Paratuberculosis (Johne’s disease) is a chronic, infectious, granulomatous enteropathy caused in domestic and wild ruminants by Mycobacterium avium subsp. paratuberculosis (Map). Johne’s disease causes huge production losses and has high impact on animal industry. The control programmes are hampered by the lack of simple and efficient diagnostic tests, especially for subclinically infected animals. Various diagnostic tools are available like fecal culture techniques, culturing, radioisotyping, PCR based methods, serological approaches (Cell mediated assay and humoral based) and most new area are recombinant antigens based detection which can open a novel approach for early detection of the disease.

Key Words: Mycobacterium avium subsp. paratuberculosis; Recombinant antigen; PCR; ELISA.
genomic DNA. *M. paratuberculosis* possesses the specific insertion sequence IS 900 and the *M. avium* could be divided into two distinct biotypes according to the presence of IS 901. Thorel *et al.* (1990) proposed a classification of *M. avium* based on a large number of biochemical tests into three subspecies, viz., *M. avium* subspecies *paratuberculosis*, *M. avium* subspecies *avium* and *M. avium* subspecies *silvaticum* (wood pigeon mycobacteria). *M. paratuberculosis*, causative agent of paratuberculosis in the ruminants is now known as *M. avium* ssp. *paratuberculosis* (*M. a. paratuberculosis*) or simply Map, belonging to the *M. avium* complex (MAC) group of organisms. The disease is ubiquitous and distributed throughout the world including India. Numerous reports of the infection are available on cattle, sheep and goats. Motiwala *et al.*, (2004) have reported occurrence of the disease in wild ruminants and birds. To date no effective therapeutics or vaccines are available and early detection along with good management practices is the only way to control paratuberculosis (Harris and Barletta, 2001). Unfortunately, control programmes are hampered by the lack of simple and efficient diagnostic tests, especially for subclinically infected animals. Serological and cell mediated immunity based assays remain most promising but so far specific immuno-dominant antigens are lacking (Bannantine *et al.*, 2004). A postgenomic analysis of Map proteins identified specific antigens that could potentially improve the diagnosis of paratuberculosis (Leroy *et al.*, 2007). Currently used antigens for diagnostic tests of Map have variable success as regard to specificity and sensitivity. So there is no matter to surprise that presently used conventional methods for diagnosis are inadequate and unreliable. Advanced DNA technology has opened a new era for exploring various recombinant antigens of Map which can develop specific and sensitive diagnostic tests.

**Epidemiology and Pathogenesis**

Ingested Map bacteria enter the intestinal wall through the small intestinal mucosa, primarily in the region of the ileum, via M cells covering the Peyer’s patches (Tiwari *et al.*, 2006). Map are phagocytosed by resident subepithelial macrophages, where they are able to inhibit phagolysosomal fusion, resulting in resistance to intracellular degradation. While the bacteria are in the mucosal tissue and submucosal macrophages, there is little or no detectable immunological reaction to the infection (Tiwari *et al.*, 2006). The delayed humoral immune response is one reason for the poor sensitivity of serological tests for Map. Eventually the infected macrophages migrate into local lymphatics, spreading the infection to regional lymph nodes. In the regional lymph nodes, the organisms are capable of stimulating inflammatory and immunological responses. There are several characteristics that animals affected by Johne’s disease can display sub-clinically and clinically. In the initial stages of disease in deer, the animal fails to thrive, stops growing and the weight and condition both decrease. In spring the affected animal only partially moult. A large part of the animals affected by the disease also exhibit diarrhea. The total time span of the clinical stage of the disease is a couple of weeks. There is a variation between younger and older animals. Animals which are infected at a younger age seem to die earlier of the disease (Mackintosh *et al.*, 1998). Johne’s disease causes significant losses due to clinical disease in deer <15 months of age (Machackova *et al.*, 2005). The most common route of infection is ingestion via contaminated pasture, food and water contaminated by Map infected faeces (Machackova *et al.*, 2006). To prevent infection, it is recommended that sheep or cattle should not be grazed on the deer farm, unless they are known to be ‘negative animals’. Other measures include keeping a closed herd and the use of artificial insemination. Better control of this disease requires a greater knowledge of the biology of Map, its epidemiology, interspecies transmission, strain diversity and contribution of wildlife reservoirs of disease (Thibault *et al.*, 2007).
Diagnostic methods

