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One Health Diagnostic Strategies For Bovine TB: Conventional Platforms To Cutting-Edge Technologies

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ABSTRACT
Bovine tuberculosis (bTB) is a chronic zoonotic disease, the causative agent of which is one of the components of the *Mycobacterium tuberculosis* complex (MTBC): *Mycobacterium bovis*. In India, this disease affects almost 7.3% of cattles and buffaloes, which may pose a major economic loss. Most commonly, humans contract this disease through the consumption of unpasteurised milk or milk products and undercooked or contaminated meat. The diagnosis of this disease is difficult for several reasons, including a lack of awareness among farmers, rural residents, and veterinarians; the presence of varied clinical signs; and inadequate laboratory infrastructure. Even the animal remains asymptomatic at the initial stage of infection but can still transmit the disease to humans. Timely diagnosis and appropriate control measures are therefore essential. Diagnosis uses a spectrum of methods: conventional approaches (culture, microscopy, tuberculin skin testing, such as TST/SIT/CIT) remain important in low-resource settings but are time-consuming. Faster molecular and cutting-edge tools (PCR variants, RFLP, LAMP, NGS, WGS, and Digital PCR) provide higher resolution, speed and support vaccine and therapeutic research, though each method has trade-offs in cost, expertise, and infrastructure needs. Combining appropriate diagnostic, vaccination, treatment, and control programmes is necessary to reduce the burden of bovine tuberculosis in both animals and humans.

KEY WORDS: Bovine tuberculosis (bTB), Disease diagnosis, Molecular methods, Zoonosis

INTRODUCTION

Tuberculosis is an infectious disease caused by the bacterium *Mycobacterium tuberculosis*, which mostly affects the respiratory system, primarily the lungs, and it is an important disease in both humans and animals worldwide. Similarly, bovine tuberculosis (bTB) is a chronic zoonotic disease caused by bacterial infection of *Mycobacterium bovis* (*M. bovis*) and the *Mycobacterium tuberculosis* complex (MTBC), which is comprised of a number of other bacteria such as *M. africanum*, *M. orygis*, *M. microti*, *M. caprae*, and *M. tuberculosis*. *M. bovis* shares high genomic similarity with *Mycobacterium tuberculosis* [1,2]. The most common route of infection of bovine tuberculosis is the inhalation of respiratory droplets from infected domestic animals. Another way of getting infected is by consuming contaminated or unpasteurized milk and milk products, as shown in Fig. 1 [3,4].

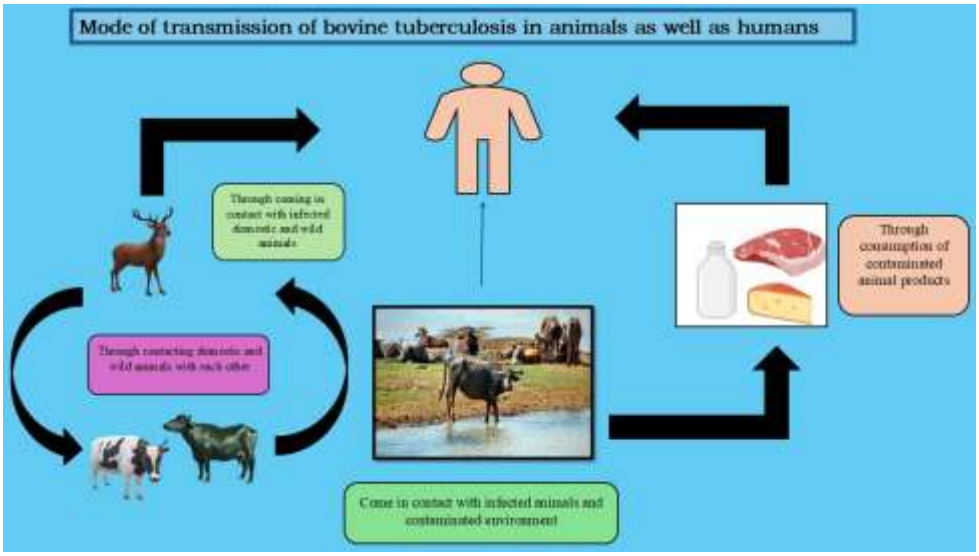

Fig. 1. Mode of Transmission of Bovine Tuberculosis

In countries, especially developing countries, since humans are living in close contact with animals, the chances of getting zoonotic infections are therefore high in these countries [5]. Infected bovines shed the pathogens in their milk, and populations that are reliant on that milk for their economy and life sustainability will have high chances of getting infected. Furthermore, bovine dung is used as a fertilizer in fields, and the presence of pathogens in the dung also increases the risk of spreading this disease by dissemination of pathogens in healthy humans as well as in animals [2]. The infection spreads early in immunocompromised persons because of their weak immune system. In humans, symptoms like productive cough, fever, night sweats, chest pain, and loss of appetite can be seen at the early stages of infection (Fig. 2) [5-7].

