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Review Article

A critical review on diagnosis of paratuberculosis in domestic animals

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Abstract

Paratuberculosis, caused by *Mycobacterium avium* subspecies *paratuberculosis*, is a bacterial disease of domestic and wild ruminants. It progresses very slowly and, hence, takes several years to develop clinical signs. The importance of the disease is ignored because of its slow progression and subtle influence on production. The prevalence of the disease in different parts of the world has been confirmed; it is high enough to control and minimize losses. A number of tests are used to diagnose, but no one seems too ideal since most diagnostics are unable to diagnose infected animals before the animal contaminates the environment. New advanced techniques no doubt increase diagnostic efficiency with reduced time, but they fail to diagnose the disease before shedding bacilli. Most of the diagnostics explore the body response against the pathogen for its detection. These strategies should not be applied in paratuberculosis diagnosis. Host response against bacilli is so late that the detection of infection based on it is of no use as animals contaminate the environment by that time. The present review summarizes the available diagnostic techniques in domestic animals.

Keywords: Diagnostic tests, Johne's disease, *Mycobacterium avium* subsp. *paratuberculosis*

Highlight

- Paratuberculosis is a slow-progressing disease of ruminants, and hence, its effect on production goes unnoticed.
- Diagnosis of paratuberculosis in early stage at field level is very difficult and hence their presence is unnoticed and disease establishes itself in the herd.
- Only prevention or eradication of paratuberculosis is advisable in the present scenario.
- Diagnosis of the disease at an early stage is important for the control of paratuberculosis.
- Present molecular diagnostic tools have become so advanced that we can identify the pathogen of paratuberculosis within 2 hours.

INTRODUCTION

Paratuberculosis, caused by *Mycobacterium avium* subspecies *paratuberculosis* (*Map*), is also known as "Johne's disease" (JD), as it was first reported by Heindrich A Johne and Frothingham in 1895. Intestinal granulomatous inflammation is the main pathological finding in the disease. The disease is worldwide in distribution and spreading insidiously. It is considered a threat as well as a burden (Garvey, 2020) to the dairy, beef or meat industry. There is a significant economic loss to cattle, sheep, and goat husbandry (Tripathi *et al*., 2002). Livestock trade restriction is also imposed due to possible transmission to other countries.

Map is a facultative aerobe, non-motile, acid-fast and weakly gram-positive bacilli (0.5-1.5 µm in length). It comes under the genus *Mycobacteria* as a slow-growing bacilli. The colonies of mycobacteria are generally seen after seven days or more under suitable conditions. Some species under this genus are pathogenic for both human and animals [examples are M. *tuberculosis*, *M. bovis*, *M. leprae*, *M. avium* complex (MAC)]. There are three subgroups of MAC: i) *Mycobacterium aviam* subspecies *avium (Maa)*, ii) *M. avium* subspecies *silvaticum (Mas)* and iii) *Mycobacterium aviumv* subspecies *paratuberculosis*. *Map* is virtually identical to *Maa;* they share 95% of their genes and exhibit homologies of more than 99% between their genes (Li *et al*., 2005). However, phenotypic features are different from *Maa*. *Map* grows more slowly with a generation time of 22 to 26 hrs as compared to 10-12 hrs for *Maa* (Thorel *et al*., 1990).

DIAGNOSIS

Diagnosis of paratuberculosis is essential because treatment is not promising and complete. Early diagnosis of disease is of prime importance as it helps in its control and prevention. Th1 and Th2 immune

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markers are important in diagnosis, but are unreliable in predicting persistent infections. The macrophage-based assay is a good diagnostic for persistent infections. Identification of bacilli in faces is the best diagnostic for *Map* infection and also predicting stages in rapidly progressing undergoing infection. However, in slow or non-progressing infections, it is of negligible significance, but macrophage assay could be superior (Magombedze *et al*., 2017). *Map* has been detected from macrophages collected from all sources in last stage (stage IV) of disease (Ramírez-García and Maldonado-Estrada, 2013). Therefore, diagnostics available for confirmation of paratuberculosis are categorized in four groups. These are 1) diagnosis on the basis of postmortem examination and histopathological lesions, 2) identification of etiological agent, 3) measurement of host response and 4) confirmation of *Map* genome.

Diagnosis on the basis of postmortem examination and histopathology

Macroscopic or characteristic postmortem lesions of bovine paratuberculosis are the thickening of the intestinal wall and corrugation of the mucosa (Gonzalez *et al*., 2005). The associated lymph nodes and tissues are enlarged several times (Whitlock and Buergelt, 1996). The intestinal wall presents a "cerebroid" due to the presence of numerous 5-8 mm folds, which do not disappear while pulling. These folds are due to thickening of the wall by infiltration of macrophages, epithelioid cells and giant cells. Variable numbers of acid-fast bacilli (AFB) in giant cells are noted depending on the specific immunopathological form. There is also lymphadenomegaly and edema of the mesenteric lymph nodes, together with lymphangiectasia. The intestinal lymph flow is restricted by the presence of macrophages, which obstruct the subcapsular sinus in afferent lymphatic vessels. Developing lesions in the intestinal wall gradually result in a malabsorption syndrome. Clinical signs may not present a linear relationship with these changes.

It is rare to find lesions outside the intestinal tract. However, hepatic injury, atherosclerosis of the aorta, myoatrophy, emaciation, atrophy of body fat, alopecia, renal infarction, edema, serous exudates in body cavities and anemia (Tiwari *et al*., 2006) can occur in advanced stage of disease. Caseous necrosis and tubercle formation (a feature of mycobacterial infection) is not feature in cattle but may be seen in sheep, goat or deer (Barker *et al*., 1992).

Lesions of paratuberculosis are classified on the basis of type and their number in the lesion as well as the size of the lesion involved. These lesions are: i) tuberculoid (focal or multifocal, lymphocytic or paucibacillary), ii) lepromatous (diffuse multibacillary), and iii) intermediate type (Gonzalez *et al*., 2005). Focal lesions are expected to appear first and are linked with a strong cellular immune response. Tuberculoid and multifocal lesions develop together and hence compress and obliterate the intestinal crypts. The adjacent villi are fused together, resulting in a net decrease in absorptive surface, hypoproteinemia and edema, leading to weight loss. The lepromatous type lesion appears in some animals and is related to the altered profile of the immune response. Microscopically, the lesion is chronic diffuse catarrhal enteritis, characterized by hyperplasia of macrophages, lymphocytes, plasma cells, epithelioid and multinucleated giant Langerhans cells in the lamina propria. Their accumulations are most often extended to intestinal submucosa and are also seen in the paracortical region of regional lymph nodes.

Histopathological diagnosis has the advantage of identifying animals with focal lesions associated with subclinical stages in which faecal and/or milk secretions are insufficient for bacterial culture or PCR amplifications. However, there are disadvantages to having trained personnel for sample processing, and it is associated with high costs. Whitlock and Buergelt (1996) recommend to take samples from at least 100 sites of the gastrointestinal tract of animal to see the true picture of disease in host. The amount of *Map* DNA isolated from faeces can also help in prediction of histopathological changes in ileocecal region and hence help in prediction of disease progression (Taniguchi *et al*., 2020). Histopathology is not an economical but is the most trusted tool for the diagnosis of paratuberculosis (Sikandar *et al*., 2021).