Conventional tools

Overview of diagnosis of Map

When it comes to (post mortem) JD diagnosis, culture of the organism from tissue samples taken at necropsy is still widely considered as the ‘gold standard’ reference test as, even before faecal shedding occurs or a humoral immune response has had a chance to develop, highly sensitive, radiometric culture techniques (BACTEC) are capable of detecting growth of Map in multiple organs including the intestinal mucosa and submucosa and regional lymph nodes (Whipple et al., 1992, Whittington et al., 1999). BACTEC culture provides a growth index of viable organisms and the number of viable organisms (Reddacliff et al., 2003). However, as Map grows extremely slowly in culture, it can still require a period ranging from weeks to months to obtain results from culture and, other than cost, this is considered to be the primary limitation of bacterial culturing as a diagnostic method. Ante mortem tests include faecal culture, serological immunoassay to detect reactivity to mycobacterial antigens by ELISA and, increasingly, nucleic acid amplification based approaches to detect mycobacterial DNA in samples. Molecular analysis of nucleic acids using Polymerase Chain Reaction (PCR) assays have been applied for detection and of Map in faecal samples, milk and tissue (Kawaji et al., 2007, Cook and Britt 2007, Schonenbrucher et al., 2008). These PCR assays have shown to be sensitive and specific for the detection of MAP and have reduced the time for detection to 2-3 days compared to other diagnostic assays, including enzyme-linked immunosorbent assays (ELISA) and faecal culture, which are limited by lack of sensitivity, specificity and long incubation times (Cook and Britt 2007, Schonenbrucher and Britt, 2008).

Direct microscopic methods

In direct method, clinical samples are examined for detection of organisms. Fecal sources are the main route of disease transmission and thus first choice as clinical sample. Staining of fecal samples for detection of acid fast bacilli may reveal mycobacterial bacilli, but the sensitivity of this reaction is very low as it is difficult to distinguish accurately, Map from nonpathogenic mycobacteria (saprophytes). As faecal contamination is considered one of the primary routes of infection, it means that when the animal is a high shedder, it can take 2-4 months before the actual cause of JD is determined and by that time the animal is responsible for significant faecal spreading of MAP on the pasture giving the opportunity to other animals in the herd to become infected. A faster method of diagnosis is necessary to prevent spreading of the disease (Cook and Britt, 2007).

Culturing of the organism

Collected fecal samples are treated in such a way that it kills all other organisms leaving Map live for culture. The main constrains with conventional culturing system is that, it takes around 12-16 weeks to detect the growth also diverse strain of the species required different growth media for their propagation in in-vitro culture. Clinical samples including feces and tissue samples like intestine, Mesenteric lymph node, liver, testes, udder, uterus etc. though blood and milk can also be used to identify Map from infected animals. Herrold’s egg media or modified Lowenstein-jensen media supplemented with iron chelator especially mycobactin are preferred by diagnostics laboratories to isolate Map (Whipple et al., 1991). By culturing techniques we can detect infected animals shedding more than 100 CFU/g of feces. Disadvantages with this method are long incubation period (2-4 months), lack of reproducibility and non homogenous distribution in feces.

Radioisotopic culture

In this method the culture can be radiolabelled with radioisotope indicator system along with antibiotics and permits detection of as few as 3 organisms per gram of sample. By this technique we can detect the organisms within few weeks to few days depending on the load of
organisms in the samples. Some examples of this radiometric culture are BACTEC 12B and BACTEC 460. Disadvantages associated with BACTEC method are, it is expensive, requires sophisticated instrumentation and it is hazardous due to complicity of radioisotope cultures.

