Review Article

Trends and advances in the diagnosis and control of paratuberculosis in domestic livestock

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ABSTRACT
Paratuberculosis (pTB) is a chronic granulomatous enteritis caused by Mycobacterium avium subsp. paratuberculosis (MAP) in a wide variety of domestic and wild animals. Control of pTB is difficult due to the lack of sensitive, efficacious and cost-effective diagnostics and marker vaccines. Microscopy, culture, and PCR have been used for the screening of MAP infection in animals for quite a long time. Besides, giving variable sensitivity and specificity, these tests have not been considered ideal for large-scale screening of domestic livestock. Serological tests like ELISA easily detects anti-MAP antibodies. However, it cannot differentiate between the vaccinated and infected animals. Nanotechnology-based diagnostic tests are underway to improve the sensitivity and specificity. Newer generation diagnostic tests based on recombinant MAP secretory proteins would open new paradigm for the differentiation between infected and vaccinated animals and for early detection of the infection. To higher seroreactivity of secretory proteins vis-à-vis cellular proteins, the secretory proteins may be used as marker vaccine, which may aid in the control of pTB infection in animals. Secretory proteins can be potentially used to develop future diagnostics, surveillance and monitoring of the disease progression in animals and the marker vaccine for the control and eradication of pTB.

1. Introduction

Mycobacterium avium subsp. paratuberculosis (MAP) is a slow-growing, obligate intracellular fastidious pathogen, with the ability to survive in a wide range of environmental conditions (Deb & Goswami 2011). MAP infection in animals leads to paratuberculosis (pTB) or Johne’s disease (JD) in a wide variety of domestic and wild life species (Table 1) (Haghkhah et al. 2008; Gupta et al. 2012; Sonawane & Tripathi 2013; Vinodhkumar et al. 2013; Singh, Singh, et al. 2014). Increasing evidence suggest the association of MAP with human diseases such as Crohn’s disease, diabetes type I, and thyroiditis (Sisto et al. 2010; Singh, Chauhan, et al. 2012; Singh, Thakur, et al. 2014). Live MAP bacilli have been detected in food products such as paneer, milk powder (both fresh and pasteurized) and milk powder (Table 1).

pTB is a chronic granulomatous enteritis characterized by weight loss, infertility, reduced productivity and productivity, progressive emaciation and untimely death, resulting in huge economic losses to the livestock industries worldwide (Hutchinson 1996; Johnson-Ifearulundu & Kaneene 1997; Ott et al. 1999; Otte & Chilonda 2000; Groenendaal et al. 2002; Groenendaal 2005; Hasonova and Pavlik 2006; Vinodhkumar et al. 2013; Rawat, Chaudhary, Kumar, et al. 2014). Control and eradication of pTB is difficult due to its insidious nature, long incubation period and lack of rapid and accurate diagnostic tests.

Early lesions following pTB infection occur in the wall of the small intestines (Sigurdardottir et al. 1999) and the draining mesenteric lymph nodes since infection is localized to these sites in the early phase (Saxegaard & Fodstad 1985). As the disease progresses, gross lesions may be observed in the jejunum, ileum, caecum, colon, and in the mesenteric lymph nodes where the organism can be detected. Lesions in the intestines result in protein leak and a protein malabsorption syndrome, ultimately leading to wasting especially muscle tissues (Maroudam et al. 2015). The disease can occur in animals at any age over 1–2 years. However, it is most frequently reported in the 3–5 years of age group in dairy cattle. Within a few weeks following infection, multiplication of MAP begins...
Table 1. Host range of *Mycobacterium avium* subspecies *paratuberculosis*.

| No. | Species | Country | References |
|-----|---------|---------|------------|
| 1   | Goat (*Capra egagrus hircus*) | India | Singh et al. (1996), Singh, 1998, Singh, Singh, et al. (2014) |
|     |         |        | Williams et al. (1979) |
| 2   | Sheep (*Ovis aries*) | Spain | Aduriz et al. (1993) |
|     |         | India | Singh, Singh, et al. (2012, 2014) |
|     |         |        | Shroff et al. (2014) |
| 3   | Cattle (*Bos taurus*) | USA | Chiodini et al. (1984) |
|     |         |        | Benedictus et al. (1988) |
|     |         | India | Singh SV, Singh AV, et al. (2007), Singh, Singh, Singh, Gupta, Chaubey, et al. (2013), Singh, Singh, et al. (2014) |
|     |         | Czech and Slovak Republic | Pavlas et al. (1997) |
|     |         | Iran | Haghkhah et al. (2008), Sharmordi et al. (2008) |
| 4   | Buffalo (*Bubalus bubalis*) | India | Singh, Singh, et al. (2014); Yadav et al. (2008) |
|     |         | Italy | Desio et al. (2013) |
| 5   | Camel (*Camelus dromedarius*) | Saudi Arabia | Ailuuwai (2008, 2015) |
|     |         |        | Alhebab and Ailuuwai (2010) |
|     |         |        | Hussain et al. (2015) |
| 6   | Pigs (*Sus domesticus*) | Portugal | Miranda et al. (2011) |
|     |         |        | Other domestic animals |
| 7   | Dog (*Canis lupus familiaris*) | Hungary | Ganemann et al. (2008) |
| 8   | Cat (*Felis catus*) | USA | Palmer et al. (2005) |
|     |         | Portugal | KuKanich et al. (2013) |
|     |         |        | Wild ruminants and other animals |
| 9   | Fallow deer (*Dama dama*) | Czech Republic | Pavlik, Pavlas, et al. (1994), Pavlik, Bärtl, et al. (2000) |
|     |         | Germany | Von Weber and Gurke (1992), Von Weber et al. (1992) |
|     |         | USA | Riemann et al. (1979), Tempel et al. (1979) |
| 10  | Axis deer (*Axis axis*) | USA | Riemann et al. (1979) |
| 11  | Tule elk (*Cervus elaphus nannodes*) | USA | Jessup et al. (1981) |
| 12  | European red deer (*Cervus elaphus*) | Ireland | Fawcett et al. (1995) |
|     |         | Scotland | Power et al. (1993) |
|     |         | New Zealand | De Lisle et al. (1993) |
|     |         | Canada | Rohonczy et al. (1996) |
| 13  | White-tailed deer (*Odocoileus virginianus*) | USA | Chiodini and Van Kruiningen (1983) |
| 14  | Roe deer (*Capreolus capreolus*) | Czech Republic | Pavlik, Bärtl, et al. (2000) |
|     |         | USA | Temple et al. (1979) |
| 15  | Sika deer (*Cervus nippon*) | USA | Manning et al. (1998) |
| 16  | Elk (*Cervus elaphus*) | Canada | Rohonczy et al. (1996) |
|     |         |        | Other domestic animals |
| 17  | Moose (*Alces alces*) | USA | Soltys et al. (1967) |
| 18  | Pudu (*Pudu pudu*) | Belgium | De Meurichy et al. (1985) |
| 19  | Antelope kudu (*Tragelaphus strepsiceros*) | Czech Republic | Pavlas et al. (1997) |
| 20  | Feral goats (unspecified) | New Zealand | Ris et al. (1988) |
| 21  | Pygmy goat (*Capra hircus*) | Germany | Von Weber et al. (1992) |
| 22  | Rocky Mountain goat (*Oreamnos americanus*) | USA | Williams et al. (1979) |
| 23  | Capricorn (*Bhe bhe*) | Germany | Von Weber et al. (1992) |
| 24  | Capricorn (*Cervus cylindricornis*) | Czech Republic | Pavlik et al. (1999) |
| 25  | Moufflon (*Ovis musimon*) | Czech Republic | Pavlik, Bärtl, et al. (2000) |
|     |         | USA | Boever and Peters (1974) |
|     |         | Germany | Von Weber et al. (1992) |
|     |         | USA | Boever and Peters (1974) |
|     |         |        | Other domestic animals |
| 26  | Aoudads (*Ammotragus lervia*) | USA | Von Weber et al. (1992) |
| 27  | Bighorn sheep (*Ovis canadensis*) | USA | Williams et al. (1979) |
| 28  | Cameroon sheep | USA | Williams et al. (1979) |
| 29  | Jimela topi (*Oryctolagus cuniculus*) | Germany | Steinberg (1988) |
| 30  | Sicilian ass | USA | Dierckins et al. (1990) |
| 31  | Pygmy ass (*Equus asinus form. dom.*) | The Netherlands | Van Ulsen (1970) |
| 32  | Alpaca (*Lama pacos*) | Australia | Ridge et al. (1995) |
| 33  | Bactrian camel (*Camelus bactrianus*) | USA | Thoen et al. (1977) |
| 34  | Rabbit (*Oryctolagus cuniculus*) | UK | Greig et al. (1997, 1999) |
|     |         | India | Singh, Singh, et al. (2012) |
| 35  | Fox (*Vulpes vulpes*) | UK | Beard et al. (1999) |
| 36  | Stoat (*Mustela erminea*) | UK | Beard et al. (1999) |
| 37  | Stumptail macaques (*Macaca arctoides*) | USA | McClure et al. (1997) |
| 38  | Wild Bison (*Bos gaurus*) | India | Singh, Singh, Singh, et al. (2011) |
| 39  | Blue Bull (*Boselaphus tragocamalus*) | India | Kumar et al. (2008, 2010) |
| 40  | Monkey (*Rhesus macaques*) | India | Singh, Singh, Singh, Kumar, et al. (2011) |
|     |         |        | Human beings |
| 39  | Human beings (*Homo sapiens*) | USA, Italy, Czech Republic | Chodnicki et al. (2009), Sechi et al. (2008), Pavlik, Bejčková, et al. (1994) |
|     |         | London, UK | Sanderson et al. (1992) |
|     |         | India | Singh et al. (2008, 2009), Singh, Singh, Singh, Sohal, et al. (2011), Singh, Chauhan, et al. (2012), Singh, Thakur, et al. (2014) |
|     |         |        | Milk and milk products of domestic livestock |
| 40  | Cattle milk | India | Shankar et al. (2008), Sharma et al. (2008) |
| 41  | Buffalo milk | Italy | Desio et al. (2013) |
| 42  | Goat milk | India | Singh and Vihan (2004), Raghuvanshi et al. (2013) |
| 43  | Semi hard cheese | Switzerland | Stephan et al. (2007) |
| 44  | Goat paneer | India | Raghuvanshi et al. (2013) |
| 45  | Hard cheese | Czech republic | Ikonomopoulos et al. (2005) |
| 46  | Semi hard cheese | Czech republic | Ikonomopoulos et al. (2005) |
| 47  | Curd | USA | Clark et al. (2006) |
| 48  | Milk powder | Czech Republic | Hruska et al. (2005) |
in the endothelial macrophages of the small intestines, depending on the natural resistance of the individual. At this stage, infection is eliminated or the animal remains infected as a healthy carrier. During later phase of infection, multiplication of the organism in a proportion of carriers leads to the extension of lesions and hence results in interference with gut metabolism and development of clinical signs of the disease. Subclinical carriers excrete variable numbers of MAP in the faeces, as clinical disease develops and larger numbers of organisms are excreted. For histopathological diagnosis, tissue samples can be obtained from the ileum, ileocecal valve, mesenteric lymph nodes and biopsies or scrapings of the rectal mucosa. Microscopically, MAP-infected tissues exhibit chronic diffuse catarrhal enteritis characterized by hyperplasia of macrophages, lymphocytes, plasma cells, epitheloid and giant (multinucleated) Langerhans cells in the lamina propria, intestinal sub-mucosa and para-cortical region of the regional lymph node along with atrophy and fusion of intestinal villi and thickening of the mucosa. In some cases, granulomatous lymphangitis can also be observed. In the lymph nodes, the subcapsular and peritrabecular cortical sinuses have been found to contain numerous macrophages. Ziehl–Neelsen (ZN) stained impression smears show acid-fast bacilli in clumps or within macrophages. The advantage of the histopathological diagnosis is that it allows identifying the animals with focal lesions associated with subclinical stages, faecal and/or milk excretion, which is insufficient for bacterial culture or PCR (Waller 2000).