Identification of etiological agent

Ziehl–Neelsen staining: Ziehl–Neelsen (ZN) staining of faeces is routinely used to detect mycobacteria. But, other acid-fast organisms, such as nocardia, corynebacterium, cryptosporidium, etc., are also stained. Therefore, differentiation between different mycobacterial species is not possible. However, confirmation of paratuberculosis is based on the presence of clumps (three or more microorganisms) of AFB (acid-fast bacteria) (Manning and Collins, 2001). The clumps are formed due to the hydrophobic features of the *Map* cell wall. Direct ZN staining of faecal samples is difficult to interpret and has a poor detection limit (Manning, 2001). The success of ZN staining

depends on the number of bacteria present in the sample. Direct observation of AFB is insufficiently sensitive or specific, but it is a quick, simple and economical technique. Direct ZN staining of faecal samples may reveal *Map* organism in clinical cases, but the sensitivity is low. Sensitivity of ZN staining of tissue is variable, which depend upon different stages of disease (Hamid *et al*., 2018; Thakur *et al*., 2019). In spite of that, ZN staining is most often used as a primary test at field level (Jatav *et al*., 2018) and in prevalence study in combination with another test. Similarly, ZN-stained impression smears of tissues collected at necropsy or through biopsy of ileum and/or mesenteric lymph node have been commonly used for the confirmation of JD. Detection of AFB in paucibacillary cases is difficult using this method (Tripathi *et al*., 2002). The presence of AFB in tissue smears indicate but do not confirm the infection.

Immunoperoxidase test (IPT): The immunoperoxidase test (IPT) technique uses a *Map*-specific antibody conjugated with enzymes. It allows the visualization of reaction with substrate. It enables to recognize spheroplasts and *Map* in tissue (Coetsier *et al*., 1998). It has good sensitivity in animals with subclinical JD, but can cross-react with *M. smegmatis*, *M. bovis*, *M. tuberculosis*, *M. leprae*, etc. The efficiency of the method depends on the anti-*Map* antibody used (Mundo *et al*., 2008); sensitivity is low as compared with bacterial culture (Moravkova *et al*., 2008). An indirect IPT for the histological diagnosis of JD (Nguyen and Buergelt, 1983) is described as an alternative to ZN staining. The sensitivity of IPT in paucibacillary cases is better than cultures (Sonawane and Tripathi, 2019) and superior to the ZN staining on tissues. Its superiority has been verified in paucibacillary and multibacillary in sheep (Sonawane and Tripathi, 2016). The method was sensitive, particularly in cases in which there were no JD lesions, and the ZN technique failed to detect acid-fast bacilli (Kumar *et al*., 2006).

In situ hybridization (ISH): *In situ* hybridization (ISH) is an indirect molecular technique that uses a labelled probe to specific nucleic acid sequences (DNA or RNA) on a histologically processed tissue section, allowing tissue localization. ISH uses a precise DNA probe of different sizes. The use of a small DNA probe easily penetrates tissues and reaches its target sequence, but it is unlikely to give specific reactions or may give weak staining that may impair the assay reading. In contrast, a larger DNA probe may have difficulty in penetrating tissue and finding its target sequence. Specific nucleic acid sequences (markers) used are tagged with radioactive compounds or fluorescent compounds or enzymes. The first two allow the detection of a sequence of interest, but with the loss of detail of tissue structure, enzymatic markers allow better observation. ISH is a technique that has been used primarily to detect spheroplasts in animal samples, samples from Crohn's disease (Secchi *et al*., 2001) and unicellular parasites where *Map* can grow (Mura *et al*., 2006). Sensitivity of rRNA ISH is almost equal to ZN staining (Mangalakumari *et al*., 2006). The advantage of the technique is the identification of *Map* in tissue.

Bacterial culture: Culture of the *Map* from faeces is widely used as well as accepted for diagnosis of paratuberculosis. This method is also considered as a reference assay for other tests. Faeces, colostrums, milk or intestinal mucosal scrapings are most often screened for the presence of viable *Map*. In cases of necropsy, the culture of the lymph node close to the macroscopic lesion and scrapings from the ileocecal valve and ileocecal or jejunal lymph nodes or the colon and rectum are the best samples. Infected cattle can excrete 10^{8-12} CFU/g of faeces and contaminate the environment; therefore, culturing pasture soil samples from manure and/or delivery areas is also recommended (Whittington *et al*., 2004; Windsor and Whittington, 2010). The bacterial culture requires at least 100 CFU/g of faeces (minimum detection limit) (Merkal, 1970). Since this amount is exceeded by animals in the clinical stage of the disease but not by subclinical low and/or moderate faecal shedders, moreover, only 15-25% of them can be detected by bacterial culture (Whitlock and Buergelt, 1996). Bacterial culture of milk from animals at this stage is difficult because excretion is 2-8 CFU/50 mL (Sweeney *et al*., 1992; Metzger-Boddien *et al*., 2006). *Map* excretion in faeces and milk may not be simultaneous; therefore, a significant proportion of positive animals may not be diagnosed if samples are collected from only one of these sources. For this reason, the simultaneous culture of both excretions is recommended (Gao *et al*., 2009). To improve the method's sensitivity, it has also been suggested that the bacterial culture be performed on samples collected from the same animal over a few days.

The sensitivity of the bacterial culture from faeces in clinical stages can be 91% (Alvarez *et al*., 2009), a value that can be reduced to 45% to 72% (Crossley *et al*., 2005; Alvarez *et al*., 2009) in subclinical stages, whereas the specificity is very good (100%) in all stages (Ayele *et al*., 2001). Cultures of intestinal tissue

and/or regional lymph nodes have a sensitivity of 70% and a specificity of 95% (Tiwari *et al*., 2006). The possibilities of huge number of different contaminants in faecal samples increase the challenges in culturing bacilli. However, challenges can be addressed by decontamination of faecal samples. This is based on *Map* inherent property of slow growth rate and hardiness of its cell wall (mycolic acid). It is possible to kill the fast-growing competitors by decontaminating the samples prior to inoculation into culture media. There are different methods to decontaminate the sample, but the most popular procedure uses hexadecyl pyridinium chloride (HPC) (Dundee *et al*., 2001). This chemical decontamination requires a day of work and can reduce the *Map* number to a range of 10^3 CFU (Reddacliff *et al*., 2003). It is estimated that, after the decontamination procedure, the expected contamination rate in the feces culture is 7% and 0.2% in the tissue culture (Whittington, 2009). Even after decontamination, chances of contamination during culture may be encountered. This may be addressed by use of a number of antibiotic cocktails. The most commonly used cocktail is the combination of polymyxin B, amphotericin B, nalidixic acid, and trimethoprim-sulfamethoxazole, supplemented with vancomycin and fungizone. This strategy is too expensive to be routinely applied in field diagnostics.

There are several *Map* culture media, all supplemented with mycobactin J, sodium pyruvate, antibiotics and antifungals. The preliminary step is the culture on liquid medium (Middlebrook 7H9, 7H12) added with oleic acid, bovine albumin and dextrose. Catalase may be used to hasten the detection of *Map* and the activation of *Map* prior to the culture on solid medium. Cultures upon solid media, such as Herrold Egg Yolk Medium (HEYM) with mycobactin J, Löwenstein-Jensen (LJ), or synthetic media, such as Middlebrook (7H10, 7H11) are most commonly used. The colonies are small, hemispherical, about 1 mm in diameter, smooth and shiny. The criteria to confirm *Map* are the slow growth rate, morphology of the colonies, ZN staining, and mycobactin dependency in primary culture. As the bacterial strain cannot be known prior to the culture, it is recommended to carry out the culture in HEYM-mycobactin-sodium pyruvate or LJ with mycobactin or Middlebrook 7H11. The use of these three media allows 100% of type I/III strain and 98% of type II strain detection (de Juan *et al*., 2006).