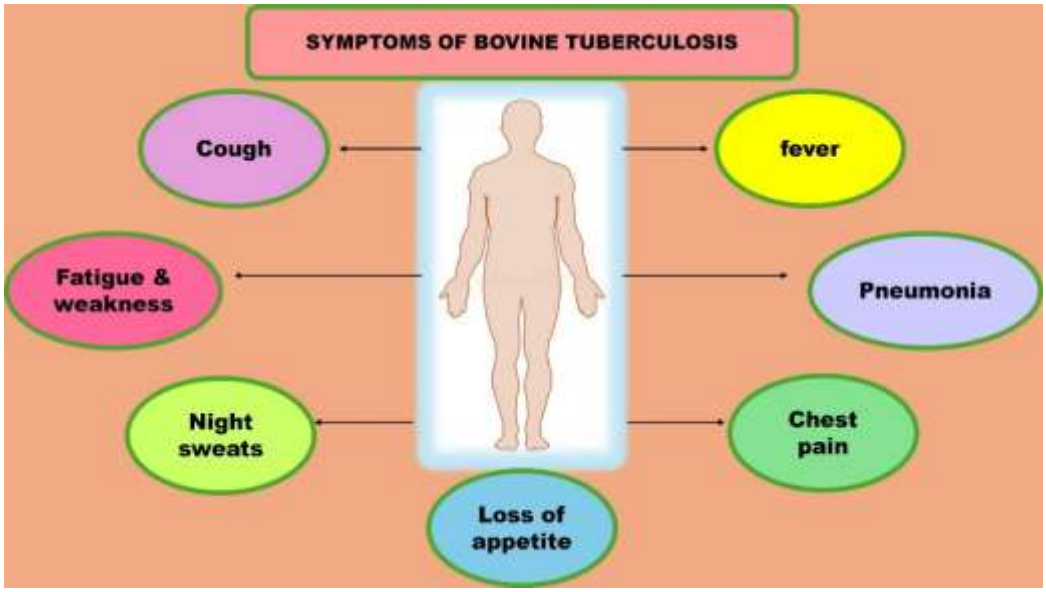

Fig. 2. Major symptoms of Bovine Tuberculosis in humans

India is a country having a large number of livestock species, ranging from small ruminants, such as sheep and goats, to large ruminants' cattle and particularly the country's buffalo (*Bubalus bubalis*), which is primarily found in Southeast Asia's tropical and subtropical regions [8-13]. In India's dairy sector, a major contributor among livestock is buffalo, accounting for more than 45% of India's total milk production and in addition to that significant impact has also been demonstrated in meat production as well. Other livestock, like cattle, etc., play an imperative role in sustaining the livelihoods of farmers and rural agricultural communities that directly pose a significant contribution to the nation's economy [14-20]. India is having a 7.3% prevalence rate with an estimation of 21.8 million bovines are affected with bovine tuberculosis. States and Union territories with high to low prevalence of bovine tuberculosis are given in Fig 3. Due to the complexity of the clinical spectrum and epidemiology, accurate diagnosis of bovine tuberculosis (bTB) is more difficult and challenging. In India, lack of awareness and insufficient laboratory diagnostic facilities hinder accurate estimation of the true disease burden [21-25]. Consequently, bovine tuberculosis remains significantly underreported due to being underdiagnosed in most of the regions of the country [21,25-27]. The best way to resolve this issue is to address the need for better monitoring of the disease, more awareness among farmers, veterinarians, clinicians, common people,

and public health professionals, along with the availability of sensitive and advanced diagnostic setups. Therefore, in this article we are discussing and highlighting almost up-to-date available conventional, advanced, and cutting-edge technologies utilized for the diagnosis of bTB. This article discusses the advantages, limitations, and future scope of these technologies, providing an up-to-date overview of diagnostic approaches for bTB that can help to reduce the disease's burden and improve public health and livestock productivity in India.

