to serum antibodies. The polystyrene (latex) beads that are functionalized with antigens extracted from a MTB, reacted with serum samples in mycobacterial positive sample. The latex beads become coagulate showing a positive test. The sensitivity of this test is not high enough. Also, the serum could contain antibodies, due to vaccinations like BCG that might interfere with the test adding further to the shortfalls of the assay [6, 56].

Other serological test like rapid diagnostic assay and antibody ELISA test have sub-optimal sensitivity and specificity are not recommended as a regular diagnostic assay.

6. Flow Cytometry Diagnosis

Over the past few years, use of flow cytometry in tuberculosis research has been increased. This technique is based on the ability of viable MTB bacteria to absorb fluorescein diacetate (FDA) and to hydrolyse it into fluorescein. The accumulation of fluorescein in metabolically active bacterium is successfully be detected by flow cytometry. The reproducibility of this test is high and it does not require active cell division of mycobacteria. The technique, however, needs logistic support from a specialized laboratory that makes it difficult to implement in developing countries [28].

7. Pathological Diagnosis

7.1 Gross /Post-mortem Diagnosis-

Grossly TB lesions typically appear as yellowish, caseous, necrotic areas in nodules surrounded by firm white to light grey fibrous tissue [30, 60]. Gross lesions indicative for tuberculosis are most commonly detected at routine meat inspection or during the post-mortem examination. The degree to which tubercles are surrounded by fibrous tissue

varies between species. Nodular lesions may be purulent (less common) or dry, with caseation or fibrosis, and may be calcified or caseo-calcified [31].

Conventionally used TB diagnosis is made on the basis of lesions found in the retropharyngeal lymph nodes, thoracic cavity, mesenteric lymph nodes and other visceral organs [45]. However, because tuberculous lesions may occur in a range of other sites, full critical post-mortem examination is important. The main sites of lesions may vary between species but the lungs and associated lymph nodes are common sites of lesions. Skeletal lesions sometimes occur, particularly of the cervical vertebrae in horses. Thin-walled purulent abscesses may be found in some species such as cervids. Tuberculous bite wounds may be seen in species such as *Meles meles* - eurasian badger [12]. Gross tuberculous lesions are not indicative of only TB, as there are many other etiological agents that may form tubercle hence, diagnosis must not be confirmatory only on the basis of gross appearance.

7.2 Histopathological Diagnosis

Histopathology can differentiate TB from *Actinobacillus bovis*, fungal lesions, hydatid cysts, tumor and other bacterial infections, all of which can grossly resemble TB. A histological diagnosis of tuberculosis can be made on the basis of the characteristic tissue reactions such as central necrosis, which is often mineralized, surrounded by a mononuclear epithelial reaction and frequently containing intracellular *Mycobacterium* in giant cells [26, 32]. *Rhodococcus equi* form comparatively same histological lesions of tubercle. Therefore, histology cannot produce a definitive diagnosis of specific *Mycobacterium* spp., in majority of the situations [51].

8. Molecular Diagnosis

Conventionally used molecular diagnostic tests are, highly specific, and can predict drug resistant mycobacteria. These diagnostic methods are generally, semi-automated, non-integrated. PCR that amplify and detect mycobacterial rRNA or DNA directly from any clinical sample (blood, sputum, bone marrow, tissue, etc.) shows higher sensitivity and specificity for smear-positive *Mycobacterium*, but poorer sensitivity and specificity for smear negative *Mycobacterium* [35, 44]. However, concentration and purity of nucleic acids derived from clinical specimens or unequal distribution of mycobacteria in the specimens may affect the sensitivity of molecular diagnosis [10, 25, 42]. Some highly recommended and commonly used molecular methods in diagnosis of TB are Loop isothermal amplification PCR (LAMP), Xpert MTB/RIF assay, Line Probe Assay (LPA).

8.1 Loop isothermal amplification PCR (LAMP)

Loop isothermal amplification PCR (LAMP) (Eiken Chemical, Tokyo, Japan) technique can amplify target *Mycobacterium* DNA with high specificity, efficiency and rapidity under isothermal conditions requiring only a heat block to detect visually DNA directly from clinical samples. This technique doesn't require any purification process of DNA and detect *Mycobacterium* in less than 2 h with minimal equipment and enhanced sensitivity and specificity [47, 49]. LAMP has higher sensitivity and lower specificity for smear-positive sample compared to smear-negative sample. However, there is still need to improve the assay to make it simpler, cheaper and more efficient to make it competitive against other PCR methods already available [29].