An automated system for the recognition of bacterial growth in a liquid medium has been developed. Examples are; radiometric BACTEC 460 system (Becton Dickinson Inc.) and BACTEC™ 12B (Middlebrook 7H12), supplemented with egg yolk and mycobactin. These systems contain a radiolabeled precursor (C^{14} labelled palmitate), and bacterial metabolism produces $^{14}CO_2$; hence, its detection confirms the presence of bacteria. Other automated systems use fluorometric, barometric and colorimetric methods (Reddacliff *et al*., 2003; Stich *et al*., 2004). These methods reduce the time required for results and is considered more sensitive than the conventional culture methods on solid media for the recognition of both ovine and bovine strains of *Map* (Eamens *et al*., 2000). Radiometric culture is more sensitive than histopathology in detecting *Map* infection in sheep and goats and more sensitive than culture on HEYM for the recognition of the infection in cattle (Whittington *et al*., 1998). Culture sensitivity may be further increased by the decontamination procedure that involves twice incubating faecal samples in HPC with a combination of antibiotics (Eamens *et al*., 2000). BACTEC™ system is being radiometrically based and is not feasible for use in some laboratories. The evaluation of the usefulness of alternative culture systems based on liquid media such as MGIT (Becton Dickinson), ESPII (Difco) and MB/BacT Alert (Organon Teknika) that do not use radioactive material for the recognition of *Map* may be recommended.

Growth and multiplication of mycobacteria can also be assessed by analyzing the volatile molecule produced during its growth. The analysis of headspace of culture tube results in identification of different volatile organic compounds (VOC) which varied with density of bacterial growth. Example includes 2-ethylfuran, 2-methylfuran, 3-methylfuran, 2-pentylfuran, ethyl acetate, 1-methyl-1-H-pyrrole and dimethyldisulfide (Trefz *et al*., 2013). Analysis of VOC from culture tubes has now been explored for early diagnosis of infection. The concentration levels of 2,3,5-trimethylfuran, 2-pentylfuran, 1-propanol, and 1-hexanol were indicative for *Map* before observable growth was noticed (Vitense *et al*., 2021). Additional validation studies are required to boost the robustness of VOC-based diagnostic systems.

Fluorescent microscopy: Fluorescent microscopy uses fluorigenic compounds. There is a conversion of fluorogenic compound into carboxyfluorescein by the enzymatic action of viable cells. This is the key step in fluorescent microscopy and hence, allows its identification. This method was used in pasteurized milk, spiked with 10^2 CFU/mL and attained a sensitivity of 73% (D'Haese *et al*., 2005). Akineden *et al*. (2015)

used a combination of different fluorigenic compounds *viz.* 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and auramine orange (AO). The combined CTC and AO staining had a detection limit of 10^2 CFU/mL for spiked ultra-high-temperature milk and 10^3 CFU/mL for spiked raw milk (with a greater than 95% likelihood of detection). Within eight hours, the total number of acid-fast bacterial cells and the number of respiratory active cells in milk can be ascertained using combined CTC and AO staining.

Bioluminescence technique: Bioluminescence uses the principle of oxidation of Lucifer, which is catalyzed by the enzyme luciferase, resulting in oxyluciferin. This reaction requires ATP, which is provided by viable bacteria. Sasahara *et al*. (2004) proposed the use of plasmid-phages labelled with luciferase for rapid detection of multiplying *Map*. PhAE85 was found to be the most effective of the three mycobacteriophages tested; it could identify 10^2 CFU/mL in skim milk and 10^3 CFU/mL in whole milk in just 24 to 48 hours. It is a quick and accurate approach for food samples, but using it calls for certain resources that most diagnostic labs don't have.

Reverse transcription polymerase chain reaction assays: Viable cell detection is also possible if we are able to identify the active gene transcription. The transcription products are mRNAs; its detection via reverse transcription PCR assays is possible nowadays. Amplification of mRNA from clinical samples of a gene of interest further selectively identifies the pathogen. Reverse transcription (RT)-PCR assays can be implied to detect amplifying mRNA of IS*900* of *Map* (Mishina *et al*., 1996). Similarly, different genes (Timp1, Hp, Serpine1, Tfrc, Mmp9, Defb1, Defb10 and S100a8) have been explored in RT-PCR for early diagnosis of disease (Park *et al*., 2017). The diagnostic efficacy was variable in different stages of disease. RT-PCR methodology is difficult to apply to the routine testing of bacteria in food samples or other complex sample matrices because of the difficulty in extracting cells from the matrix as well as dealing with contaminants (Hanna *et al*., 2005).

RT-PCR in combination with lateral-flow biosensor assay or microtiter plate assay: In the preceding section RT-PCR is being used to detect viable *Map* in clinical samples. Kumanan *et al*. (2009) used RT-PCR in combination with membrane-strip-based lateral-flow (LF) biosensor assay or microtiter plate assay on faecal samples spiked with 10^1 -10^6 *Map* cells. In both assays, the target molecule is the RNA of IS*900*, amplified by RT-PCR and subsequently used in LF biosensor or microtire assay. In the former assay, targeted oligonucleotide is combined with an immobilized probe and detected by a second DNA probe coupled with liposome encapsulating the dye, sulforhodamine B. Similar principle was used in microtitre assay in plate. The sensitivity of the combined test is to detect 10 viable cells within 10 h. The specificity of tests has also been checked against other mycobacteria, such as *M. avium complex*, *M. ulcerans*, *M. marium*, *M. kansasii*, *M. abscessus*, *M. asiaticum*, *M. phlei*, *M. fortuitum*, *M. scrofulaceum*, *M. intracellulare*, *M. smegmatis*, *M. bovis* and found specific to *Map*.

Diagnostics based on host response

Cell mediated immunity based assay: Tests based on cell mediated immunity (CMI) include measurement of delayed-type hypersensitivity (DTH) responses (Johnin test) to purified protein derivative (PPD) antigens and *in vitro* assay for IFN-γ production as a result of PPD stimulation. The major limitations of these tests are the cross-reactivity of *M. bovis* PPD and related bacteria, especially *Maa*. Detection of a cellular immune response by either skin testing or stimulation of leukocytes for IFN-γ release is suitable for earlier diagnosis of infection, but these assays suffer from high variability and lower specificity (Huda and Jensen, 2003).

Johnin test: An intradermal test (also known as Johnin test), a DTH, is a diagnostic test available at the field level, and it has been generally used for screening of paratuberculosis. It is mostly used as a single intradermal test, 0.1 mL Johnin or avian PPD (0.5 mg/mL or 25000 U/mL) on caudal fold or in middle of neck. An absolute increase in the skin thickness over 2 mm (WOAH, 2022) or 3 mm (Manning and Collins, 2001) after 72 hrs after inoculation is interpreted as positive. PPD used in test is a complex, undefined *Map*-secreted proteins. This test is prone to false-positive results due to cross-reactivity with similar proteins present in other mycobacteria. It has an estimated sensitivity of 54% and a specificity of 79% (Ayele *et al*., 2001; Kalis *et al*., 2003). The performance of this test may also be significantly affected by minor antigenic differences that may occur in different batches of antigens (Kalis *et al*., 2003). Cut off values of skin thickness also affect the specificity of test like 88.8%, 91.3% and 93.5%, respectively at ≥2 mm, ≥3 mm and ≥4 mm (Kalis *et al*., 2003).

A variant of comparative intradermal can be

performed by concurrent inoculation of PPD of *M. bovis* and *M. avium* in two separate areas in the same neck. The JD positive animals react more to both PPDs, but with more intensity to PPD of *M. avium*, because of similar antigenic make up among the *Map* and *M. avium*. This will help in differential diagnosis among the two closely related pathogens.

Lymphocyte stimulation test: Proliferative responses of lymphocytes either in peripheral blood or lymphocyte culture following mycobacterial antigens stimulation have been utilized as lymphocyte stimulation test and may be used as a diagnostic tool of JD (Tripathi *et al*., 2002). Test gives positive results before faecal culture becomes positive (Kurade and Tripathi, 2008); hence, it claims early detection of JD. Lymphocyte stimulation test is a sensitive *in vitro* T-cell mediated test.