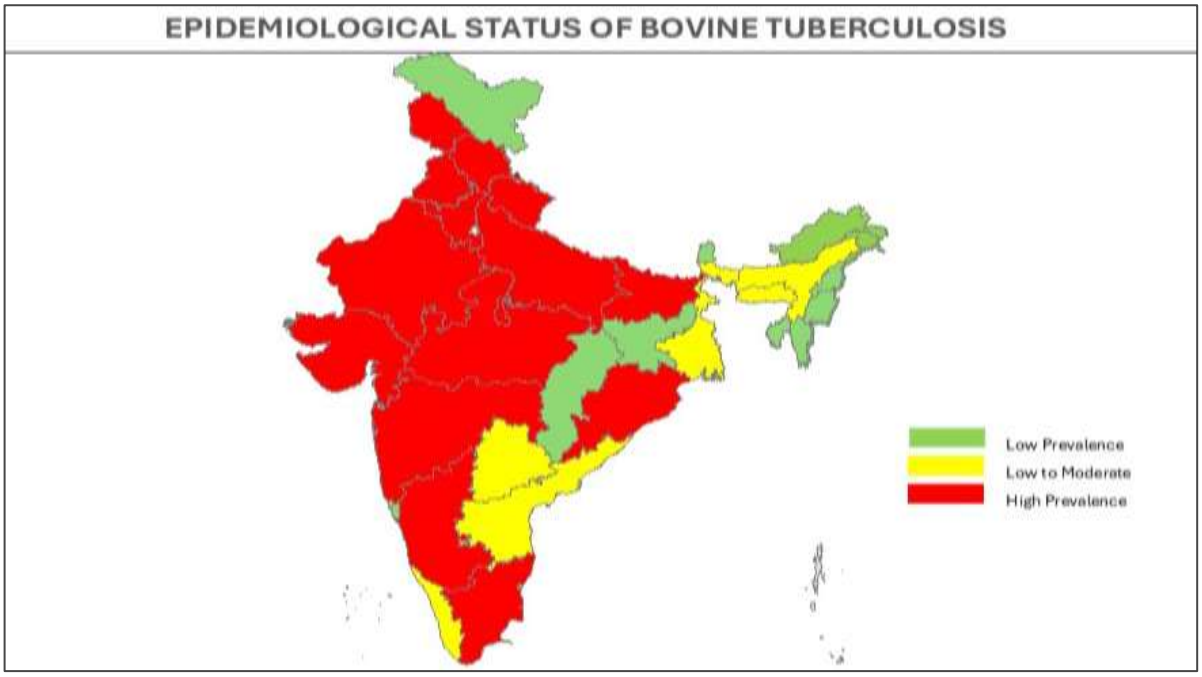

Fig. 3. Epidemiological status of Bovine Tuberculosis in India

DIAGNOSTIC METHODS

There are several diagnostic techniques to diagnose *M. bovis* which include conventional, advanced and cutting-edge methods:

CONVENTIONAL METHODS

Microscopy and Staining: Microscopy is an early and most widely used conventional diagnostic method to see small objects or microorganisms that cannot be seen with the naked eye. Several microscopic techniques are available for the identification of different target sources, such as simple microscopy, which is used for the observation of basic biological properties of any clinical or biological sample; compound microscopy may be utilized for the observation of cells' and tissues' morphology. On the other hand, stereoscopic microscopes are majorly involved in dissections and entomology studies [28], and brightfield microscopes may be explored for examining the stained thin specimens under bright field. Fluorescent microscopes and electron microscopes are mainly employed for visualizing specific structures within cells using fluorescent dyes, nanometer-scale resolution, and producing detailed 3D structures of material [29]. Microscopy is being explored in routine diagnosis of *M. bovis* via direct smearing methods from prepared positive tissue materials and clinical samples. Then, smears are stained with Ziehl-Neelsen (ZN) stain and observed under a light microscope. Staining techniques play a crucial role in microbial identification so that the morphology of these microbes can be properly examined under a microscope. The Ziehl-Neelsen (ZN) stain is an acid-fast stain particularly used for examining acid-fast bacilli in clinical samples (blood or sputum) via the smear preparation technique. The diagnosis of positive clinical sample with this stain indicates red coloured Acid-Fast bacilli, which can be present single or in clump formation [30]. Acid-fast stain is most preferred to differentiate between acid-fast or non-acid-fast bacteria that have a waxy lipid-rich outer layer that contains high concentrations of mycolic acid; that's why it makes them resistant to other staining methods like Gram staining, etc [31].

Bacterial Culture: *M. bovis* diagnosis in humans is being conducted routinely by performing various culture methods using blood and sputum samples [32]. In this strategy, clinical samples are collected, in which sputum is the most preferable sample source for mycobacterial culture, and are cultured on Löwenstein-Jensen (LJ) medium. In this, strains are suspended in sterile normal saline to achieve a good enough inoculum containing the required volume of colony-forming units (CFUs) and incubated first with loosened caps at 37°C temperature for 24 hours, followed by tightened closed caps and incubated for about 12 weeks to allow the detection of slow-growing bacteria. In addition to Löwenstein-

Jensen medium, other solid media like modified Middlebrook 7H11 agar, Stonebrink's medium, tuberculosis bovine blood agar, and egg-yolk-based media are also utilized for primary isolation and differentiation of *M. bovis* [3,30,32,33].