**Molecular tools**

*Molecular tools*

*Polymerase chain reaction (PCR) based diagnosis*

Various mycobacteria infection can be diagnosed by PCR (Hawkey, 1994). It provides a rapid and specific detection of Map within 24 hours to 2-3 days in some cases. It is reported that due to presence of PCR enzyme inhibitor sometime detection of IS gene probe become difficult (Stevenson et al., 1997). Again the genetic probe based on 16s rRNA is identical for both M. paratuberculosis and M. avium and so it could not be considered as specific probes for paratuberculosis detection. Although an improved version of immunomagnetic PCR is available for diagnosis of Map in milk samples (Grant, 1999) still PCR is less sensitive than culture when applied to screen clinical samples either due to presence of inhibitory substances or non recovery of DNA. Another aspect is nonspecificity of IS 900 due to presence of IS 900 like sequence in non-Map mycobacteria (Collins, 1996). Most progress made in improving diagnosis has been made in direct detection of Map by virtue of the specific sequence IS 900 (genetic probes) and advent of gene amplification techniques like PCR. The IS900 sequence is the most commonly targeted sequence for molecular confirmation of Map, despite some concerns that the sequence may not be 100% unique to Map (Englund et al., 2002). Due to homologous, IS900-like elements, in related mycobacterial species, cross-reactions can potentially result false-positive results in rare cases (Fang et al., 2002). The IS900 sequence is 1,451 base pairs in length and occurs in 14 to 20 copies per genom (Collins et al., 1989). For greater specificity of PCR of Map, other genes, which have a lower copy number in the Map genome, and thus a potentially lower analytical sensitivity, than IS900, such as F57 (Poupart et al., 1993), hspX (Bannantine et al., 1993), ISMav2 (Strommenger et al., 2001) ISMap02 (Stabel et al., 2005) and IS1311 (Kaur et al., 2010) have been suggested.. However, the reliability of these methods is dependent upon overcoming inherent shortcomings that nucleic acid-based methods have, namely cell lysis efficiency, DNA absorption to surfaces and PCR inhibition from co-extracted compounds present in environmental samples (Cook et al., 2007). Improved DNA extraction protocols, that effectively remove inhibitors will likely result in a increased sensitivity for PCR based methods (Schonenbrucher et al., 2008). PCR based tests are an attractive prospect in terms of speed and cost but, like any diagnostic assay, must be carefully controlled for both false-positive and false-negative results. For management of JD, early identification of heavy shedders from a faecal sample would be a useful tool in preventing further pasture contamination and spread of infection; such a quantitative assay may be accomplished with a quantitative PCR (qPCR) approach. Recently qPCR (Real time PCR) has been used to detect two different Map sequence viz. F57 and ISO900 in paratuberculosis infection in Deer (Buijs 2009). A qPCR assay should fulfill criteria such as a high detection probability in the tissue to be examined, straightforward sample preparation and DNA extraction, high specificity, robustness, reproducibility and working with a user-friendly protocol (Bustin et al., 2005). While several articles have suggested that PCR analysis cannot compete with the sensitivity of faecal culture of Map (Taddei et al., 2004). Others report that detection of MAP in ovine faeces by direct quantitative PCR has similar of greater sensitivity compared to radiometric culture (Kawai et al., 2007). As methodologies for extracting high-quality, inhibitor-free DNA from faecal material improve, sensitivity and reproducibility of faecal PCR diagnostic tests will likely improve.
Other Molecular Tools

With the development of standardized procedure of PFGE (Pulsed Field Gel Electrophoresis) it is now possible to characterize and phylogenetically analyzes Map (Hughes et al., 2001). PFGE also revealed two different types of organisms viz., type 1 (pigmented), natural host sheep, and type 2 (non-pigmented) which has broad range of hosts (Stevenson et al., 2002). Again it is demonstrated that different strain of Map like C1-C5 (cattle), S (sheep) and I (intermediates) have been described by means of RFLP (Restriction Fragment Length Polymorphism) using various enzymes (Whipple et al., 1990; de Lisle et al., 1992 and Baufriend et al. 1996). Patel et al. (2006) sequenced K-10 genome of Map by means of RNA isolation and DNA microarray as well as RT-PCR.

Serological approaches

In the infected individual, the level of humoral infection is related with fecal shedding of the organisms. In early stage of infection strong cell mediated immune response is elicited, which leads to a delayed type of hypersensitivity reaction, but when the disease is progressed gradually, CMI become fleeted and there is development of a strong humoral immune response and at the edge of final stage due to lack of antigen specific CMI, there is rapid dissemination of infection throughout the host (Benedixen, 1978). The AGID (Agar Gel Immunodiffusion) assay is an inexpensive method of serological diagnosis in ruminant paratuberculosis, but it can detect antibody only 3-9 months after shedding of microbes (Colgrove et al., 1989). As the specificity of the test is 100% it is a reliable test to detect Map. Another reliable serological test to detect Map is ELISA (Enzyme Linked Immuno sorbet Assay), which is superior to AGID as it has both high sensitivity and specificity. Numerous modified techniques of PCR are available for detection of Map infection. To reduce the chance of cross contamination it is necessary to characterise and isolate Map species specific antigens. In the terminal stages due to immune anergy, sensitivity of serological tests may be as low as 10 to 25% (Coussens., 2001).

Cell mediated immunity (CMI) based diagnosis

CMI assay involves Delayed type hypersensitivity assay; lymphocyte stimulating assay and gama interferon assay. As already cited, CMI response is present only in early stage of infection; tests mentioned above are negative during advance stage of infection. Skin test by intradermal inoculation using johnin is not so reliable test for detection due to low specificity. An increased thickness of skin at the site of inoculation (4 mm or more) within 72 hours is considered as positive. This test is less specific as well as less sensitive, also has poor correlation with the infectious status of animals (Hremel et al., 1998). Gamma interferons are better indicator of CMI assay for both research and diagnostic purposes, which is released by peripheral blood mononuclear cells in response to antigen. Gamma interferon can be estimated by various approaches like, ELISA using monoclonal antibody, enzyme linked immunospot (ELISPOT) (Lalvani et al., 2003) , RT-PCR based analysis. Two assays known as a bioassay (Wood et al., 1989) and sandwich enzyme immunoassay (SEIA) have been evaluated (Rothel et al., 1990). But some results indicated that this test is not so specific and reliable for diagnosis of paratuberculosis infection (McDonald et al., 1999 and Ridge et al., 1995).