Interferon gamma assay: Interferon Gamma Assay mesures specific cytokine IFN-γ production by T lymphocytes after PPD stimulation. Quantitative detection of IFN-γ can be implied in 1 to 2 yrs old animals (Ayele *et al*., 2001; Jungersen *et al*., 2002; Huda and Jensen, 2003; Huda *et al*., 2004; Speer *et al*., 2006; Alvarez *et al*., 2009) with positive results. The IFN-γ assay has been shown to be superior to humoral antibody tests in the detection of subclinical infection in both cattle (Stabel and Whittlock, 2001) and sheep (Gwozdz *et al*., 2000). Sensitivity of this assay in sub-clinical JD in cattle ranged from 50-93% when compared with repeated faecal culture results. Sometimes, in some experimental interpretations, its sensitivity as well as its specificity may reach 100% (Corneli *et al*., 2021). Clinically affected cattle may give negative results (Stabel and Whitlock, 2001). The sensitivity of IFN-γ test is higher than that of serological tests in sub-clinical stage, but low (41%) in absolute terms (Stabel, 1996; Gwozdz *et al*., 2000). It may be possible for animals that are not infected to occasionally test positive, which could indicate that the assay is less specific.

Intradermal sensitization of subclinically infected cows with Johnin dramatically increased IFN-γ secretion, suggesting that the sensitivity of this assay can be increased for diagnostic purposes (Stabel *et al*., 2007). The sensitivity of the IFN-γ assay drops to 20% in mixed infections (tuberculosis and para-tuberculosis); these variations may be caused by the strains that are present in each herd or the host species (Aranaz *et al*., 2006; Alvarez *et al*., 2009). A study conducted by Walravens *et al*. (2002) compared the IFN-γ response when cattle inoculated with *M. bovis*, *Map* and *M. phlei*; the response obtained was of low intensity as well as slow onset (4^{th} to 5^{th} week) in those animals which were inoculated with *Map*. The stimulation of lymphocytes with recombinant proteins viz. *Map* 0210c, *Map* 0268c, *Map* 1297, *Map* 1365, *Map* 1693c, *Map* 2020, *Map* 3651c, and *Map* 3651cT(it) have been explored and found useful for more specific and early detection of the disease (Hughes *et al*., 2013; Köhler *et al*., 2021).

The advantage of the IFN-γ test is that it is used during the early stages of infection, and hence, it may be an attractive tool for detecting infection in the subclinical stage (Corneli *et al*., 2021). However, it has a number of disadvantages: i) the potential cross-reactions, ii) as early as possible sample processing, as cells died after sometimes (Stabel, 1996), iii) higher expenditure and iv) lower sensitivity. Due to all of these factors, IFN-γ is a test that is not commonly used, but it can be used in control programs to find infected animals before they become sick and to lessen transmission to adult animals (Stabel, 1996; Jungersen *et al*., 2002; Paolicchi *et al*., 2003).

Humoral immunity based assays: A number of serological methods have been developed to identify the presence of an antibody response against the JD. Diagnostic tests measuring antibody response lack sensitivity in early infections, and hence, their use has been limited to the diagnostic confirmation of suspected clinical cases and herds for the absence of JD infection (Tripathi *et al*., 2002). Serological tests including complement fixation tests (CFT), agar gel immunodiffusion (AGID) test and enzyme-linked immunosorbent assay (ELISA) have been developed to perceive antibodies against *Map* in animal sera (Colgrove *et al*., 1989). These assays can show false-positive results and may not be consistent in detection of infected animals in the early stages of infection (Hilbink *et al*., 1994).

Complement fixation test: Complement fixation test (CFT) has been most often used in the earlier period (Ratnamohan *et al*., 1986), as it is satisfactory to identify animals in advance stage of paratuberculosis, but its use is restricted in control programmes because of uncertain result in early stage of infection. The complement fixation test (CFT) confirmed poor diagnostic potential and is no longer recommended in cattle for diagnosis (WOAH, 2022). Sensitivity of CFT is as good as of ELISA test (Kaba *et al*., 2008) in clinical cases, but is less in subclinically infected carriers. CFT

is now phased out, as its reagent preparation and standardization are laborious and time-consuming.

Agar gel immunodiffusion test: Immune complexes formed between antibodies (from serum of infected animal) and soluble antigens (from a protoplasmic extract of *Map*) in a gel matrix (agarose) and its precipitation is known as AGID. It has good diagnostic potential in advanced clinical stages in cattle, sheep (Shulaw *et al*., 1993) and goats (Rajukumar *et al*., 2001). AGID is very simple to perform, can be adopted in laboratories with limited infrastructure and resources, and has relatively low input cost, but it possesses low sensitivity in the initial stages of disease. The test is found to be highly sensitive as well as specific in clinically affected goats (Tripathi *et al*., 2006). The use of AGID tests is usually recommended for the confirmation of the advanced stage/clinical stage of paratuberculosis (Ferreira *et al*., 2002), with 96.9% of concordance with faecal culture (Sherman *et al*., 1984). The sensitivity is very good in advanced clinical stages (90% - 95%) but poor in subclinical stages of infection (30% - 18.9%) (Ayele *et al*., 2001).

Hope *et al*. (2000) found 38 to 56% sensitivity and 99 to 100% specificity for AGID. In one of the reports of Sergeant *et al*. (2003), AGID was found to be more sensitive and specific than ELISAs. The specificity of 99-100% (with a 95% confidence interval) and sensitivity of 38-56% (with a 95% confidence interval) of AGID has been determined against histological results (Hope *et al*., 2000). Tripathi *et al*. (2006) recorded the sensitivity of AGID as 96.2% in multibacillary cases and 50% sensitivity in paucibacillary cases.

Enzyme linked immunosorbent assay: Para-tuberculosis screening in the herd, most frequent by the use of various forms of ELISAs. Its diagnostic sensitivity during the early phases of the infection is low, but it is a useful tool for control programme because of its low cost, high throughput, standardized protocols and correlation with *Map* faecal-shedding levels. ELISA is most often applied in conditions where the possibility of positive results remains high, for example, animals are in clinical or advanced clinical stages with clinical signs of paratuberculosis (Nielsen *et al*., 2002; Kudahl *et al*., 2004; Nielsen and Toft, 2008). In other words, animals after 2nd or 3rd delivery or calf of age >2 years develop a humoral response, which is also correlated with the presence of bacilli in dung and/or milk and hence give good sensitivity. Milk can be used in ELISA (Milk-ELISA), because antibodies in the milk, which is moderately correlated with serum antibodies. Factors like antibody concentration in serum, milk production influenced by genetic make-up of dam, days of milk collection in lactation, number of calving, etc. (Raymond *et al*., 2006). Therefore, Milk-ELISA has lesser sensitivity than Serum-ELISA (Hendrick *et al*., 2005; Garg *et al*., 2015). To ascertain the stability of Milk-ELISA, it is suggested that milk be collected at two different times of lactation and at least two different ELISA determinations be used.

Antigens used in ELISAs are the main determinant for test's sensitivity. Commonly used antigens are protoplasmic antigen (PPA), lipoarabinomannan (LAM), p34 protein carboxy-terminal (P34-cx), PPD, heat shock proteins (Hsp), lipoarabinomannan affinity-purified antigen (APA) etc. PPA is one of the most often used antigens in ELISA (Collins *et al*., 2005; Nielsen and Toft, 2008; Gilardoni *et al*., 2012) because of the higher antigen yields (Tripathi *et al*., 2002) and ease of preparation (Koets *et al*., 2001). Accordingly, the sensitivity and specificity of ELISA vary (Köhler *et al*., 2022). In addition, age of animals, level of *Map* shedding in faeces, etc., also influence the ELSA's sensitivity and specificity. In different infection stages, sensitivity of ELISA is different: 7% in silent stage, 15% in sub-clinical stage, and 85%-98% in the clinical or advanced stage of the disease (Sweeney *et al*., 1995; Ayele *et al*., 2001; Nielsen *et al*., 2001; McKenna *et al*., 2005; Eda *et al*., 2006; Nielsen and Toft, 2008;). In different ages of animals, sensitivity of the ELISA is 1.2%, 8.9% and 11.6%, respectively, in 2, 3 and 4 years old, but remained between 20 and 30% in older age groups (Jubb *et al*., 2004). The ELISA detects about 30-40% of cattle identified as infected by a culture of faeces on solid media. However, overall actual sensitivity for all age groups is around 15% (Whitlock *et al*., 2000). Commercially available ELISA kits have variable sensitivity; the range varies between 9-32% for low bacilli-faecal shedders and between 47-63% for moderate bacilli-faecal shedders (Whitlock *et al*., 2000).