***In vivo* and *in vitro* immunological assays; TST, SIT, CIT, and IFN-γ:** *In vivo* immunological assays are mostly used to detect infection with *Mycobacterium* species in animals as well as in the humans and is comprised of tuberculin skin test (TST), single intradermal tuberculin tests (SIT), comparative intradermal tuberculin test (CIT), and Interferon-gamma (IFN-γ) release assay. The TST involves intradermal injection of purified protein derivative to assess cell-mediated immune sensitization and is commonly applied to determine whether a person or animal has been infected with tuberculous mycobacteria. A positive TST suggests the presence of mycobacterial infection, whereas a negative result generally indicates the absence of detectable infection, but there might be a chance of false positive results too. This test is cheaper in cost and availability of this test is higher in nowadays, but limitations in test interpretation sometimes required confirmatory or second-step investigation to improve diagnostic precision [34-36]. In cattle, SIT uses bovine tuberculin PPD injected intradermally, typically at the caudal fold, with the absence of a local immune response indicating a negative animal and the development of a characteristic reaction leading to classification as a suspect or reactor. The CIT refines specificity by injecting both avian and bovine tuberculin at separate intradermal sites, usually in the neck; a stronger bovine reaction compared with the avian reaction is interpreted as positive for bovine tuberculosis, whereas equal or lesser bovine reactivity is interpreted as negative or non-specific [35]. Interferon-gamma release assays provide an additional *in vitro* immunodiagnostic approach: leukocytes from individuals or animals exposed to *Mycobacterium* antigens are incubated with specific mycobacterial antigens and controls, and sensitized cells release IFN-γ, which is subsequently quantified. For the diagnosis of bTB, whole blood (heparinized) is incubated with the controls and test antigens. Post-incubation, plasma supernatants are collected, and the concentration of IFN-γ is measured by using enzyme-linked immunosorbent assay (ELISA) results, which are classified as positive, intermediate, or negative, reflecting the likelihood of infection [34,37].

Histopathology: Assessment of necrosis and characterization of granulomatous lesions can be done through histopathological analysis of tissues suspected of *Mycobacterium bovis* infection. It includes fixation of tissue samples collected or extracted from the affected organs by using appropriate fixative routinely practiced by formalin followed by graded dehydration using different concentrations of ethanol clearing in xylene or acetone and embedding in paraffin wax. Thin sectioning of paraffin blocks of generally 5 μm in thickness is conducted and the sections are mounted on glass slides with Dibutyl phthalate Polystyrene Xylene (DPX) mounting medium. The slides are then stained with hematoxylin and eosin to allow evaluation of tissue architecture. Microscopic examination typically focuses on the presence of tuberculoid granulomas with central caseous necrosis, surrounded by epithelioid macrophages and, in many cases, multinucleated giant cells; such lesions are consistent with tuberculosis compatible pathology, whereas sections without necrosis and with preserved normal morphology are interpreted as negative or nonspecific [30,33].

ADVANCED MOLECULAR DIAGNOSTIC METHODS

CBNAAT, Truenat, Conventional PCR and RT-qPCR, and ELISA:
A number of advanced nucleic acid-based assays are increasingly used for the fast and accurate diagnosis of tuberculosis in animals as well as in humans. The Cartridge-Based Nucleic Acid Amplification Test (CBNAAT) is a rapid, fully automated molecular platform that analyses the DNA from clinical samples, preferably sputum, to detect the *Mycobacterium tuberculosis* complex and rifampicin resistance, able to deliver the results within a short turnaround time and often serving as a primary or initial diagnostic test in high-burden settings; for better results, proper sample collection and handling by trained personnel are essential during testing [38,39]. Truenat is a chip-based real-time polymerase chain reaction (PCR) assay that utilizes the clinical samples for the detection of tubercle bacilli. Following sample collection, a dedicated device (e.g., Trueprep-MAG™) is employed for the DNA extraction, which is then loaded onto a microchip containing stabilized reagents for amplification, and the time of delivery of the result is about one hour. Its performance has been evaluated by using different statistical tools such as ROC analysis, Bayesian sensitivity or specificity estimation, and forest plots, and the tests have shown comparable results with culture, even though there might be a chance of providing false positive results by this assay, and this could be due to the presence of genetic mutations in target DNA regions [40-42]. Conventional endpoint PCR amplifies specific DNA segments by using repeated cycles of denaturation, annealing, and extension, and amplified DNA is further visualized on agarose gel electrophoresis by the application of intercalating dyes such as ethidium bromide under ultraviolet light. This method is useful for cloning specific sequences, detecting mutations, and identifying bovine genomic DNA in the given clinical samples, but this approach provides only qualitative information, and using staining dyes such as ethidium bromide is carcinogenic, due to which proper precautions should be taken during handling agarose gel [43-48].
Reverse transcription PCR (RT-PCR) extends this approach by synthesizing complementary DNA (cDNA) by using RNA as a template with the application of an enzyme, reverse transcriptase, which proceeds further with qualitative results of gene expression; it can be a one-step or two-step protocol. In a one-step protocol, reverse transcription and PCR can be performed in the same tube, while in a two-step protocol, cDNA synthesis is carried out in one tube, followed by PCR in a separate or another tube, and is majorly applicable in genetic studies, virology, forensic science, and diagnostic medicine. For quantitative measurement of nucleic acids, RT-PCR is combined with real-time PCR (qPCR). The resulting RT-qPCR