Control and eradication measures include hygiene, test-and-cull methods, restriction of animal movements and vaccination. Test-and-cull policy has been frequently used in developed countries but has only limited success due to vertical transmission of disease from infected parents to their offsprings through colostrum, milk, semen, transuterual, etc. (Lu et al. 2008). pTB vaccine development has been reported (Hagan 1935; Kalis et al. 2001; Windsor et al. 2003; Munoz et al. 2005; Scandurra et al. 2010; Kabra & Coussens 2012; Faisal et al. 2013; Rawat, Chaudhary, Gupta, et al. 2014; Singh 2015; Singh et al. 2015). Vaccination with pTB vaccine has been shown to improve the productivity and reproductive performance of the animals and has potential to replace the ‘test-and-cull policy’ for the control and eradication of the disease. However, since vaccination also induces antibodies, it would be difficult to differentiate between antibodies induced due to MAP infection or due to vaccination.

This review is aimed to specifically address the new generation diagnostics and marker vaccines based on recombinant secretory proteins of MAP and will construct new horizons in the field of pTB research, including early detection of the MAP infection, differentiation of infected and vaccinated animals as well as control of pTB infection in domestic livestock.

2. Diagnosis

Like other infectious diseases, diagnostic tests are based on (1) direct detection of the infectious agent (like MAP) by culture, microscopy, PCR, in situ hybridization and immunofluorescence (including infected tissues), (2) indirect detection based on cell-mediated and humoral immune response to infection which includes lymphocyte proliferation assay (T-cells), delayed-type hypersensitivity (DTH) reaction, altered cytokine (IFN-γ) level as well as anti-MAP antibodies (ELISA), (3) pathology (gross and histopathology) which may be performed to visualize the degenerative changes in the infected tissues. Each of these diagnostic tests has a variable sensitivity and specificity which is crucial for both the initial screening test and confirmatory assays. All diagnostic methods are fraught with difficulties that have impeded the control and eradication of pTB (Wood et al. 1989; Collins 1996; Pavlas et al. 1997; Cho et al. 2007; Leroy et al. 2007; Martinson et al. 2008; Shin et al. 2008; Fernández-Silva et al. 2011; Mon et al. 2012; Wadhwa et al. 2012; Singh 2015). In chronic infection like pTB, local strains are key to the development of efficient diagnostic tests and vaccines; therefore, the use of commercial diagnostic kits globally has led to underreporting of the infection whenever used. When animals are tested positive, their breeding programmes get disturbed due to restrictions on
movement and confinement. Similarly, a false-positive result is extremely disruptive for the livestock industry.

2.1. Direct detection of MAP in faeces, blood, milk and tissues

2.1.1. Ziehl–Neelsen (ZN) or acid-fast staining-based microscopy

Microscopy is the direct examination of clinical (faeces, blood, milk, tissues) and necropsy samples for the detection of MAP. Ziehl–Neelsen (ZN) or acid-fast staining-based microscopy has the advantage of being simple, fast and inexpensive, but cannot be performed in an ordinary laboratory or under field conditions. Faeces is a major source of infection for disease transmission, and hence considered the first choice as a clinical specimen, though blood, milk and tissue samples are equally good for MAP detection. A presumptive identification of pTB can be made if clumps of acid-fast bacilli (AFBs) are seen under the microscope in ZN-stained smears. Concentration of bacilli in faeces by centrifugation and subsequent decontamination by hexa-decyl pyridinium chloride (HPC) improve the sensitivity of this test (Eamens et al. 2000). In smears or sections of tissues (ileo-ceccal valve/intestinal lymph nodes with gross lesions), visualization of groups of brightly pink coloured bacilli within the resident macrophages in the lesions is highly suggestive of pTB. Gilmore and Wood (1991) reported that microscopy (faecal and tissue impression smears) correctly identifies 20%–60% of clinically infected animals depending upon the quantum of shedding MAP bacilli in faeces. Quite often animals with subclinical paratuberculosis, where there is lower excretion of bacilli in faeces, are likely to be missed by microscopy. Singh, Singh, Gupta, et al. (2013) have shown that microscopy has been excellent as a screening test and accuracy depends on the experience of the examiner. Furthermore, difficulties to differentiate between MAP and saprophytic AFBs (Manning & Collins 2001) compromise specificity.

2.1.2. Fluorescent microscopy

Fluorescent antibody test (FAT) is based on the use of a fluorogenic component, which, by enzymatic action in live cells, might be transformed into carboxy fluorescence. This method has been used to detect very low (<102 CFU/ml) number of MAP bacilli present in pasteurized milk/tissue samples. In pasteurized milk, the sensitivity of the FAT test was reported as 73.0% (D’Hasee et al. 2003). The FAT may also be useful for confirmation of the MAP in raw and pasteurized milk samples, vaginal smears, and tissue specimens. Gilmore and Angus (1976) have reported that serological response by the FAT was specific in cattle experimentally infected with M. avium subspecies avium and M. avium subspecies paratuberculosis. Gouldsward et al. (1977) suggested that the FAT, with utilization of the defined antigen substrate spheres (DASS) system, will be a good projection for routine examination for antibodies against Mycobacterium species.

2.1.3. Bioluminescence

Enzyme luciferase catalyzes the oxidation of protein luciferin resulting in oxyluciferin. This reaction needs ATP, which is only provided by live bacteria. Nevertheless, Sasahara et al. (2004) have reported the luciferase enzyme-based diagnostic method for rapid detection of MAP.