Uses of reference parameters for determining the efficacy of tests also influence the sensitivity and specificity. Taking histological results as a reference, the specificity and sensitivity of an in-house ELISA were 99% and 21.9%, respectively (Sergeant *et al*., 2003). In the same way, a comparison of commercial ELISA kits for JD illustrated that the assays performed comparably overall, with diagnostic sensitivity ranging from 27.9 to 44.5% for faecal culture-positive cattle (Collin *et al*., 2005). In herds that appear healthy or have a low prevalence, a bacterial culture should be

performed to confirm the infection stage if ELISA produces positive results. In six to twelve months, ELISA should be reexamined if the results are negative (Tiwari *et al*., 2006). In herds with low prevalence, ELISA combined with bacterial culture yields a high sensitivity (Adaska *et al*., 2002; Nielsen *et al*., 2002; Nielsen and Toft, 2008; Nielsen, 2008), and hence, it is the most reliable diagnostic strategy.

Production of *Map*-specific immunoglobulin isotypes switches during the disease progression (Koets *et al*., 2001) with Th1 responses being related to IgM and IgG2, and Th2 responses being related to IgG1 (strong correlation with clinical signs of JD) and IgA in cattle (Estes and Brown, 2002). Accordingly, diagnostic meant for detecting specific IgG1 yields superior diagnostic value (sensitivity and specificity) in clinical paratuberculosis. For example, conventional PPA-ELISA, detecting IgG, is to identify clinically infected animals and PPA-ELISA, detecting IgG2, is better for subclinically infected cattle (Fernandez *et al*., 2012).

Speer *et al*. (2006) designed a type of an ELISA known as SELISA by adsorbing ELISA plates with *Map* antigens that had been subjected to formaldehyde treatment and sonication. This allowed them to attain 100% specificity and 96% sensitivity in calves that had been experimentally infected or low shedders in particular. Ethanol Vortex-ELISA (ELISA) is another variation in ELISA where antigens from *Map* bacilli were extracted by ethanol (Eda *et al*., 2006). Here, the stability of antigens was increased, and hence, the ELISA plate prepared could be safely stored for up to 7 weeks without a change in specificity or/specificity. Species-specific lipid molecules in ethanol extract were surface lipopeptides (e.g., Para-LP-01). Eckstein *et al*. (2006) claimed the high sensitivity of EVELISA. The author classified cattle that tested positive for faecal culture as low (less than 10 colonies), moderate (10 to 50 colonies), or high (more than 50 colonies) shedders based on the results of their blood sample testing by EVELISA. Further, the sensitivity of EVELISA was also evaluated as 96.6% (for low shedders) and 100% (for middle and high shedders) in these categories of animals. However, some instances of serological false-positive reactions have been found four years later. Antigens from numerous environmental mycobacteria are responded to antibodies, revealing the presence of cross-reaction. This has been resolved by absorption (EVA-ELISA) of sera with *M. phlei* antigens. further, its sensitivity and specificity of EVA-ELISA has been estimated as 97% and 100%, respectively. This has proven that the pre-absorption step increased the test's sensitivity and specificity by eliminating the possibility of cross-reaction (Milner *et al*., 1987; Milner *et al*., 1990). The absorbed ELISA, designed by Yokomizo *et al*. (1983; 1985) and modified by Milner *et al*. (1988), was developed into a commercial kit by Cox *et al*. (1991).

Most of the time, ELISA uses protoplasmic antigen as a coating antigen; therefore, possible exposure to immune cells is lower in comparison to secreted proteins. Taking this difference as the basis of ELISA, a JTC-ELISA was designed for the diagnosis of paratuberculosis. Here, coating antigens were made using secreted proteins of low passage JTC-303 strain of *Map* in the early to mid-stage of growth on liquid media. The performance of JTC-ELISA is good compared to other commercial ELISA available at that time (Shin *et al*., 2008), both in serum and milk in the sub-clinical stage of infection. Alanine and proline-rich antigen (Apa) has been confirmed its presence in feces of JD infected cattle; it is a secretory antigen of *Map* bacilli. An ELISA directed toward the Apa antigens has been assessed in faecal samples and claims to be of good diagnostic value with good specificity (de Souza *et al*., 2018). ELISA using host biomarker viz. SPARC (secreted protein acidic and cysteine rich), ABCA13 (ATP binding cassette subfamily A member 13) and MMP8 (Matrix metallopeptidase 8) reliably identify infection, greatly improving early detection when antibody responses and faecal shedding are undetectable (Blanco-Vazquez *et al*., 2020).

ELISA with recombinant proteins (Chimeric poly protein) *viz*. MAP0038, MAP0209c, MAP2513c, and MAP1589c confirmed better diagnostic efficacy and lower cross-reactivity in comparison to protoplasmatic antigen (Moyano *et al*., 2021). Recombinant protein cocktail of seven antigens *viz*. *Map*2513, *Map*1693, *Map*2020, *Map*0038, *Map*1272, *Map*0209c and *Map*0210c used in ELISA resulted in good sensitivity without cross reactivity (Mon *et al*., 2012). Similarly, six antigenically distinct recombinant *Map* cell envelope proteins (SdhA, FadE25_2, FadE3_2, Mkl, DesA2, and hypothetical protein *Map*1233) as well as an extract of *Map* total cell envelope protein, were demonstrated good diagnostic potential (Karuppusamy *et al*., 2021). Lipopentapeptide (L5P, or Lipopeptide-I or Para-LP-01) characterized from C-type (bovine) strains, when incorporated in ELISA, its sensitivity is low as compared to commercial ELISA, but no cross-reactivity has been established even with *M. avium* subsp. *hominissuis* (Bay *et al*., 2021).

Conductometric biosensor and bioelectronics sensor: Conductometric biosensor and bioelectronics sensor are same in basic architecture; only author preference to name is different. PPD from *M. avium* was used in conductometric biosensor with assumption that both *Map* and *M. avium* produced identical PPD, whereas PPA from *Map* was used in bioelectronics sensor. Tracing conjugate was prepared by coating species specific antibody on electrically active polyaniline coated magnetic (EAPM) nanoparticles. A polyaniline (Pani)-based conductometric biosensor in an immunomigration configuration was designed by Okafor *et al*. (2008) to identify serum IgG against *Map*. Immobilized *M. avium* purified proteins in the capture membrane were used to detect *Map* IgG. Similarly, Karthik *et al*. (2013), with PPA in bioelectronics sensor, evaluated the sensitivity and found that the new test was as sensitive as absorbed ELISA and faecal PCR. In addition, it was performed within 2 min and can be incorporated at the point of care.