enables the quantitative analysis of nucleic acid in real time with the application of probes or fluorescent dyes, allowing assessment of the kinetics of the ongoing reaction and precise quantification of DNA or RNA [49]. It is more sensitive and faster than conventional PCR and can generate large numbers of copies within a few hours, and in this variant of PCR there is no need for post-PCR processing like running target nucleic acid fragments on agarose gel, etc., but it is very obvious that there is a requirement of appropriate biosafety, specialized molecular laboratories, and trained and well-experienced personnel for handling and working while using this technology. For the bovine tuberculosis diagnosis, the MPB70 gene is one of the important genes that are targeted and have been successfully applied to tissue samples from infected animals, demonstrating higher diagnostic sensitivity and rapid diagnosis of bTB with a higher accuracy rate. Enzyme-Linked Immunosorbent Assay (ELISA), originally described by Engvall and Perlmann in 1971. It is an antigen-antibody interaction test which is used for the detection and quantification of proteins or peptides by using enzyme-labelled conjugates and substrates [50]. This method utilises an antigen, an antibody, an enzyme (mostly horseradish peroxidase), a substrate and a stop solution for the generation of a coloured product whose intensity is measured with a spectrophotometer or ELISA reader at particular specified wavelengths. Biological samples like milk, urine, saliva, and serum can be used as the source of antigen; these antigens bind labelled antibodies (for example, IgG/IgM) to form complexes. Removal of non-bound material can be done by washing; a substrate is added, and colour development is read spectrophotometrically [51]. There are different variants of this technique, viz., sandwich, indirect, and competitive ELISA, which use secondary antibodies and have differing strengths, including the ability to detect multiple targets. But in this case, the number of incubations and multiple washes make this approach time-consuming, and there are chances of false positive results also due to cross-reactivity [52]. For the diagnosis of bovine tuberculosis, ELISA is widely used for the detection of antibodies against *Mycobacterium bovis* proteins such as MPB70 and MPB83 [53]. A biotinylated protein G-based ELISA has been applied to detect anti-MPB70 antibodies across species, using antigens secreted by *M. bovis.* Results from *M. bovis* ELISA are read spectrophotometrically, commonly in the wavelength range of 450-620 nm, and interpreted with cutoffs and categorised accordingly into positive, negative and intermediate or doubtful [54,55].

LAMP, Digital PCR, Nested PCR, BACTEC MGIT 960, LED Fluorescence Microscopy, and Line Probe Assay (LPA):

LAMP, Digital PCR, Nested PCR, BACTEC MGIT 960, LED Fluorescence Microscopy, Line Probe Assay (LPA) are important diagnostic methods that play an important role in the diagnosis and characterization of bovine tuberculosis caused by *M. bovis* and other members of the *Mycobacterium tuberculosis* complex (MTBC).