8.2. Xpert MTB/RIF assay

The Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) is the more advanced fully automated tool available. This assay system is integrated with NAAT platform that include sample preparation, amplification, and detection of DNA [7]. The Xpert MTB/RIF is carried out in a closed system that amplifies and confirms the presence of *Mycobacterium* and also the rifampicin resistance-conferring mutations within 2 h [40, 59, 66].

The WHO endorsed its use in 2010 [34]. World's 69% countries were recommended Xpert MTB/RIF as the initial diagnostic test for individuals suspected of having DR-TB, and 60% recommended it as the initial diagnostic test for individuals suspected of living with HIV by 2015 [69]. However, it's maintenance in developing countries is quite difficult, the machine requires a stable and uninterrupted electrical power supply and is linked to a computer for data analysis, which of course requires security against theft. The instrument requires at least annual calibration and needs to be performed by a trained technician using specialized calibration equipment. The most commonly-deployed GeneXpert device (GX4) has a limited throughput, and larger systems (or linked devices), with throughputs of up to 1000 tests/day, that carry higher capital costs.

8.3 Line Probe Assay (LPA)

Line Probe Assay (LPA) can detect TB and MDR-TB rapidly from smear-positive samples and *Mycobacterium* isolations. This technique is endorsed by the WHO in 2013 [68]. LPA system has ability to detect genetic mutations and can confer antibiotic resistance from clinical samples by a DNA hybridization technique within 2 days. However, scientists from India reported after analysis, that it is difficult have LPA to be 100 per cent sensitive in its target detection. Therefore, an additional test may be the solution in the existing set-up [15].

Tubeculosis diagnosis by electrophoresis method mainly relies on the difference in electrophoretic mobility of mutated DNA fragments of mycobacteria especially of resistant strains. The PCR amplified DNA can be compared with the electrophoretic mobility of a wild type/reference MTB DNA, however this technique is not able to determine any resistance.

These conventionally used modern TB diagnostic methods still have their limitations of being time consuming, expensive, having requirements of sophisticated laboratory infrastructure and highly trained personnel. None of these commercially available TB tests qualifies as an ideal TB diagnostic method.

9. Nanodiagnostics

Researchers have proved that nanotechnology can improve diagnostic techniques and has huge impact in early diagnosis. Since a long time, majority of the nanodiagnostic work has been carried out in diagnosing cancer, however now the thrust area has extended to diagnosis of infectious diseases. This seminar is focused on applications of nanotechnology embarking upon diagnosis of deadly disease tuberculosis [16, 37, 41, 43]. With the critical review of researches, this section discusses how nanotechnology is helpful for early diagnosis of tuberculosis in short time with increased sensitivity and specificity through nanosized biomolecular interaction viz. enhanced visualization of fluorescent signals, visual colorimetric signals of amplified DNA product, nanofluidic

technology, nuclear magnetic resonance technology (NMR) and prototype miniaturized device etc.

10. Lab on Chip

Lab-on-Chip (LoC) devices integrates biochemical operations, chemical synthesis, DNA sequencing onto a single chip which otherwise would have been performed in laboratory taking sufficient amount of time. Due to the miniaturization of these biochemical operations, better diagnostic speed, cost efficiency, ergonomy, sensitivity and so on can be achieved. Scientist evaluated the performance of the molecular lab-on-chip-based VerePLEX Biosystem for detection of tuberculosis, obtaining a diagnostic accuracy of more than 97.8% compared to sequencing and MTBDR*plus* assay on clinical isolates and smear-positive specimens. The high speed, user-friendly interface, and versatility make it suitable for routine laboratory use [25].

11. Conclusions and Future Prospects

Tuberculosis (TB) has long been neglected and under diagnosed with conventional tools but now the modern diagnostics tools has given a wide path to tackle this difficult task, so that the management of TB becomes rapid, easier, cheaper and more sensible in poor resource countries. The development of molecular tools in mycobacterial fields of research, introducing new and revolutionary approaches for molecular detection. The obvious advantages of molecular diagnostics based schemes are their ability to provide results within hours, with increased sensitivities and specificities at a fraction of a minimum cost when compared to conventional microbiological methodologies. It is expected that in the next few years, some of the strategies depicted throughout take their rightful place at the front line of fighting *Mycobacterium* infection and do the needful for eradication of TB in future.

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