Test based on surface-enhanced Raman scattering: Roughened metal surfaces improve the Raman scattering of an adsorbed organic molecule in surface-enhanced Raman scattering (SERS). The primary cause of this improvement is an increase in the electromagnetic field at the roughened coinage metals' (silver or gold) nanometric asperities. Same process worked at the surface of metallic nanoparticles. Surface-enhanced Raman scattering along with potential contributions from chemical effects, an enhancement of up to 10^{14} was determined (Kneipp *et al*., 2002). Extrinsic Raman labels (ERLs) incorporate the intrinsically strong Raman scattering of aromatic compounds (reporter molecules). Yakes *et al*. (2008a; 2008b) explore the potential of surface-enhanced Raman scattering (SERS) to detection of *Map* at low levels. Here, organic molecule was first coated on the gold nanoparticles followed by tethering of antibody (a molecular recognition element) to the organic molecule coated nanoparticle surface. Similar principles were used to prepare the capture substrate with another specific antibody against *Map* bacilli. Presence of amine residues throughout the protein's structure indicates a more random distribution of orientations (Dong and Shannon, 2000). Lastly, by removing the antigen from solution, labelling the captured antigen with ERLs and quantifying the tagged antigen using SERS, a sandwich immunoassay can be completed in less than 24 hours.

Compared to conventional signal transduction techniques like radioisotope decay, colorimetry and fluorescence, SERS detection offers a number of advantages. First, excitation in the red spectral range is employed when using gold nanoparticles; this reduces the possibility of interference from native fluorescence. Secondly, SERS intensity for immobilized molecules is starting to resemble fluorescent dyes and also have a lower photobleaching susceptibility. Lastly, there is less chance of spectral overlap from several labels because the widths of Raman spectral bands are 10 to 100 times less than those of fluorescence.

Bead-based microfluidic immunoassay: Ethanol extracted antigens of *Map* were coated on magnetic bead, used to capture the antibody if present in animal sera and hence whole complex was traced by secondary species-specific antibody conjugated with florescent dye. Solution containing beads was subjected to flow cytometric analysis and microfluidic analysis (Wadhwa *et al*., 2012). The result obtained with known bovine samples (both positive and negative) was promising in comparison with commercially available immunoassay. Similarly, multiplex bead-based assays were designed and found superior in the diagnosis of paratuberculosis in comparison to ELISA, even in early detection (Li *et al*., 2017). In another design, the detection of HRP reaction with its substrate may be measured quantitatively electrochemically using a redox-active probe, ferrocyanide. When compared to a traditional colorimetric testing method, the novel electrochemical detection approach could discriminate samples of *Map*-infected calves from those of uninfected cattle with a higher separation between the two sets of samples (Hatate *et al*., 2021).

Molecular detection

PCR assay: PCR assay is an advanced technique for precise detection of genomic sequence of concern. Colostrum, milk, excrement and tissues from the ileocecal valve, ileum, jejunum, or ileocecal lymph nodes can all be used as samples (Buergelt and Williams, 2004). Some sample components may inhibit Taq polymerase, leading to false negative results, and hence, due consideration is given before interpretation of test results (Tiwari *et al*., 2006). It has been discovered that the IS*900*-based PCR is a quick and accurate way to find *Map* in cultures, feces, blood and tissues (Tripathi *et al*., 2006; Singh *et al*., 2010). Direct PCR on feces has been used for paratuberculosis diagnosis in cattle flock (Whipple *et al*., 1991). In general, faecal PCR tests were found to be less sensitive than faecal culture but offered great advantage in terms

of rapidity of results without assessment of viability of bacteria in sample. Sensitivity of faecal culture has been reported to be 10^2-10^3 bacilli/g, whereas, those of faecal IS*900* PCR was 10^4 bacilli/g in bovines (Whipple *et al*., 1992). Culture slant (CS)-PCR offered some advantages (Mohan *et al*., 2013) over the antemortem faecal testing as well as postmortem tissue testing (Woodbury *et al*., 2008). PCR is now a day used in various formats in addition to traditional ones, *viz*; nested PCR, multiplex PCR, real-time or quantitative PCR (qRT-PCR), Triple real-time PCR (TRT-PCR) etc.

Nested PCR assay: Nested PCR assay performs two-times amplification of the target sequence with two different primer sets in a successive manner, thus allowing increased sensitivity of the reaction as well as quick (Erume *et al*., 2001). First amplification in nested PCR generates the site for the second pair of primers. Vansnick *et al*. (2004) applied two sets of primers both for IS*900* and *f57* regions of *Map* and achieved a sensitivity of 1CFU/PCR assay for both amplifications. Similarly, nested PCR for ISMap02 region has been established with comparable sensitivity to conventional PCR and qPCR (Stabel and Bannantine, 2005; Pithua *et al*., 2010; Soltani, 2018). The ISMap02 element demonstrated a detection sensitivity of 7.6 fg/µL of the standard genomic DNA and was specific for *Map* only (Rani *et al*., 2018). This test is now adopted for different samples in different animals for the diagnosis of JD (Okuni *et al*., 2013; Correa-Valencia *et al*., 2017; Sadeghi *et al*., 2020).

Real-time or quantitative PCR assay: Two types of chemistry are used in Real-time, also known as quantitative PCR (qRT-PCR). One technique uses SYBR green (Brand name of N', N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene) methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine) which will bound to PCR products and the second method detects the cleavage of fluorogenic (brand name: Taqman) probe bound to a target sequence during primer extension phase. Each PCR cycle's fluorescence emission is measured, and this measurement is correlated with the sample's DNA content. Both the assays are sensitive and able to detect 3-4 fg of DNA from *Map* strains (Ravva and Stanker, 2005). These assays can also quantify cells directly from *Map* cultures without any extractions or processing and detect 0.07 cells per assay. Media ingredients do not interfere with the assay. The sensitivity of direct detection was ten times more than that of extracted DNA detection. These assays are helpful in recognition and differentiation of *Map* from other mycobacteria. The benefit of qRT-PCR is that, it is more sensitive and enables instantaneous monitoring of the target's amplification and quantification in comparison to bacterial culture (Bogli-Stuber *et al*., 2005; Rajeev *et al*., 2005) or gross- and histopathological examination (Acharya *et al*., 2017; Hamid *et al*., 2018) or PCR (Selim and Gaede, 2015; de Albuquerque *et al*., 2017), ELISA (Sonawane and Tripathi, 2013). Even qPCR has shown similar results with optical densitometer enumeration of *Map* bacilli (Kralik *et al*., 2012). Since, IS*900* is contained in multiple copies across the bacterial genome; therefore, IS*900* sequence amplification in qPCR is not accurate for quantification of CFU of *Map* bacilli in the sample. However, qRT-PCR was a highly sensitive test in comparison to conventional PCR (Jae-Ik *et al*., 2015), ELISA and bacterial culture on infected tissues, especially from paucibacillary paratuberculosis in sheep (Sonawane and Tripathi, 2013; Mathevon *et al*., 2017).

When using *f57* in qPCR, it was somewhat less sensitive than our main IS*900*-system and did not provide any false positive results (Herthnek and Bolske, 2006). The application of qRT-PCR using the insertion sequences IS*Mav2* showed a 76% and 4% sensitivity in faeces samples, respectively from animals belongs to large and low/moderate faecal shedder groups (Wells *et al*., 2006). Similar findings were also confirmed by Alinovi *et al*. (2009). Schonenbrucher *et al*. (2008) developed and validated a TaqMan qPCR with target sequence of *f57* and the ISMav2 in bovine faecal samples. Further, in comparison to TagMan from six different suppliers, the detection probability of *Map* was 100% achieved, and it was 0.1pg *Map* DNA per PCR. The three-copy insertion sequence ISMav2 had an amplification efficiency of 97.8%, while the single-copy gene *f57* had an efficiency of 98.2%. The selection of *Map*-specific Mptb52.16 target in qPCR provides the additional possibility of differentiation of viable and dead bacilli in samples (Dzieciol *et al*., 2010).