2.1.4. Culture-based diagnostic test

Culture of MAP is considered ‘gold standard’ for the detection of MAP infection. Faeces, milk, blood and tissues from infected animals may be used for the culture of MAP. MAP form colonies on solid media usually within 2–4 months but sometimes it may take even longer, up to 6–8 months (Deb & Goswami 2011). Liquid culture is considered much faster, i.e. about 7 weeks, but always there is risk of contamination when incubating liquid culture for months. Faeces and milk (clinical and suspected animals), tissues (necropsied animals) and environmental samples from heavily populated areas may be screened for culture to set up a contamination status of the premises. One of the important drawbacks of culture is its sluggishness associated with requirement of mycobactin J and high costs. The addition of mycobactin J in the medium is the primary requirement for in vitro culture of MAP enabling iron acquisition and its dependence can be used as discriminatory tests for confirmation of MAP in culture (Whittington et al. 2011). Use of automated systems such as BACTEC MGIT 960 shortens the time of detection (4–7 weeks) and can detect as low as 10 CFU/ml (Shin et al. 2007). If >100 CFU/g tissue is being detected, the status of the animals are considered as MAP infected (Whitlock & Buergelt 1996). Shedding of MAP is irregular/intermittent in faeces (Nielsen & Toft 2008), and therefore serial sampling must be taken into consideration for conclusive results.

2.1.5. Radioisotopic culture

In radioisotopic culture method, culture of the MAP is being radiolabelled with radioisotope indicator system. It permits recognition of as low as 3 organisms/gram of sample (faeces/milk/tissues). On the basis of load of the organisms present in the samples, time of detection by radioisotopic culture method vary from few weeks to few days. BACTEC 12B and BACTEC 460 are the radiometric culture systems used for the detection of MAP (Collins et al. 1990; Whittington et al. 1998). Due to high costs, requirement of sophisticated instruments and being hazardous in nature, the BACTEC radioisotopic culture method is usually not preferred for the detection of MAP (Deb & Goswami 2011).
2.1.6. Molecular techniques

Diagnostic methods based on 16s rRNA genetic probes have been unsuccessful as it is identical in both MAP and *M. avium*. The characterization of the IS900 insertion sequence which has 1451 base pairs and is present with 15–20 copies in the MAP genome has enabled the specific identification of the bacterial DNA by the PCR. Although some studies have described elements similar to IS900 (IS900-like sequences) in other bacterial genome, they can be differentiated through the characterization of the amplified segment by sequencing or genotyping via methylation-restriction (Mundo 2005).

2.1.6.1. PCR. Though culture is considered as ‘gold standard’ (Chiodini et al. 1984; Cocto et al. 1994), the slow growth and fastidious nature of MAP hamper its use as routine test (Thorel 1984). Several types of PCR have been developed and validated for detection of MAP-specific genome in faeces, milk, blood and tissues (Wood et al. 1989; Hawkey 1994; Grant et al. 1999; Coussens 2001). IS900 PCR has better sensitivity than culture and can detect 10–100 CFU/ml in milk samples (Giese & Ahrens 2000; Pillai & Jayarao 2002). However, due to presence of PCR enzyme inhibitors, detection of IS gene probe is not always easy (Stevenson & Sharp 1997) and hence could provide false-negative results (Whipple et al. 1992). Moreover, presence of IS900-like sequence in some of the mycobacteria other than MAP further compromises the test efficacy (Collins 1996). Therefore, culling decisions cannot be made solely on PCR results (Lu et al. 2008). Isolation of DNA is very crucial for performing PCR; clinical specimens usually have very few organisms; therefore, each laboratory has to standardize its own protocols for DNA isolation and hence optimum performance of the test (Fernández-Silva et al. 2011; Kaur et al. 2011; Gupta et al. 2012).

2.1.6.2. RT-PCR. Real-time or quantitative PCR (RT-PCR) uses fluorochrome/fluorochrome-labelled primers or, probes complementary to an intermediate fragment of the target sequence that is amplified. The quantity of fluorescence emitted during every PCR cycle is directly proportional to the amount of amplifying product. It allows the immediate examination of the target amplification and quantification with greater sensitivity than bacterial culture (Bogli-Stuber et al. 2005). IS900-specific RT-PCR is very sensitive to identify very low numbers of MAP, but is insufficient to give accurate quantification of the organism (CFU) present in the sample, since it is present in variable copy numbers within the bacterial genome. It can quantify the starting material and relatively or absolutely allows for comparative studies and optimization by the use of standard curves. By these features, RT-PCR revealed a high sensitivity (Greiner et al. 2001; Jauregui et al. 2001; Fang et al. 2002; Larsen et al. 2002; O’Mahony & Hill 2002; Patel et al. 2003) that makes it a preferred tool for detection.

2.1.6.3. Multiplex PCR. Luminex technology has got the competence to examine up to hundreds of analytes simultaneously. On the basis of IS900, IS901, IS1245 as well as dnaJ gene, a multiplex PCR has been developed for the detection of certain *Mycobacterium* species, including MAP, viz. *Mycobacterium hominisissus, M. silvaticum*, etc. This multiplex PCR has a sensitivity of 10³ CFU for each of the strains of bacteria in a single reaction tube. It can detect a pathogen by multiple gene targets simultaneously, as an alternative to successive corroboration of positive samples. Cousins et al. (1995) and Tasara et al. (2005) used multiplex PCR for MAP detection in conventional PCR setups which can be combined with real-time PCR (Schönenbrücher et al. 2008; Slana et al. 2008). However, it makes its optimization complex and shows lower sensitivity due to reagents interference and primer dimer formation (Rachlin et al. 2005).

2.1.6.4. PCR-restriction enzyme analysis (REA). Single nucleotide polymorphism (SNP) in MAP IS900 elements has been exploited to develop PCR-REA (restriction enzyme analysis) for genotyping of MAP (animal species-specific MAP), which has enabled to track the sources and the pattern of transmission for improving pTB control programmes (Whittington et al. 2000; Castellanos et al. 2007, 2010).

2.2. Pathological diagnosis

2.2.1. In situ PCR

*In situ* PCR may be used to detect MAP specific sequences in formalin-fixed, paraffin-embedded tissue samples (Delgado et al. 2011). This method may also be useful for the detection of spheroplasts (cell wall-deficient forms of MAP) and for the detection of the DNA in mycobacteria-infected tissues (Delgado et al. 2011).

2.2.2. In situ RT-PCR

*In situ* RT-PCR has a high specificity enabling detection of transcripts in the cells that originally lead to their synthesis and is capable to detect mRNA expression inside mycobacteria-infected tissues. *In situ* method is also popular and frequently used for research purposes (Chen & Fuggle 1993; Nicol et al. 2008; Rocca et al. 2010).

2.2.3 In situ hybridization (ISH)

ISH utilizes a labelled probe to specifically label DNA or RNA on a histologically processed tissue section, allowing localization of MAP genome in tissues (Maroudam et al. 2015). ISH has been successfully used to detect spheroplasts in specimens derived from animals as
well as specimens from Crohn’s disease patients (Sechi et al. 2001).

Besides these above-mentioned techniques, other genome-based assays such as pulsed field gel electrophoresis (PFGE) have made it possible to characterize and phylogenetically analyze MAP (Hughes et al. 2000; Stevenson et al. 2002), as well as loop-mediated isothermal amplification (LAMP), which does not require the use of a thermocycler (Enosawa et al. 2003), has also been developed for the detection of MAP.

### 2.2.4. Immunohistochemistry (IHC)

This technique uses a MAP-specific antibody marked with enzymes, which allows visualizing the reaction on the enzymatic substrate. The advantage of this method is that it enables identifying spheroplasts and MAP in tissues (Maroudam et al. 2015). It shows a good sensitivity in tissues from subclinically infected animals. However, false-positive reactions due to cross reaction with other mycobacteria may occur. Sensitivity is usually low as compared with bacterial culture (Martinson et al. 2008).

### 2.2.5. Flow cytometry

This technique allows detection of subclinical infection. A sensitivity of 95% and specificity of 97% was observed in experimentally infected animals (Eda et al. 2005). This technique is rapid (less than 4 hours), but is expensive and requires sophisticated equipment. In this assay, intact MAP bacteria serve as the test antigen and measuring particle at the same time. The complete repertoire of unmodified MAP surface antigens is available for recognition by antibodies in bovine serum samples in this setting. In naturally infected adult cattle, the FC assay can detect MAP earlier than faecal culture. It retains high specificity and allows detection of MAP-specific antibodies as early as 170 days after experimental infection in calves (Eda et al. 2005). Schillinger et al. (2013) has reported 78% sensitivity and 100% specificity of FC assay for IgG1 in adult cattle. MAP antibodies can only occasionally be detected in infected calves less than 12 months of age (Schillinger et al. 2013).

### 2.2.6. Immune-based test platforms

Measuring specific cell-mediated immunity (CMI) and humoral immunity are relevant alternatives to faecal culture and other diagnostic tests based on identification of the agent (Maroudam et al. 2015). CMI assay involves DTH assay, lymphocyte stimulating assay and IFN-γ assay. Subclinical stage of infection is typically characterized by robust cellular immune response, clinical stage by a strong humoral immune response and advanced stage by anergy where diagnostic tests of cellular immunity become negative and serological tests are less reliable (Maroudam et al. 2015).