Loop-mediated isothermal amplification (LAMP) is a DNA-amplification technique that follows a uniform pattern of temperature, which means it works at a constant temperature and is designed in such a way that helps in the detection of specific bacterial or viral DNA sequences, including those from *M. bovis* and other pathogens; it uses mainly multiple sets of primers, generally six in the initial step and four in later steps, to improve specificity and avoids the need for conventional thermal-cycling instruments [56]. Different LAMP formats, viz., paper-based microfluidic LAMP, reverse-transcription LAMP, and digital LAMP, have been developed so far for molecular studies and rapid detection of infectious agents in clinical and environmental samples. Although this technique is proven as a good approach for diagnosing the pathogen infection, the use of several primers can increase the chance of primer-dimer formation and may disturb the amplification process [57]. After conventional and real-time PCR, Digital PCR refines nucleic acid detection by partitioning the reaction mixture into thousands of small compartments in two different manners: either droplets in an oil-based system or wells in a plate, each treated as an individual PCR reaction. After completion of amplification, the absence or presence of sequences of target DNA can be read with the help of fluorescence, which allows accurate quantification of even low-level targets of pathogen DNA. Algorithm-based optimization and advanced imaging systems are further improving Digital PCR, despite its relatively high cost and unsuitability for routine point-of-care settings [58-61]. Nested PCR is another variant of PCR, which is a more advanced form of conventional PCR. It carries out two successive amplification rounds with two different sets of primers; the first round basically generates the primary product employing fewer cycles, and the second round utilizes internal primers and generally a greater number of cycles (25-35 cycles) in order to amplify a smaller region within the initial product, thereby reducing background and improving detection of low-abundance targets such as *M. bovis* DNA in tissue samples. In bovine tuberculosis studies, nested PCR has been applied to the samples taken from cattle and buffaloes. By the application of specific primer pairs, for example, JB21/JB22, detection and verification of MTBC can be done to declare animals positive or negative based on PCR-amplification patterns, which makes nested PCR faster and more specific than conventional PCR and other culture-based identification [62-67]. BACTEC MGIT 960 is an automated, non-radiometric culture system also known as a Mycobacteria Growth Indicator Tube. It is a sensitive and widely used strategy for detecting MTBC, including *M. bovis* in milk and other body fluids, and for performing drug susceptibility testing as part of the culture-based workflow. It uses Middlebrook 7H9 broth supplemented with an antibiotic mixture and an oxygen-sensitive fluorescent sensor to monitor the growth of mycobacteria from many clinical specimen types except blood. Higher fluorescence indicates greater oxygen consumption by growing organisms and is interpreted as a positive result, making it a powerful tool for diagnosing *M. bovis* infection [68-70]. Light-emitting-diode (LED) fluorescence microscopy replaces or complements conventional light-microscopic examination of Ziehl-Neelsen-stained smears. In this approach, tissue-smear preparations are viewed under light-emitting-diode (LED)-based fluorescence microscopes. This strategy improves the sensitivity, especially for low-grade positive samples, and shortens the time required for slide reading, and in addition to that, it is cheaper and easier to maintain than traditional mercury-based fluorescence systems, and these advantages make this technique convenient

for even resource-limited labs that are engaged in tuberculosis or bovine tuberculosis diagnosis even though pathogen identification abilities are comparable with conventional microscopy [71-74]. The Line Probe Assay (LPA) is a PCR-based molecular approach which is being utilised for the amplification of MTBC DNA, and further analysis can be done by hybridising the products to membrane-bound probes and analysing the banding pattern. Along with the amplification of nucleic acids of MTBC, it has also been explored for the detection of mutations which are associated with resistance to first-line drugs such as rifampicin (rpoB region) and isoniazid (katG, inhA regions), thus delivering faster results and a higher rate of accuracy than conventional culture-based drug susceptibility testing and therefore allowing early adjustment of treatment regimens. Furthermore, in order to reduce manual workload and accelerate interpretation, some laboratories have begun using machine-learning-assisted software and automated image capture to read and analyse LPA strips, improving precision and speeding up result dissemination [75-78].