Multiplex PCR assay: Multiplex PCR amplify multiple target sequences simultaneously in the same reaction mixture with the help of multiple primer sets. It is used to identify and differentiate mixed infections, viz. MAH (*M. avium* subp. *hominissuis*), MAA (*M. avium* subp. *avium*), MAS (*M. avium* subp. *silvaticum*), etc., by simultaneously amplifying different sequences like IS*900*, IS*901*, IS*1245*, *dnaJ* gene, etc., (Moravkova

et al., 2008). Additionally, it improves the test's specificity by combining data from multiple loci in a single reaction. Simultaneous amplification of IS*6110* of *M. bovis* and IS*900* of *Map* in duplex technique with detection limits of 92.98 pg and 110.27 pg, respectively, has been achieved (Zhang *et al*., 2013).

Irenge *et al*. (2009) designed a Triple real-time PCR (TRT-PCR). Here, target sequences were IS*900*, *f57* and IS*Map02* and were used in faecal samples. In comparison to bacterial culture and ZN staining, the test demonstrated a better sensitivity with a detection limit of $2.5x10^2$ CFU/g of feces. Faecal samples from animals in the subclinical stage can be evaluated quickly and precisely using this technique, but it needs to be validated using a large number of samples. Similarly, Schonenbrucher *et al*. (2008) achieved 94.4% sensitivity from spiked bovine faecal samples with 98.9% accuracy by TRT-PCR.

Loop-mediated isothermal amplification (LAMP) assay: Another variant of PCR amplification system is the loop-mediated isothermal amplification (LAMP). It is devoid of the need for a thermocycler (Enosawa *et al*., 2003), the process is also not lengthy, and the test has high diagnostic value. Therefore, the LAMP assay is a comparatively cheaper test. Final detection of product can be assessed by the turbidity in the vial since the by-product (Magnesium pyrophosphate) accumulates in reaction mixture that imparts the turbidity (Mori *et al*., 2001). Additionally, products can be observed under UV light or more precisely, in agarose gel following restriction enzyme digestion (Notami *et al*., 2000). Enosawa *et al*. (2003) designed a LAMP to identify IS*900*, *HspX* and *f57* in samples from culture of *M. avium*, *M. bovis*, *M. intracellulare*, *M. kansasii*, *M. scrofulaceum* and *M. smegmatis*. The result was obtained within 2 hrs, and the sensitivity of the reaction was 2 pg DNA/tube. This test has also been adopted for milk samples and has shown a perfect agreement with culture, microscopy, and PCR, as well as early detection (Singh *et al*., 2020). In some cases, the results were also completed within 1 hrs (Safi *et al*., 2015). The detection limit of 10 fg DNA/µL has been achieved by LAMP (Punati *et al*., 2019; Singh *et al*., 2020). Likewise, LAMP technology has been utilized to detect *Maps* via ISMap02, and it has been shown to be suitable for good sensitivity and specificity (Sange *et al*., 2019).

LAMP technique in lateral flow device (LAMP-coupled LFD) has also been incorporated for the recognition of *Map* at point of care with limted resources. When compared to the culture, the LAMP-coupled LFD assays achieved 100% sensitivity and 97.02% specificity, respectively (Punati *et al*., 2019). The coupled assay's specificity was 100% and its detection limit was 10 fg/µL. This technique was successful in detecting *Map* in both faecal samples and purified bacterial DNA.

Bioluminescent assay in real-time loop-mediated isothermal amplification (BART-LAMP) test: Detection of LAMP product was improved by incorporation of heating block containing a photo-diode light detection system (Gandelman *et al*., 2010). It was a computer-assisted real-time detection system. The reaction mixture contains ATP sulfurylase (APS) and a heat-tolerant firefly luciferase/luciferin. Upon pyrophosphate synthesis, it reacts with the APS to release ATP, which results in a very strong light signal resulting from the luciferase and hence detected by detection system.

Basra (2013) uses mycobacteriophage (D25 and FF47) for rapid identification of *Map*. It was based on the assumption that phages, within 9 min, release their progeny in huge amounts after infection with their host. Detection of phage genome indirectly can correlate with the presence of host bacilli. He combined the BART-LAMP with mycobacteriobpage for fast detection of *Map*. The result summarizes that the lowest concentration of DNA detected by FF47 was 100pg/µL from pure DNA (10^7 PFU/mL equivalent from crude DNA), whereas D29 detected 1pg/mL from pure DNA (10^4 PFU/mL equivalent from crude DNA).

Recombinase polymerase amplification technique: Recombinase polymerase amplification (RPA) is an isothermal DNA amplification technology, best operating at temperatures of 37-42°C and can work at room temperature. Therefore, all the steps of DNA amplification in this technique can be performed in a single tube at a constant temperature (Piepenburg *et al*., 2006). The RPA assay is quicker and much easier to handle than real-time PCR. All RPA reagents are cold-chain independent. With a simple sample DNA extraction procedure, RPA may be incorporated at the point of care. With the use of the IS*900* gene, Hansen *et al*. (2016) were able to detect 16 DNA molecules in 15 minutes. There was no evidence of cross-reactivity with other mycobacterial strains or environmental-associated bacterial strains. The *Map* RPA assay had 100% specificity and 89.5% sensitivity when compared to the qPCR assay. In another study, a combination of the RPA technique and lateral flow dipstick (LFD) assay was able to detect DNA in 30 min

at 39°C with a detection limit of up to 8 copies/reaction, which was equivalent to that of qPCR assay. In comparison with the qPCR, the RPA-LFD assay showed 100% sensitivity, 97.63% specificity and 98.44% concordance rate (Zhao *et al*., 2018).

PCR along with enrichment of Bacilli: Since shedding of bacilli in milk and feces are erroneous, particularly in milk, its amount is low to detect in PCR. Therefore, an enrichment of bacilli is required to make it up to the detection level. Different researchers use different strategies to enrich bacilli in milk. Grant *et al*. (2000) use an immunomagnetic PCR (IMS-PCR) technique for the detection of *Map* in milk (Gilardoni *et al*., 2016). IMS is nothing but magnetic beads coated with *Map* specific antibody. Magnetic property of beads helps in separation of attached bacilli on beads from rest of clinical samples. It may bypass the decontamination and additionally enrich the sample with bacilli. The specificity and sensitivity of IMS-PCR were found to be 95% and 100%, respectively. Sensitivity of PCR without enrichment was 23% (Grant *et al*., 2000). This method's advantage is in its higher specificity, which was contributed by microorganism's concentration, which enables good assay reproducibility and removes any potential Taq polymerase inhibitors from the samples (Grant *et al*., 2000; Metzger-Boddien *et al*., 2006). Similarly, with help of chicken-IgY coated beads, IMS capture assay was performed. Its detection level for *Map* bacilli in spiked bovine faeces was 200 cells/ assays (Chui *et al*., 2010).

Apart from use of immunoglobulin specific for *Map* (Khare *et al*., 2004; O'Mahony and Hill, 2004) for spiking of samples, other like peptide-mediated capture (Stratmann *et al*., 2002) followed by PCR can be adapted to high-throughput testing using standard laboratory automation. Use of phage display-derived peptide, aMptD (Stratmann *et al*., 2004) for the capture of *Map* in milk samples was applied (Stratmann *et al*., 2006). aMptD peptide binds MptD protein (surface-exposed protein) of *Map* (ORF 3733C) (Li *et al*., 2005); this protein belongs to *Map*-specific pathogenicity island (Stratmann *et al*., 2004) and hence method designed with aMptD was the defined receptor-ligand interaction (aMptD-MptD interaction). Another way to concentrate the bacilli is via the use of magnetic nanoparticles with immunoglobulin; it enhances the diagnostic efficacy of PCR with rapid identification and is cost-effective (Kim *et al*., 2016). A rhodamine-hydrazone-immunosensor was designed to detect *Map* in samples (feces, milk and colostrum), which was enriched by immunomagnetic beads. Here, combination of separation enrichment and imunosensor was fast, sensitive, as well as cost-effective in detecting *Map* in bovine faeces, milk and colostrums (Khosravi *et al*., 2021).