#### 2.2.7. Measuring specific cell-mediated immunity

MAP-infected macrophages present antigens in association with MHC class I and II on the CD8+ and CD4+ T-cells which leads to activation of the CD4+ TH1-cells resulting in production of a range of cytokines, including interferon-γ (IFN-γ) (Coussens et al. 2004). IFN-γ production by TH1-cells are, therefore, major mediators of specific immunity during early infection (Stabel 2000). Peripheral and intestinal CMI responses are reduced in symptomatic compared to asymptomatic animals (Koets et al. 2002). Disease development appears to be linked with local loss of CD4+ T-cells and γδ T-cells (Chiiodini 1996; Koets et al. 2002). The fundamental defect that could be connected with a reduction in CMI response is the deregulation in MAP-mediated macrophage function (Tooher et al. 2002; Weiss et al. 2002; Zur et al. 2003). Interruption in gene activation and signalling breakdown in the immune response are responsible for controlling MAP infection through genes encoding tumour necrosis factor alpha (TNF-α), interleukin-1 (IL-1), IL-12, and IFN-γ and their receptors (Jouanguy et al. 1999; Dorman & Holland 2000; Ottenhoff et al. 2000) and this could also affect effector activity of CD8+ T-cells (Canaday et al. 2001).

#### 2.2.8. Delayed-type hypersensitivity

Skin test for DTH is a measure of CMI response (Maroudam et al. 2015). In this test, 0.1 ml intra-dermal inoculation of antigen was administered into a clipped or shaven site (usually middle third of the neck). In the beginning, avian PPD tuberculin or Johnin was used as an antigen as it was thought that these are of comparable sensitivity and specificity. Thickness of the skin is measured by slide calipers before and 72 hours after inoculation of the antigen. Increased thickness of the skin (≥2 mm) indicates presence of DTH in infected animals. Rather than producing a discrete swelling, some animals, like deer, produce a diffuse swelling which makes interpretation of the result complicated (Kalil et al. 2003). The specificity of Johnin in skin test is 88.8% at the cut-off value of ≥2 mm, 91.3% at the cut-off value of ≥3 mm and 93.5% at the cut-off value of ≥4 mm (Whittington & Sergeant 2001). However, these cut-off values did not significantly enhance the sensitivity of the test nor detected changes in the test performance by minor antigenic differences in different batches of antigen. Therefore, further research is required for increasing the value of skin test.

#### 2.2.9. IFN-γ assay

IFN-γ is released by peripheral blood mononuclear cells (PBMCs) in response to antigen. It can be estimated by various approaches like enzyme-linked immunosorbent assay (ELISA), enzyme linked immunospot (ELISPOT) (Lalvani 2003; Veerasami et al. 2011) and qRTPCR-based analysis (Rothel et al. 1990). IFN-γ assay requires whole blood sample cultured with MAP
antigens in a proliferation assay, where released IFN-γ level is measured in the supernatant by ELISA (Wood et al. 1989; Jungersen et al. 2005). Fluctuating IFN-γ responses to purified protein derivatives (PPDs) in calves younger than 15 months questions the usefulness of the IFN-γ assay in its current form (Jungersen et al. 2002; Huda et al. 2003). As per Kohler et al., cross-reactive antigens (PPD), rather than specific (MAP) antigens, are responsible for poor specificity and sensitivity of CMI-based assays (Flynn et al. 1993; Kohler et al. 2001; Gwozdz et al. 2000; Mikkelsen et al. 2011a). The advantage of the IFN-γ test is the significant secretion of IFN-γ during the early stages of disease and may be used to detect animals in the subclinical stage. However, it has several disadvantages such as the possible cross reactions, the need to process the sample quickly since cells must be alive, and its high costs and low sensitivity (Stabel & Whitlock 2001).

2.2.10. Lymphocyte transformation test
Lymphocyte transformation test (LTT) measures the ability of PBMCs in recognizing and responding to MAP antigen and can be investigated at different days post-vaccination when pulsed with protoplasmic antigen. The Simulative Index value of vaccinated goats has been found to be significantly higher at 30 days post-vaccination and onwards. There is a considerable variation in the lymphocyte transformation response in case of animals that are heavily infected. In animals that are noninfected, the number of positive reactions recorded depends on the population. A greater proportion is found in herds that have got a proven history of paratuberculosis (Stabel & Goff 2004; Singh et al. 2010).

2.3. Serology-based diagnostics
As the infection progresses, CMI response wanes and an antibody-mediated CD4+ T-helper type 2 (TH2) response becomes predominant. CMI, but not antibody response, is considered important for protective immunity against pTB (Toman et al. 2003; Waters et al. 2003; Maroudam et al. 2015). Serum antibody titres are quite consistent and higher during clinical disease. Serology-based tests are low in costs, rapid and highly sensitive in subclinical and clinically infected animals (shedding large numbers of MAP in their blood, milk and faeces) (Maroudam et al. 2015). Different methods such as complement fixation test (CFT) (Morris & Stevens 1977; Maroudam et al. 2015), agar gel immune-diffusion (AGID) (Sherman et al. 1990; Reichel et al. 1999; Gumber et al. 2006) and ELISA (Reichel et al. 1999; Collins 2002; Gumber et al. 2006; Maroudam et al. 2015) can be employed in serology (diagnostic identification of anti-MAP antibodies in serum) or antibody-based detection of MAP infection (detection of MAP in clinical samples using anti-MAP antibodies). Of these tests, AGID has a high specificity, which can reach up to 100% but the sensitivity is lesser than ELISA (Robbe-Austerman et al. 2006; Alvarez et al. 2015).

2.3.1. Complement fixation test
CFT has been the standard diagnostic method used for screening of animal populations suspected for pTB for several years (Maroudam et al. 2015). Morris and Stevens (1977) used improved antigen for paratuberculosis CFT. Due to less specificity of CFT, its use is limited for screening of general animal population for control purposes. However, it is frequently demanded by countries that import animals. Varieties of CFT protocols exist and are adopted internationally, but there are no universal pattern sera with standardized complement fixation units for use as a reference. Singh et al. (2005) has reported that the specificity of the CFT is less than AGID and ELISA. It can detect antibodies 15 months later than ELISA and it has intermediate sensitivity to AGID and ELISA.

2.3.2. Agar gel immune-diffusion/precipitation test
Agar gel immune-diffusion/ precipitation test (AGID/AGPT) is a very cost-effective test for the confirmation of the MAP infection in clinically suspected animals for pTB. As the specificity of the test is reported as 100%, it is a reliable test to detect MAP. It can detect antibodies only 39 months after shedding of microbes (Colgrove et al. 1989). AGID has shown higher sensitivity and specificity than ELISAs in small ruminants of New Zealand and Australia (Gwozdz et al. 2000; Hope et al. 2000; Sergeant et al. 2003). The specificity and sensitivity of the AGID measured against histological results were 99%–100% (95% CI) and 38%–56% (95% CI), respectively (Hope et al. 2000). In another study, it is indicated that ‘Indigenous ELISA’ and AGPT using two antigens from different sources could be a better method for screening and diagnosis of MAP infection in herds and flocks (Pahangchop et al. 2014).

2.3.3. Enzyme-linked immunosorbent assay
ELISA is a simple, rapid, cost-effective and the most frequently employed assay to determine the infection status (Maroudam et al. 2015). ELISA is efficient of detecting small amounts of antibodies and therefore has highest sensitivity among the serological tests used for MAP (Harris & Barletta 2001). Removal of cross-reacting antibodies by absorbing sera with Mycobacterium phlei has improved the specificity of ELISA (Yokomizo et al. 1983). Some studies have reported 98.8% (Cox et al. 1991), 99.7% (Reichel et al. 1999) and 97.4% (Eda et al. 2006) specificity, but it compromises with assay sensitivity. In general, highest sensitivity of serological tests have been recorded in animals with clinical signs of the disease since presence of antibodies is higher in the later stages of infection (Sweeney et al. 1995; Nielsen & Toft 2008). However, these tests lack ability to detect animals in early stages of
infection. A major benefit of these tests is easy implementation in large-scale screening of animals without any costly equipment or well-trained personnel. Selection of antigens is the major challenge in development of ELISA that is pathogen specific and permits sensitive recognition. Antibodies produced against shared epitopes of closely related species of organism can contribute to cross-reactivity resulting in false-positive results and fluctuations in antibody titres and hence development of ELISAs for optimum specificity for the entire mycobacterial diseases, together with human tuberculosis (Mycobacterium tuberculosis), leprosy (Mycobacterium leprae), bovine tuberculosis (Mycobacterium bovis), as well as pTB has been a tedious task. For instance, at the Central Institute for Research on Goats (CIRG), Mathura, India, an ELISA was initially standardized by harvesting antigen from an advanced case of clinical pTB in a goat followed by using ‘Tepes strain’ of MAP used for making Johnin at IVRI, Izzatnagar, India and subsequently used own culture-based antigens (Singh et al. 1998; Singh, Singh, et al. 2014). Based on our experience in chronic infections where organism takes long time for multiplication, use of semi-purified whole cell protoplasmic antigen from locally available strains has been the best test for the diagnosis of MAP infection in domestic livestock (Singh, Singh, et al. 2014). The test has since been successfully employed for screening of serum and milk samples of domestic livestock and human beings for the detection of MAP infection and also for monitoring of the vaccine response in vaccinated animals. Use of the likelihood ratio method of Collins (2002) has helped to convert this assay into an ‘Indigenous ELISA kit’.