CUTTING EDGE DIAGNOSTIC METHODS

16s rRNA sequencing, Next-generation sequencing (NGS), Whole-genome sequencing (WGS), and CRISPR-Cas:
Cutting-edge diagnostics like 16S rRNA sequencing, NGS/WGS, and CRISPR-Cas are needed because they detect and differentiate *Mycobacterium bovis* rapidly and with much higher sensitivity and specificity than conventional tests. They identify strain-level variation, antimicrobial-resistance markers, and mixed or low-abundance infections that culture, microscopy, and immunoassays often miss. These methods enable faster, evidence-based treatment and control decisions and support epidemiologic tracing and surveillance for outbreak containment.
Unlike slow, labor-intensive culture (up to 12 weeks) and cross-reactive immunological assays, these technologies deliver results in hours to days with minimal sample requirements. All these methods are being utilized for identifying and characterizing bacterial pathogens, including members of the *Mycobacterium tuberculosis* complex. The 16S rRNA gene is comprised of both the conserved and hypervariable regions. In this approach, the variable regions are targeted using conserved sequences by PCR primers, followed by sequencing and comparison with reference databases for species identification being carried out. This method is useful for bacterial detection from a wide range of sample sources like food, clinical, and environmental samples but is unable to identify viruses [79-82]. Next-generation sequencing platforms use a metagenomic approach as well as carry out massively parallel sequencing of mixed DNA without prior culture [83,84]. Whole-genome sequencing provides a clear picture by offering better resolution for genotyping and phylogenetic analysis through the sequencing of entire genomes. For *M. bovis*, the most-used reference genome is AF2122/97. The basic workflow for WGS includes culturing of bacteria followed by extraction of genomic DNA; PCR library preparation; sequencing using different platforms like Illumina, MiSeq, etc.; and, in the last, downstream bioinformatic analysis. For the detection of drug resistance patterns, surveillance, and genomic research, WGS is proven to be a powerful tool, but it faces challenges for cost, complexity, need for bioinformatics expertise, limited reference databases for veterinary pathogens, difficulty with mixed infections diagnosing [85-89].
The novel drug targets of any disease can be discovered using transcriptomics by studying the expression of genes that play important roles in different pathways and specific mechanisms of that particular disease. The information about the pathogenesis of *Mycobacterium bovis* can be revealed by studying before and after (pre & post) *M. bovis* infection in animals, and this kind of study helps in developing the biomarkers that might make bovine TB tests better. Transcriptomic studies have a number of applications in agriculture, food safety, and disease diagnostics [90-93]. By applying single-cell RNA seq and bulk RNA seq, host responses, which means how host cells react when there is an infection of *Mycobacterium bovis* at various cellular levels, are documented [94]. Single-cell RNA sequencing (scRNA-seq) is a powerful diagnostic approach in molecular biology that helps to understand gene expression levels at the individual cell level by analysing transcriptome profiles [95]. In tuberculosis (TB) research, scientists have applied several related techniques, including CITE (Cellular Indexing of Transcriptome and Epitopes) sequencing, ATAC (Assay for Transposase-Accessible Chromatin) sequencing and TCR (T-cell receptor) sequencing. CITE sequencing combines measurement of cell-surface proteins with intracellular transcriptome data, converting transcript signals into quantitative sequencing values, thereby helping investigators to explore the underlying mechanisms of biological processes [96]. In a similar way, ATAC sequencing uses the Tn5 transposase enzyme to insert DNA transposons into open or accessible chromatin regions, making it useful for building differential gene-regulation pathways and identifying functionally distinct cell populations [97]. Likewise, TCR sequencing enables precise, large-scale profiling of T-cell receptor repertoires at the single-cell level and is widely used to study immune-related diseases and to support rational drug design [98]. In TB diagnosis, single-cell RNA sequencing is employed to capture intracellular dynamic changes, analyze the expression of infection-associated proteins and uncover molecular mechanisms that can guide the development of new potential therapeutic agents against tuberculosis. Various sample types such as peripheral blood, brain tissue and bone marrow have been examined using single-cell RNA sequencing in tuberculosis studies, enabling detailed characterization of infected and bystander cells. Overall, single-cell RNA sequencing contributes significantly to understanding tuberculosis progression and to the discovery of novel targets and therapies that may enhance the quality of *M. tuberculosis* diagnosis. The technique is now widely applied in developmental biology, clinical research, and evolutionary and ecological studies, but a key limitation is that it can generate sparse data, which may reduce the depth of downstream biological interpretation and the resolution of cellular heterogeneity [99-100].
Clustered Regularly Interspaced Short Palindromic Repeats Cas (CRISPR-Cas) is an adaptive immune system naturally present in many bacteria and forms the basis of modern gene editing technology and now has a number of applications,

including disease diagnosis [101]. The Type VI system of CRISPR-Cas (CRISPR-Cas13a) has been applied to detect bacterial pathogens, including species of *Staphylococcus*, *Streptococcus*, *Salmonella, Listeria*, and *Mycobacterium*, therefore playing an important role in disease diagnosis. *Mycobacterium bovis* can be identified in milk samples using CRISPR-Cas13a by targeting the *M. bovis* MPB70 transcript (mRNA), which is of particular concern because consumption of contaminated or fermented milk from animals can transmit tuberculosis infection to humans. CRISPR-Cas13a is considered a more practical and reliable method for diagnosing *M. bovis* in milk compared with conventional PCR, since PCR demands skilled personnel and the calcium ions present in milk can interfere with magnesium-dependent amplification, thereby reducing efficiency. In milk-based tuberculosis detection, CRISPR-Cas13a-coupled lateral flow assays show higher accuracy and sensitivity for *M. bovis*. The system relies on RNA-based fluorescent reporter probes that emit fluorescence when the target RNA activates Cas13a; once activated, a single Cas13a molecule can cleave thousands of these reporter RNAs, greatly amplifying the signal from the target RNA in the reaction. In addition to Cas13a, advanced CRISPR-Cas variants such as CRISPR-Cas12a-based lateral flow detection have been developed to identify bacterial and viral infections in various body fluid samples. As a result, CRISPR-Cas has become one of the most widely applicable tools in molecular research, clinical diagnosis, and food safety testing; however, challenges include the requirement for specialized equipment, optimization of reporter probe design, and potential cross-reactivity with closely related species [102-106].