Another method uses the conjugation of recombinant lysin Gp10 (derived from the mycobacteriophage L5) with magnetic beads, and its use in the separation and concentration of bacilli from complex media was demonstrated by Singh *et al*. (2014). This approach along with qPCR amplification removes the requirement for protracted culture, permits *Map* cell pre-concentration, and gets rid of different PCR inhibitors. As a result, it provides a general decrease in the amount of time needed to test milk for *Map*.

Hybridizing magnetic relaxation nanosensors: A polymer-coated iron oxide nanoparticle on which affinity ligands are attached to enable binding and magnetic detection of a specific target makes up magnetic nanosensors (Kaittanis *et al*., 2009; 2011). The sample's magnetic resonance signal is altered by a target's particular binding to ligands on the magnetic nanoparticle; this is shown in the water proton relaxation time, which is correlated with the target concentration in the solution. Measurement of the changes in proton relaxation times by a sensitive detection method called magnetic relaxation nanosensors (MRS). Use of MRS binding-based assays with low-valency nanoparticle system or in another words, hybridization of these two results in development of more sensitive assay called Hybridizing-Magnetic-Relaxation-Nanosensors (hMRS). When used to diagnose the *Map* genome in various clinical samples, hMRS worked noticeably faster than traditional culture approaches. Furthermore, the intracellular bacterial target in clinical samples will be detectable at the genomic and epitope levels using a hMRS and one nanoparticle preparation (Kaittanis *et al*., 2012).

Fluorescent semiconductor quantum dots (QDs) and magnetic beads (MBs): Through a sandwich hybridization reaction, two biotinylated oligonucleotide probes are utilized to identify and detect particular complementary mycobacterial target DNA. To provide a fluorescent signal, streptavidin-conjugated cadmium selenite QDs and species-specific probes are utilized. To separate and concentrate the DNA targets in samples, however, MBs conjugated with streptavidin and genus-specific probes are employed. Florescent emitted by quantum dot after

excitation with UV light is analyzed in terms of target sequence. The assay's minimal detection limit was established at 12.5 ng of diluted DNA in a 20 µL sample volume. By avoiding DNA amplification, the suggested diagnostic technique provides a straightforward, quick, accurate and economical way to directly detect and identify mycobacterial DNA in clinical samples (Gazouli *et al*., 2010).

Use of mycobacteriophage for detection of *Map*: An established method for the quick identification of human tuberculosis in sputum is the FAST Plaque TB assay (FPTB) (Rees and Loessner, 2005). In brief, phage is combined with samples containing *M. tuberculosis* cells and incubated to allow for infection. At this stage, a virucide is added to ensure that only phages protected by the host cell survive. It destroys any phages that have not infected cells. The sample is combined with *M. smegmatis* and agar to create a lawn, and plates are then incubated for an entire night in order to identify protected phage. Phages are released with the lysis of the infected target cell, and they subsequently infect *M. smegmatis* cells to create plaques. In human sputum, this is most likely *M. tuberculosis*. Each plaque indicates the presence of a mycobacterial cell that is capable of being infected by the phage in the original sample. The fact that the mycobacteriophage (D29) utilized in this experiment can infect different kinds of mycobacterial cells in addition to *M. tuberculosis* is an important aspect of it. Stanley *et al*. (2007) successfully used FPTB assay reagents to detection of *Map*. Further, to increase the specificity of FPTB assay, PCR-based identification method was incorporated in the system. Similarly, for differentiation, a multiplex PCR method was incorporated in the system. The combination of plaque PCR and phage-based detection assay confirmed the *Map* in 48 hrs.

Similarly, Foddai *et al*. (2009) optimized the procedure of a commercialized FPTB involving D29 mycobacteriophage to permit accurate counting of *Map* in milk. Phage amplification's primary benefit is its ability to count live cells in 24 to 48 hours. Furthermore, since the D29 phage would only infect live mycobacterial cells, there is no need to perform chemical cleaning of the sample prior to the phage assay, increasing the test's sensitivity for detection. For these reasons, when conducting inactivation tests with samples that have been spiked with *Map*, the optimized phage amplification approach may be utilized to expedite the acquisition of findings. For inactivation research employing *Map*-spiked samples, an optimized phage assay can be used in place of traditional culture on HEYM to expedite the acquisition of findings (48 hours as opposed to at least 6 weeks) (Foddai *et al*., 2010a).

A PMS-phage (peptide-mediated magnetic separation-phage) assay to rapidly detect viable *Map* was used (Foddai *et al*., 2009; Foddai *et al*., 2010b). The phage amplification assay allows for the quick enumeration of viable *Map* within 24 hours; before that, PMS efficiently removes the great majority of contaminating microorganisms from a sample by selectively capturing and concentrating *Map* (Foddai and Grant, 2020). In artificially contaminated milk samples, PMS and the phage amplification assay combination are selective for low numbers of live *Map*, negating the requirement for chemical cleaning (Foddai *et al*., 2010b). In comparison to culture with 0.75% with HPC, PMS-phage assay detected more number of viable *Map* (Foddai *et al*., 2011). However, feces cannot be directly subjected to the phage amplification assay because an unidentified component of feces suppresses phage infection, preventing the development of plaque. PMS-phage assay may be used as a diagnostic tool if fresh milk and faeces are used, but it needs further validation.

The phagebead qPCR (PBQ) based assay is another modification of technique in which phages are legend to magnetic beads. The limit of detection (LOD) of PBQ was superior and has been achieved 10 *Map* per 10 mL milk as compared to PMS-phage assay (100 cells/10 mL). Therefore, to check the viability of *Map* bacilli in milk, PBQ and PMS-phage could be promising techniques. Nevertheless, PBQ was preferred over the PMS-phage test because of its lower limit of detection (LOD), speed, increased sensitivity, and lack of requirement for *M. smegmatis* and the ensuing virucidal treatment, which are crucial for the PMS-phage assay's ability to produce lawn and inactivate exogenous mycobacteriophages, respectively (Hosseiniporgham *et al*., 2022).

Conclusions

Now-a-days, it is possible to diagnose the viable *Map* within one day. In PCR format, detection of mRNA (RT-PCR) of IS*900* sequence is possible in one day. Fabrication of RT-PCR with lateral flow assay or microtiter plate assay, detection of viable organism can be done in 10 h.

Antigens, like PPA, LAM, P34-cx, PPDp, Hsp, ethanol, extract, APA, recombinant proteins etc., were evaluated in different serological tests including ELISA but, none of them is accurate enough to use

with certainty. Absorption of sera with *M. phlei* antigens will remove cross-reacting antibodies and improve the specificity of test but decreased the test's sensitivity. Serological tests use either PPD or PPA as antigen to capture antibodies in different techniques. These diagnostics in subclinical stage are not appropriate.

Molecular tests circumvent both the long incubation of culture and low sensitivity. IS*900* was the most often used target, but it was reported that environmental mycobacteria also have IS*900* and IS*900* like sequence in their genome. Use of more than one target in single reaction or nested approach for single sequence was tried with success. Different formats, like LAMP, qRT-PCR, TRT-PCR, etc., were designed with improved specificity and sensitivity. Enrichment of samples will improve the sensitivity of test. The most common method of enrichment is IMS. They used antibody against PPA. Some of the researchers also tried more refined antigen (aMptD) with increased specificity.

Broad spectrum mycobacteriophage (D29) was used to detect viable *Map* bacilli. FPTB with modification is able to detect bacilli within 48 h from spiked milk sample. Similarly, incorporation of IMS with Phage assay detects viable *Map* within 24 h. Therefore, rapid diagnostics are available with high sensitivity and specificity. Main thing is to adopt the most appropriate test in control programme that should be economically viable.

Conflict of interest: The authors declare that there is no conflict of interest regarding the publication of this review article.

Author's contributions: AM: Has collected the required research or review article and also prepared the outline of the manuscript; NK: Did the needful correction as per the journal guideline and final editing of the manuscript.

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