### 2.3.4. **MAP antigen candidates for diagnostics**

Identification of well-defined specific antigens is very important in contributing high test sensitivity and specificity in quantifying cell-mediated and humoral immunity. The ideal characteristics of a diagnostic antigen should be its high antigenic property, uniqueness to MAP, easy recognition in infected animals in early/late stages/subclinical/clinical stages of infection and should remain recognizable throughout the course of the infection. Theoretically, a diagnostic test of MAP should use a cocktail of antigens due to the spectral nature (Kathaperumal et al. 2009), long range of heterogeneous MHC molecules present in various outbred animals (Kathaperumal et al. 2009) and differentiated range of antigen expression of MAP (Radosevich et al. 2007). Mon et al. (2014) recommended on individual antigens that have been able to identify only a subset of pTB in clinically infected animals, but a cocktail of antigens could be a superior candidate for serological diagnosis of pTB. The same antigen may not be useful for both CMI and serology-based assays.

The antigen candidates used in screening of MAP includes secreted antigens, cell wall and membrane antigens, lipoproteins, heat shock proteins (Hsp) as well as recombinant proteins. Earlier studies have reported a range of natively purified MAP proteins to recall CMI responses in *in vitro* assay, e.g. Avi-3 (Radosevich et al. 2007), P30 (Abel et al. 1989), A36-complex (Gilot et al. 1992), MP14 (Olsen & Storset 2001; Olsen et al. 2005), alkyl hydroperoxide reductase C (AhpC) and alkyl hydroperoxide reductase D (AhpD) (Olsen, Reitan, Holstad, et al. 2000; Olsen et al. 2001), and MPB70 (Olsen et al. 2005) (Table 2). These antigens lack MAP specificity because they are orthologues in other mycobacteria species except two antigens, namely AphC and AphD. These antigens are most capable of inducing CMI antigens among the natively purified MAP antigens as they appear to be species specific with a high sensitivity (Mikkelsen et al. 2011b). In recent years, diagnostic assays based on recombinant antigen candidates have been replaced by purified antigen candidates. Recombinant antigens have higher product yield as well as high homology of antigen preparation and production. A 67-kDa heat shock protein (HspX) which is quite host-specific has been shown to induce robust CMI response (Bannantine & Stabel 2000). However, a recombinant HspX protein did not generate a CMI recall response when expressed in cell culture from 10 infected cows (Bannantine & Stabel 2000). In another study, HspX and Hsp65 (Koets et al. 1999; Nagabhushanam et al. 2001) induced low CMI response in both cattle (Koets et al. 1999) and mice (Nagabhushanam et al. 2001).

### 3. Control strategies

Control and eradication of pTB is a big challenge as MAP has a wide host range and is also present in many alternative hosts in the environment. Bovine TB has been successfully managed in nearly all developed countries, but despite large control efforts, pTB remains an unsettled problem. Many initiatives have been undertaken to control and eradicate pTB, and principally three approaches have been followed, namely management changes to restrict transmission of MAP, test-and-cull approaches in order to eliminate the sources of infection and vaccination to increase resistance to infection with the main goal of minimizing or eliminate the exposure of susceptible animals through faeces of MAP-infected animals and lessen the environmental contamination by eliminating positive animals. Biosafety appears to be the only measure to control pTB in uninfected animals. However, control of pTB in a highly diverse country like India with lack of indigenous kits and reagents and numerous breeds, huge population of domestic and wild animals and human beings (Singh, Singh, et al. 2014; Singh, Thakur, et al. 2014), unrestricted movement of animals and limited resources (both skilled human and economic) remains an indomitable task and huge challenge.
In the face of the fast increase in the population of infected animals and incalculable losses caused to domestic livestock industry and also native human population, the absence of a control policy for domestic livestock pTB has become a major challenge.

### 3.1. Management practices

The main focus of management practices is to avoid contact between infected and healthy animals and reduce the load of MAP in the environment. Separation of offspring from their mothers/dams right after birth, MAP-free Colostrum supplement and milk replacement used in calf feeding, raising replacement heifers in separate place, avoiding manure fertilization of fields where replacement heifers graze, improving general farm hygiene, and eliminating practices that may facilitate disease control programme.

#### Table 2. MAP antigens stimulating cell-mediated immune response.

| Sn. | MAP antigens | Characteristics | Test assay | References |
|-----|--------------|-----------------|------------|------------|
| 1   | P30          | Secreted protein | IFN-γ test, 2/2 MAP infected, 0/2 uninfected sheep | Burrells et al. (1995) |
| 2   | Hsp70        | Heat shock protein 70 | Positive PBMC proliferation; 88% of FC pos, 74.1% of FC neg, 30.5% of control (FC neg), and 93.3% of MAP vaccinated cows | Koets et al. (1999) |
| 3   | Ahp C        | Alkyl hydroperoxide reductase C | IFN-γ test, 3/3 infected and 0/1 control goats Serum antibody, 4/4 infected and 0/1 control goats ELISA: 13/56 positive, 0/10 negative cattle | Olsen, Reitan, Holstad, et al. (2000) |
| 4   | Ahp D        | Alkyl hydroperoxide reductase D | IFN-γ test, 3/3 infected and 0/1 control goats | Olsen, Reitan, Holstad, et al. (2000), Olsen et al. (2001) |
| 5   | SOD          | Superoxide dismutase | IFN-γ significantly higher in 20 faecal culture positive vs. 18 faecal culture negative cows | Mullerad et al. (2002) |
| 6   | MAP0261c     | Homologue protein of M. TB is immune-dominant in mice and humans | IFN-γ test: low response but significant difference between 21 naturally infected and 9 negative control cows | Huntley et al. (2005) |
| 7   | P22          | Exported lipoprotein (LppX/LprA FG family) | IFN-γ test, positive: 8/9 MAP vaccinated and 0/5 control sheep | Dupont et al. (2005) |
| 8   | MPP14        | Protein from MAA complex | IFN-γ test response. Of 14 calves during two months, 5/7 positive and 0/7 negative | Olsen et al. (2005) |
| 9   | Ag85B        | Protein, T-cell epitope | IFN-γ significantly higher of 20 faecal culture positive vs. 18 faecal culture negative cows | Shin et al. (2005) |
| 10  | Ag85C        | Protein, T-cell epitope | IFN-γ significantly higher, of 20 faecal culture positive vs. 18 faecal culture negative cows | Shin et al. (2005) |
| 11  | MAP41        | Possible T-cell Ag, homologous to PPE protein family. Hypothetical protein | IFN-γ test, positive 5/5 and 0/5 negative | Nagata et al. (2005) |
| 12  | MAP 39       | Possible T-cell Ag, homologous to PPE protein family. Hypothetical protein | IFN-γ test, positive 5/5 and 0/5 negative | Nagata et al. (2005) |
| 13  | Ag85A        | Protein, T-cell epitope | IFN-γ test, positive 5 infection mice (Ag85A) and 5 infected calves (Ag 85 complex) | Rosseels et al. (2006) |
| 14  | P35          | Associated with invasion of bovine intestinal epithelial cells, hypothetical protein | IFN-γ test, 3/3 positive and 0/3 negatives | Basagoudanavar et al. (2006) |
| 15  | 74F          | Polyprotein of MAP3527 (a peptidase) and MAP1519 (a hypothetical protein) | Proliferation of pooled spleen cells of 24 mice. IFN-γ significantly higher in MAP74F immunised vs. control mice | Chen et al. (2008) |
| 16  | Apa          | Alanine- and proline-rich Ag or Fibronectin attachment protein | IFN-γ test, 6/45 faecal culture positive and 0/45 faecal culture negative cows. Not significant. | Gioffre et al. (2009) |
| 17  | MAP1718c     | — | Proliferation assay, no significant difference between MAP FC pos (n = 12) and FC neg (n = 14) cows | Santema et al. (2009) |
| 18  | MAP3515c     | — | Proliferation assay, no significant difference between MAP FC pos (n = 12) and FC neg (n = 14) cows | Santema et al. (2009) |
| 19  | MAP1693c     | Peptidyl-prolyl-cis–trans-isomerase | IFN-γ responses lower recognized in CS7BL/6 and BALB/c mice infected with MAP | Roupie et al. (2012) |
| 20  | MAP1637c     | UbD superfamily | Strong, antigen-specific IFN-γ responses were detected | Gioffre et al. (2009) |
| 21  | MAP3743      | Saccharopine dehydrogenase | IFN-γ responses lower recognized in CS7BL/6 and BALB/c mice infected with MAP | Gioffre et al. (2009) |
| 22  | Ag5          | — | IFN-γ responses most strongly recognized both in CS7BL/6 and BALB/c mice infected with MAP | Gioffre et al. (2009) |
| 23  | Ag6          | — | IFN-γ responses lower recognized in CS7BL/6 and BALB/c mice infected with MAP | Gioffre et al. (2009) |
| 24  | MAP 0388     | Dyp-type peroxidase family | Strong, antigen-specific IFN-γ responses were detected | Santema et al. (2009) |
| 25  | MAP 2677     | Glyoxalase family | Strong, antigen-specific IFN-γ responses were detected | Santema et al. (2009) |
| 26  | MAP 3837c    | Secreted proteins | Induce a significant IFN-γ response | Thakur et al. (2013) |
| 27  | MAP 3870c    | ESAT-6 family member proteins | Induce a significant IFN-γ response | Santema et al. (2009) |
| 28  | MAP 3710c    | Latency proteins | Induce a significant IFN-γ response | Santema et al. (2009) |
| 29  | MAP 1609c    | Hypothetical protein | Induce a significant IFN-γ response | Santema et al. (2009) |
| 30  | MAP 3783c    | Antigen Ag85B | Induce a significant IFN-γ response | Santema et al. (2009) |
| 31  | MAP 3837c    | — | Strongly induced IL-10 gene transcription in bovine macrophages | Bannantine et al. (2015) |
Furthermore, introduction of new animals should not be neglected; diagnosis of pTB positive animals as well as the pTB status of the herd of origin should be taken into account. The major setback to these practices is the ability of MAP to survive in the environment for extended periods. Economic constraints of farms may further limit the success of this approach. Moreover, these measures did not yield immediate results. Hence, this kind of strategy is unlikely to be successful in Indian management conditions but definitely has a role in maintenance of bacilli in farm premises. Composting of farm excreta is another major issue, which if implemented in letter and spirit can substantially bring down environmental contamination of livestock premises.