PREVENTION AND CONTROL

For the prevention of this disease, the first step is that awareness programs should be conducted in rural as well as urban localities, and second, milk and milk products must be properly pasteurized before consumption. Furthermore, for the prevention of bovine tuberculosis, along with segregation and proper management practices, tuberculin skin testing and other diagnostic tests should be conducted routinely. In addition, infected animals should be removed from herds, hygiene must be maintained, and tuberculosis eradication programs should be monitored in rural areas to ensure the rural population remains informed about how the infection spreads. In developed countries, bovine tuberculosis cases are rare because eradication programs are implemented [25,107], and pasteurization of milk products is one of the major approaches that prevent bovine tuberculosis infection [108]. Animal husbandry practices should reduce contact between domestic livestock and infected wild animals as transmission can occur through direct contact. Control measures should be applied in a timely manner to prevent the disease [109]. With proper prevention and control measures, bovine tuberculosis can be controlled or even eliminated at the national or international level.

TREATMENT

Combining an ESAT-6 DNA vaccine with the conventional Bacillus Calmette Guérin (BCG) vaccine has been reported to enhance protective immunity against tuberculosis in cattle. ESAT-6, or Early Secreted Antigenic Target 6 kDa, and CFP10, or Culture Filtrate Protein 10 kDa, are important immunodominant antigens secreted by members of the *Mycobacterium tuberculosis* complex, including *Mycobacterium bovis*, the causative agent of bovine tuberculosis. These antigens can stimulate strong cellular immune responses, particularly T-cell-mediated immunity, which is essential for protection against tuberculosis. When used in combination with BCG, ESAT-6 DNA vaccines may improve vaccine efficacy by broadening the immune response and strengthening protection compared with BCG alone.

In animals, generally in cattle, the development of improved tuberculosis vaccines is especially important because BCG vaccination can interfere with conventional diagnostic tests such as the tuberculin skin test. Therefore, novel vaccine candidates and Differentiating Infected from Vaccinated Animals (DIVA)-compatible strategies are being explored which allows vaccinated animals to be distinguished from naturally infected animals during disease surveillance. This is crucial for bovine tuberculosis control programs, where accurate diagnosis is necessary for monitoring infection, preventing disease transmission, and supporting eradication efforts. ESAT-6 approaches, along with appropriate companion diagnostic tests, may therefore contribute to more effective vaccination and disease-control strategies in cattle.

FUTURE PERSPECTIVES

Bovine tuberculosis is caused by *Mycobacterium bovis* and spreads to humans through contact with infected animals or by drinking their milk or eating meat or dairy products. Several methods are used for the diagnosis, ranging from conventional techniques, including culture, histopathology, microscopy, tuberculin skin tests, and interferon-γ assays, to advanced methods, including CBNAAT, Truenat, conventional PCR, real-time PCR, nested PCR, and ELISA, along with cutting-edge methods, viz., LAMP, digital PCR, NGS, and whole-genome sequencing, providing a fast, more accurate, and efficient way of identifying strains and proving to be very helpful in outbreak investigation, vaccine research, and surveillance programmes. The outcomes from the aforementioned strategies will be further enhanced by the incorporation of artificial intelligence and bioinformatics in bovine TB control. The introduction of machine learning and image-analysis tools will also expedite and enhance the interpretation of radiographs, colony morphology, and lateral flow. AI-driven technology also aids in differentiating the genomic and proteomic data of *Mycobacterium bovis* from that of other mycobacteria; furthermore, it will help in creating those models, which will be able to identify the risk factors for transmission of this disease. Likewise, the surveillances which are driven by artificial intelligence when combined with computational biology as well as big data science will enable the researchers to predict the zoonotic spread in advance on a probability basis and also speed up new ways of developing therapeutics in medical research as well. But to apply the

combinatorial approach, there must be a need for accurate and strong data, proper validation, and sufficient infrastructure to work in both human and animal health.

CONCLUSION

In this review, zoonosis of bovine tuberculosis (bTB) caused by one of the *Mycobacterium tuberculosis* complexes (MTBC), *Mycobacterium bovis* (*M. bovis*), is discussed. Mainly drinking milk or eating raw dairy products from infected animals, eating uncooked or contaminated meat, or coming in direct contact with other body secretions like urine, saliva, etc. is the main route of transmission from animals to humans. At the initial stage of infection, animals are often non-symptomatic; still, they remain a danger to the human population; that's why on-time diagnosis and treatment are important, and for that, this paper summarizes the up-to-date diagnostic strategies, which include conventional, advanced, and cutting-edge methods. Conventional methods such as microscopy, culturing, CIT, TST, and SIT are routinely used for diagnosis; however, these methods are generally slow, highlighting the need for advanced or cutting-edge techniques that are more time-efficient, despite their own advantages and disadvantages, and the requirement for well-established laboratories. Finally, a combined approach that includes diagnostics, vaccination, treatment, and control programs is necessary to reduce the burden of bovine tuberculosis in both animals and humans.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this article.

AUTHORS' CONTRIBUTION

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Not Applicable

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