### 3.2. Culling strategies

Faecal culture, ELISA and PCR are three diagnostic tests that in combination may be used to detect infected animals at farms (Singh, Singh, Singh, Gupta, Chaubey, et al. 2013) and facilitate decision on culling (Lu et al. 1997; Zimmer et al. 1999; Whitlock et al. 2000; Kudahl et al. 2007). Heavily contaminated farms and the presence of super-shedder animals (10,000–10 million MAP/gram of dung) (Aly et al. 2008) as well as non-clinical and asymptomatic shedder animals will also give positive results just because the animals live in the contaminated environment and MAP bacteria pass through their gastrointestinal tract (pass through phenomenon) and hence can influence culling decisions. In such cases, blood PCR must be performed (Whitlock et al. 2000; Collins et al. 2006). However, under Indian conditions, the number of such cases (theoretically negative but positive in test) would be too low or non-existent since the number of animals with clinical or advanced clinical symptoms is too high at any point of time (as per corresponding author observations).

Different commercially available diagnostic kits have very different efficacies (Garrido et al. 2000; Dieguez et al. 2009), which therefore can severely affect the control programmes. This is more so when the commercial kits, tests and reagents used are based on MAP strains which are not locally available, therefore inducing a poor response in positive animals (Singh et al. 2015). If the costs are low and the results are obtained in less than a week, it is more easily accepted instead when positive results keep trailing for long time since it is always possible to intensify the control programme by testing more frequently and apply better monitoring of herds. The regional ELISA-specific strategies implemented up till now are rather complex and still not proven successful.

Culling has mainly been used in Western countries and is impracticable under Indian conditions due to ban in cow slaughter. Since MAP is transmitted both vertically, through colostrum, milk, semen, during pregnancy, etc. (Larsen et al. 1970; Sweeney et al. 1992; Shankar et al. 2008; Sharma et al. 2008; Desio et al. 2013), and horizontally (by environmental contamination), it is not possible to check transmission of MAP. The corresponding author has first-hand experience on culling large number of animals (positive in AFBs in microscopy) in the government farms of goats and sheep as a measure to control and restrict MAP infection and disease. However, this ‘test-and-cull’ method miserably failed and bioincidence of pTB in goats and sheep continued to increase both in intensity and number of animals infected. Such interventions are even not possible in non-government farms. Therefore, the alternative approach of ‘Therapeutic vaccination’ as developed by CIRG was used and proved to be successful. However, this methodology is still not commonly used in the non-government farms, whereas the test-and-cull method is still preferred in the government farms, though it had very limited value. The test-and-cull approach along with hygiene can be at best help in the management of pTB, but not its control.

### 3.3. Vaccination

The first pTB vaccine was formulated in 1926 (Rosseels & Huyge 2008). Vaccination is considered as a cost-effective strategy as it has both prophylactic and therapeutic effects (Fridriksdottir et al. 2000; Singh, Gupta, et al. 2014). Vaccination approaches have been consecutively launched for domestic and wild ruminants in different regions (Fridriksdottir et al. 2000). There are a range of vaccines available for pTB; live attenuated, heat killed or sonicated preparations of MAP (Table 3). The main drawback of traditional vaccines is that there is no test which differentiates between infected and vaccinated animals. Hence, these tests can interfere with serological diagnosis of pTB and tuberculosis infections. However, attempts to develop vaccines aimed at differentiation between infected and vaccinated animals (DIVA) tests have been initiated.

Vaccination strategy has huge economic benefits which may be up 20 times higher than test-and-cull strategies (Juste & Casal 1993; Van Schaik et al. 1996). Vaccination seems to be the only alternative to manage the worldwide pTB problem, and hence conservation of threatened domestic and wild ruminant species, especially. In endemic areas of Iceland, vaccination has been compulsory in sheep since 1966 and losses have been decreased significantly (Fridriksdottir et al. 2000). In Australia, introduction of a commercial killed vaccine demonstrated a more efficient method for eradication and thus vaccination got wide acceptance (Reddcliff et al. 2006). In Spain, the development of a live-attenuated vaccine kept ovine pTB in
check (Juste & Perez 2011). Goat vaccination against pTB is currently in use in USA, Spain, the Netherlands, Norway, and India (under trial) (Saxegaard & Fodstad 1985; Kalis et al. 1992; Aduriz 1993; Kabara & Coussens 1994; Jorgensen 1983; Saxegaard and Fodstad 1985). Goat vaccination against pTB is currently in use in USA, Spain, the Netherlands, Norway, and India (under trial) (Saxegaard & Fodstad 1985; Kalis et al. 1992; Aduriz 1993; Kabara & Coussens 1994; Jorgensen 1983; Saxegaard and Fodstad 1985).

As mentioned before, the prime drawback of vaccination is that vaccinated animals cannot be differentiated from infected animals. Furthermore, pTB vaccination may interfere with serological diagnosis of tuberculosis and hence can obstruct tuberculosis vaccination decreases pTB lesions (Eppleston & Windsor 2007), reduces the excretion of MAP (Van Schaik et al. 1996; Singh SV, Singh PK, et al. 2007), decreases losses in productivity and producibility (Singh SV, Singh PK, et al. 2007) as well as reduces the overall incidence of the disease (Saxegaard & Fodstad 1985), all of which in turn leads to economic gains (Van Schaik et al. 1996; Juste & Perez 2011).

Vaccination decreases pTB lesions (Eppleston & Windsor 2007), reduces the excretion of MAP (Van Schaik et al. 1996; Singh SV, Singh PK, et al. 2007), decreases losses in productivity and producibility (Singh SV, Singh PK, et al. 2007) as well as reduces the overall incidence of the disease (Saxegaard & Fodstad 1985), all of which in turn leads to economic gains (Van Schaik et al. 1996; Juste & Perez 2011).
eradication programmes. The latter is, in fact, the major hurdle affecting MAP vaccine approval for animals by medical and agricultural authorities all over the world and the major deterrent for pharmaceutical companies to design new MAP vaccines for animals. The most widely used tuberculosis diagnostic test in cattle is the single intradermal tuberculin test, and cattle vaccinated with the currently available ovine or experimental MAP vaccines would like to give false-positive results. According to legislation in many countries, these animals are banned from international trade and should be slaughtered unless it can be proved that they are not infected with tuberculosis. New tuberculosis immunological diagnostic tests, such as the gamma interferon release assay or the Enferplex™ TB assay, could help in the differentiation between MAP vaccinated and tuberculosis infected animals, but improvements of these tests might be required, since interference with tuberculosis diagnosis can still occasionally occur in MAP-infected animals (Rawat, Chaudhary, Gupta, et al. 2014). However, a modification of the single intradermal tuberculin test, the comparative intradermal tuberculin test, could solve the interference problem in the vast majority of cases. This test has been available for many years and is actually an official tuberculin test according to the OIE and EU legislation, consisting of the simultaneous intradermal injection at two different sites of tuberculins from M. bovis (PPDbov) and M. avium subspp. avium (PPDav). Higher reactivity to the avian tuberculin indicates infection or vaccination with avian-type mycobacteria and allows ruling out mammal tuberculosis infection according to standardized criteria. Mineral oil adjuvants have been routinely used in the vaccine formulation which causes abscess development at the site of vaccination being also an important limitation for the widespread use of vaccines against pTB (Chiodini & Van Kruiningen 1983).

4. Natural vaccination of domestic livestock

Mix livestock farming and keeping of goats and sheep together have been traditional practice of livestock husbandry. There are several benefits of such practice including natural vaccination. Wherever goats and sheep are managed on the same premises, the species in majority (goat/sheep) has a tendency to get infected due to adaptation of MAP and gaining in pathogenicity of bacilli due to repeated passage of MAP (virulence). The same goat or sheep adapted strain of MAP acts as vaccine in the minority species (Singh, Singh, Gupta, et al. 2013).

5. Natural resistance of animals to pTB

Natural resistance depends on natural exposure of animals to an infectious agent. Huge and diverse natural resources of domestic livestock species in terms of breeds and strains are found on the Earth. Such polymorphism and inherent capacity of individual animals to resist MAP infection can form the basis of breeding of animals for disease resistance as an alternative tool for building the ‘herd immunity’ (Singh, Dhama, et al. 2013).

6. Secretory proteins-based diagnostics and vaccine therapies

Comparing the secretory proteins with other cellular proteins showed a greater seroreactivity in MAP-infected animals. So, secretory proteins are the focus of research for DIVA-based diagnostics and vaccine development. A range of new immunologically important MAP-secreted antigens have been identified using new technologies like 2D-gel electrophoresis, chromatography, mass spectrometry and peptide mass fingerprint. Currently, crude antigen mixtures of MAP are used for immune diagnostics, and a more detailed categorization of antigen framework of MAP will be important to develop improved diagnostic tests. A whole range of antigens have in recent years been identified and characterized. These include various secretory proteins (antigens) like MAP1693c, MAP0853, MAP4308c, CobT, MAP2272c, ArgJ, MAP0628c, MAP2609, MAP2942c, MAP2168c, Mod D, MAP1022c, MAP2099, Antigen 85c, Pep A, MAP3273c, MAP0593c, MAP2281c, SOD, MAP1513, MAP2020, MAP0038, MAP1272, MAP209c, MAPPeA, MAP3547c, MAP4308c, MAP2677c, MAP0586c, MAP0210c, MAP0232c, MAP1240c, MAP1738, MAP2239, Ag3, Ag5, Ag9, Apa and MAP3641c (Romain et al. 1993; Willemsen et al. 2006; Cho et al. 2007; Roupie et al. 2008; Gioffre et al. 2009; Gumber & Whittington 2009; Souza et al. 2011; Mon et al. 2012; Roupie et al. 2012; Carlos et al. 2015). In addition, secretory proteins are either present in very scanty (low) amount or absent in vaccines, which are as a result unlikely to induce antibodies against the secretory proteins. Due to continued replication of MAP bacilli during infection, the immune system repeatedly encounters with secretory proteins and develops antibodies against those secretory proteins (Cho et al. 2006). Secretory antigens govern major impact on the development of DIVA-based novel serodiagnostic techniques with improved sensitivity and specificity (Shin et al. 2004; Malamo et al. 2006; Bannantine et al. 2011; Deb & Goswami 2011) and subunit/cocktail vaccines may aid in the immediate control and management of pTB. Therefore, secretory proteins are potentially more functional for developing future diagnostics and vaccines of pTB and monitoring of its progression.

6.1. Secreted antigen candidates for diagnostic purposes

Secreted antigens are expected to be highly immunogenic or, immune-dominant due to their presence in
the extracellular environment where they are more likely to encounter sensitized immune cells (Table 4). This was confirmed in a study comparing secreted antigens from supernatants and antigens of intracellular origin (Cho & Collins 2006). Serum from infected cattle was used for immune-blot analysis which showed that infected sera reacted more strongly to the secreted antigens compared to the antigens of intracellular origin (Cho et al. 2006). The sensitivity of serodiagnostics improved with the use of MAP culture filtrate (CF) secretory proteins and similarly for other mycobacterial pathogens, including *M. bovis* and *M. tuberculosis* (Samanich et al. 2000; Waters et al. 2006; Bannantine et al. 2006). Pradenas et al. (2009) reported that most CF proteins have low degree of difference in CF protein immunoreactivity in culture-positive cases of pTB. They observed a high immunoreactivity in immunoblots. These results suggested that a comprehensive panel of antigens were required for a more sensitive assay.

### Table 4. MAP secretory proteins inducing humoral immunity.

| Sn. | MAP proteins                                                                 | Characteristics                                      | Test assay                                                                 | References                  |
|-----|------------------------------------------------------------------------------|------------------------------------------------------|----------------------------------------------------------------------------|-----------------------------|
| 1   | Antigen D                                                                    | Peroxidase, extracted from culture                   | ELISA: 22/22 positive at specificity = 90, AGID:18/22 positive             | Sugden et al. (1991)        |
| 2   | Antigen A                                                                    | Extracted from culture filtrate and sonicate         | ELISA: 18/22 positive at specificity = 90, AGID 4/22 positive              | Sugden et al. (1991)        |
| 3   | kDa                                                                          | Putative signal peptide                              | Immunoblot positive ELISA: 13/30 positive 1/10 negative                   | Olsen, Reitan, Wilke (2000); Oslen et al. (2001) |
| 4   | SOD                                                                          | Superoxide dismutase. Induce B-cell response         | ELISA: 49/60 positive, 2/22 negative                                       | Shin et al. (2004)          |
| 5   | MAP 2942                                                                     | Ag mpt53                                             | Immunoblot: 11/11 positive                                                | Willemens et al. (2006)     |
| 6   | MAP 2609                                                                     | Conserved hypothetical proteins                      | Immunoblot: 11/11 positive                                                | Willemens et al. (2006)     |
| 7   | MAP 0210c                                                                    | Not known                                            | Immunoblot positive in 10/ 11 lymphnode                                    | Willemens et al. (2006)     |
| 8   | ModD                                                                         | Associated with MHC II and fibrinectin attachment    | Immunoblot and ELISA positive                                             | Cho et al. (2006, 2007)     |
| 9   | Pep A                                                                        | Serine protease                                      | Immunoblot and ELISA positive                                             | Cho et al. (2006, 2007)     |
| 10  | MAP 2168c                                                                    | Hypothetical protein                                 | Immunoblot and ELISA positive                                             | Cho et al. (2006, 2007)     |
| 11  | MAP 1693c                                                                    | Peptidy-l-protyl-cis–trans-isomerase                  | Immunoblot and ELISA positive                                             | Cho et al. (2006, 2007)     |
| 12  | MAP 1272c                                                                    | Putative invasin, NlpC/P60 superfamily               | Immunoblot, ELISA: 7/7 positive, 0/2 negative                             | Li et al. (2007)            |
| 13  | MAP 2677c                                                                    | Hypothetical protein, glyoxalase                      | ELISA: 4/7 positive and 0/3 negative                                       | Leroy et al. (2007)         |
| 14  | MAP 4308c                                                                    | Possible L-lactate-2-monoxygenase                     | ELISA: 4/7 positive and 0/3 negative                                       | Leroy et al. (2007)         |
| 15  | MAP 0593c                                                                    | Hypothetical protein, Degradation of proteins, peptides, and glycopeptides | ELISA: 24/41 positive and 5/41 negative                                   | Gumber et al. (2009)        |
| 16  | GreA                                                                         | Transcription elongation factor GreA                  | ELISA: 10/41 positive and 2/41 negative                                   | Gumber et al. (2009)        |
| 17  | ClpP                                                                         | ATP-dependent Clp protease proteolytic subunit. Degradation of proteins, peptides, and glycopeptides | ELISA: 19/41 positive and 2/41 negative                                   | Gumber et al. (2009)        |
| 18  | Ppa                                                                          | Inorganic, pyro-phosphatase involved in metabolism   | ELISA: 14/41 positive and 2/41 negative                                   | Gumber et al. (2009)        |
| 19  | MAP 1152                                                                     | PPE protein                                          | Significant humoral response observed                                    | Bannantine et al. (2011)   |
| 20  | MAP 1156                                                                     | Diacylglycerol acyltransferase                        | Significant humoral response observed                                    | Bannantine et al. (2011)   |
| 21  | MAP 1635c                                                                    | UbiD superfamily, hypothetical protein               | Weak MAP1637c specific antibodies were detected in *M. bovis*-infected BALB/c mice | Roupie et al. (2012)       |
| 22  | Ag3                                                                          | –                                                    | Weaker antibody response                                                  |                             |
| 23  | Ag5                                                                          | –                                                    | Weaker antibody response                                                  |                             |
| 24  | Ag6                                                                          | –                                                    | Weaker antibody response                                                  |                             |
| 25  | MAP 0388                                                                    | Dyp-type peroxidase family                           | Weaker antibody response                                                  | Gumber et al. (2009)        |
| 26  | MAP 2677                                                                    | Glyoxalase family                                    | MAP2677c was significantly recognized by Map infected BALB/c mice        | Gumber et al. (2009)        |
| 27  | MAP 3743                                                                    | Saccharopine dehydrogenase                           | Weaker antibody response                                                  |                             |
| 28  | MAP 1693c                                                                    | Peptidy-l-protyl-cis–trans-isomerase                  | Significant levels of antigen-specific antibody were induced in C57BL/6 mice in response to MAP1693c |                             |
| 29  | MAP 2513                                                                    | Alkanal monoxygenase alpha chain                     | Significant levels of antigen-specific antibody were induced              | Mon et al. (2012)          |
| 30  | MAP 2020                                                                    | Cutinase                                             | Significant levels of antigen-specific antibody were induced              |                             |
| 31  | MAP 0038                                                                    | Hypothetical protein                                 | Significant levels of antigen-specific antibody were induced              |                             |
| 32  | MAP 1272                                                                    | NLP/P60 family protein                               | Significant levels of antigen-specific antibody were induced              |                             |
| 33  | MAP 0209c                                                                    | Peptidoglycan biosynthesis                           | Significant levels of antigen-specific antibody were induced              |                             |
| 34  | MAP 0210c                                                                    | P36/P34 precursor                                    | Significant levels of antigen-specific antibody were induced              |                             |
| 35  | MAP 0217                                                                    | Secreted proteins                                    | Weaker antibody response                                                  | Thakur et al. (2013)       |
| 36  | MAP 1508                                                                    | ESAT-6 family member proteins                        | Significant levels of antigen-specific antibody were induced              |                             |
| 37  | MAP 3701c                                                                    | Lactacy protein                                      | Significant levels of antigen-specific antibody were induced              |                             |
| 38  | MAP 3783                                                                    | Hypothetical protein                                 | Significant levels of antigen-specific antibody were induced              |                             |
| 39  | MAP 1690c/Ag858                                                              | Antigen Ag858                                        | Weaker antibody response                                                  | Gumber et al. (2009)        |

Screening for antibody responses to a panel of 96 recombinant MAP antigens (Gumber et al. 2006). Antibody responses were detected as early as 70 days post-infection. However, fluctuations in antibody responses and epitope specificity were observed over 321 days (Gumber et al. 2006). Some studies suggest that secreted proteins may be better as solid-phase ELISA antigens resulting in a more sensitive assay (Cho & Collins 2006; Willemens et al. 2006). Pradenas et al. (2009) reported that most CF proteins have low molecular weight and reacted strongly with sera from culture-positive cases of pTB. They observed a high degree of difference in CF protein immunoreactivity among MAP-infected animals. Sera from cattle with clinical pTB or heavy faecal shedders of MAP reacted more intensively to CF proteins. Infected goats and sheep sera showed reactivity with CF proteins tested in immunoblots. These results suggested that a
cocktail of CF proteins of MAP could be good candidates as antigens for serodiagnosis of paratuberculosis. Dheenadhayalan et al. (2002) have also reported the immunogenicity of five recombinant antigens, namely MAP2411, ClpP (MAP2281c), Ppa (MAP0435c), MAP0593c and GreA (MAP1027c). These were tested with sera from 41 sheep with known MAP infection and 41 non-infected control sheep. Two of these antigens, MAP0593c and ClpP, reacted against 58.5% and 46.3% test positive sera and 12.1% and 4.9% of the negative control sera, respectively. MAP2411 could not distinguish between MAP positive or negative serum samples. The specificity of these antigen candidates towards MAP was not reported, and hence it is difficult to evaluate their usefulness. An earlier study tested recombinant antigens of Ag85A, Ag85B, Ag85C and SOD in ELISA with sera from 60 MAP-shedding cows and 22 non-shedding cows (Shin et al. 2004). Antigens of the Ag85 complex and SOD showed high reactivity against sera from the MAP-shedding cows and little reactivity against sera from the non-shedding cows. Conserved proteins such as antigens of the Ag85 complex (Gumber et al. 2009) are found in all mycobacteria species. At the protein level, the three Ag85 components of MAP share 99% sequence identity with M. avium subspecies avium (Rosseels et al. 2006). Gupta et al. (2015) reported that the greater part of CF proteins had a molecular mass less than 70 kDa and suggested that these immunoreactive MAP CF proteins could be the potential targets for developing diagnostics against pTB with improved sensitivity and high specificity instead of whole cell sonicated crude protoplasmic extracts (PPA). However, antigen selection remains a challenge, as there is no single MAP-specific antigen that is recognized by all infected cattle, especially those in early and subclinical stages of disease. These data suggest the need for a standardized cocktail of antigens for incorporation into a single multiplex ELISA for detection at all stages of disease in infected animals.

6.2. Secreted antigen candidates for marker vaccine development

DIVA vaccine, also termed as marker vaccine, can induce an immune response which is different from that induced by conventional vaccine. DIVA vaccination is one of the most promising strategies based on subunit technology, where well-defined and highly purified recombinant proteins (antigens) can raise a protective immune response against infection. The development of an efficacious subunit vaccine (marker vaccine) based on recombinant secretory proteins of MAP needs a combination of antigens appropriate for the broad range of varied MHC molecules present in an immune-competent outbred ruminant population (Derrick et al. 2004; Cai et al. 2005; Sable, Verma, Behera, et al. 2005; Sable, Verma, Khuller, 2005; Grover et al. 2006). Immune responses to recombinant antigens of MAP have been studied and demonstrated the protective effects of recombinant MAP antigens in mice (Chen et al. 2008) and calves (Kathaperumal et al. 2008). In the latter study, protective efficacy of recombinant antigens was assessed for 85A, 85B, SOD and Map74F antigens in a goat model. While MAP is intracellular in nature, a Th1 response mediated by IFN-γ-secreting sensitized T-cells plays a major protective role similar to other mycobacterial infections (Flynn et al. 1993; Flynn and Chan et al. 2001; Koets et al. 2002; Skeiky et al. 2004). Here, recombinant secretory antigen-specific lymphocyte proliferation was detected after 3 weeks of booster vaccination, which was significantly higher in the vaccinated groups than control groups, indicating the induction of recombinant antigen-specific cellular immunity. Immunization of goats with the recombinant secretory antigens gave a sustained antibody response for a prolonged period. The study indicated that the recombinant secretory antigens stimulated both CMI and humoral immune response. After booster vaccination, antibody response was significantly increased in animals vaccinated with recombinant secretory antigens compared with unvaccinated control group. However, humoral response was higher in animals that received the antigens in combination with an adjuvant (oil emulsions, lipopolysaccharides, polymers, saponins, liposomes, cytokines, ISCOMs (immunostimulating complexes), Freund’s complete adjuvant, Freund’s incomplete adjuvant, alums, bacterial toxins) in comparison with animals that were only administered with antigens (Rajput et al. 2007). Multi-component subunit vaccine has given significant protection from MAP infection in terms of reduction of MAP bioload in target organs as compared to sham-immunized goats. It has been reported that a multi-component subunit vaccine provided a better immune response by inducing both Th1 and Th2-cell-mediated response where subunit vaccine only induced T-cell responses (Sable, Verma, Behera, et al. 2005; Sable, Verma, Khuller, 2005). Studies showed that vaccination of goats with cocktail of recombinant secretory antigens provided better protection against MAP infection (Kathaperumal et al. 2009). Lymph proliferative response, IFN-γ response, CD4+ T and CD8+ T-cell responses used to assess the protective efficacy of the recombinant antigens indicated a significant Th1 response in immunized goats. Evaluation of protective efficacy of recombinant secretory antigens of MAP (marker vaccine) measured by the absence of clinical signs, histopathology and bacterial load of tissues collected at necropsy has been considered to be the gold standard (Gilmour & Wood, 1991; Uzonna et al. 2003; Waters et al. 2003). In accord, tissues were analyzed for histopathological lesions and burden of MAP by culture, where a significant
7. Conclusions

pTB is an economically important devastating disease of domestic livestock, and all the developed and western countries have made a lot of efforts in terms of money and resources for the diagnosis, prevention and control of the disease in their livestock.

Different studies over a period of time in different countries have helped in the development of a wide range of diagnostic tests from time to time, including microscopy, culture, molecular detection techniques using various forms of PCR, in situ PCR (hybridization), histology as well as serology-based techniques. Herd-based diagnostic tests (screening tests) have been developed and used for the determination of the prevalence as well as management, control and eradication of MAP infection.

Vaccination strategy has a high probability of having positive effect on reduction in environmental contamination and in control of pTB in animals, thereby reducing the risk of human contamination. Vaccination-based immune responses can interfere with certain diagnostic tests, which makes it difficult to differentiate between infected and vaccinated animals. Using secretory proteins in diagnostics has enhanced the sensitivity and specificity of the diagnostic tests and helped to differentiate infected from vaccinated animals (DIVA). Standardized cocktails of secretory proteins for incorporation into a single multiplex ELISA will be helpful to make diagnostic tests more sensitive and specific at all stages of infection in animals. Immunization of animals with secretory proteins has shown improved cell-mediated and humoral immune responses. Various studies reported that subunit/cocktail vaccines of secretory proteins have not shown any side effects and interference with diagnostic responses. Compared with other cellular proteins, secretory proteins of MAP showed greater reactivity with serum from MAP-infected animals. Therefore, the use of secretory proteins in development of DIVA-based diagnostics and vaccines may aid in the immediate control and management of pTB, as traditional vaccines and diagnostics are unable to do.

Acknowledgments

The authors are highly thankful to ICMR Projects, New Delhi for strengthening facilities for research at CIRG, Makhdooom.
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