Humoral immunity based diagnostic methods

This test having high specificity (>90%) in animals with clinical signs but due to lower sensitivity (30%), this test is not reliable for diagnostic purposes (Wood et al., 1989).Next of AGID is complement fixation test or CFT. The specificity of the test is less than AGID and ELISA. This test can detect antibodies 1-5 months later than ELISA (Singh et al., 2005) and it has intermediate sensitivity to AGID and ELISA. So CFT is not used in routine diagnostic tests.ELISA is one of the most valuable herd screening test for detection of paratuberculosis. Although the sensitivity of this test is higher in clinically infected animals but low at
initial and terminal stages of disease. It was showed that ELISA sensitivity for clinical and subclinical cases are 85% and 15% respectively (Wood et al., 1989). An indigenous ELISA by using protoplasmic antigen from Native “Bison type” strain of Map has been developed for screening of paratuberculosis infection in animals as well as humans (Singh et al., 2007). ELISA can be used for ‘herd screening test’ also. Pre absorption of sera with M. Pheli is a good candidate to increase assay specificity but reduces the sensitivity. It was reported that only 1/3 rd of animals shedding Map can be detected by present ELISA tests (Collins et al., 1989). So it is very much essential to search for Map specific antigens and to characterize them for sero diagnosis. A derivative of ELISA, the Paralisa test provides a customized ELISA, based on the antigen reactivity of the IgG1 isotype, for the serodiagnosis of Johne’s disease in deer and has a reported specificity of 99.5% and has a sensitivity of 84% with Purified Protein Derivative (PPDj) antigen, but the test is still 75% less sensitive for animals that Purified Protein Derivative (PPDj) antigen, but the test is still 75% less sensitive for animals that are positive in culture and have no detectable pathology pathology (Griffin et al., 2005).

Recombinant antigens based detection

A large scale post genomic analysis of Map proteins identified various specific antigens that could potentially improve the diagnosis of paratuberculosis. Cho et al., (2007) identified fourteen proteins of potential diagnostic value for bovine paratuberculosis. These proteins were ultimately designated by mass spectroscopy and BLAST analysis as ModD, PepA, ArgJ, CobT, Antigen 85c and nine hypothetical proteins. These 14 antigenic proteins from Map culture filtrate (CF) are good candidates as antigens for improvement of serodiagnostic tests for bovine paratuberculosis.

While screening genomic expression library with serum of naturally infected cattle, Willemsen and his colleagues identified and characterized three novel secreted Map antigens of 9, 15 and 34 kDa sizes (Willemsen et al., 2006). An absorption ELISA based diagnostic test using 34 kDa protein was established with high specificity and sensitivity which could be useful for screening of johne’s disease (Malamo et al., 2006). Carboxy terminal end of this 34 kDa protein is 100% specific for Map (Ostrowski et al., 2003).

Tizard MLV and colleagues (1992) reported two secretary proteins Ahp C and Ahp D having the ability to differentiate paratuberculosis and tuberculosis. Patients with chrone’s disease were reported to have higher antibody levels against a 14 kDa secreted antigen (Shin S J et al., 2004). Recently a highly specific and sensitive Ethanol Vortex ELISA (EV ELISA) was developed for diagnosis of paratuberculosis by using antigens extracted from surface of the organism. Study has shown that EV ELISA is subspecies specific and highly sensitive to detect early as well as late state of Map infections (Eda et al., 2006). Lpp34 is an unique membrane protein of Map, which is a putative lipoprotein (Gioffre et al. 2006). Lipoprotein have long been considered immunomodulators and mycobacteria are especially rich in these post translationally modified proteins. As Lpp 34 is an envelop protein of the bacteria, so it can perform numerous functions including adhesion to host tissues, nutrient acquisition and interaction with host defenses .These Lpp 34 lipoproteins can be used for diagnosis of Map specifically. Also a 35 kDa protein of Map was studied to check its ability to elicit CMI response using murine model (Basagoudanavar et al., 2006) and found that it could be used as a diagnostic test to measure delay type hypersensitivity response in paratuberculosis infection. It was also suggested that a 35 kDa based ELISA can be useful for detecting Map infection (Sung et al., 2005). A 19 kDa lipoprotein was also identified which can stimulate both T and B cell immune responses as well as induce a number of Th1 cytokines. In order to evaluate the Map 19 kDa lipoprotein can act as immune modulator in cattle with johne’s disease and has been shown to
stimulate CD4+ T cell proliferation as well as release of IFN-γ and IL-2. Acylation near the N terminal portion of the 19 kDa protein is believed to occur at amino acids 19-24 and contributes to its immunogenecity. Further more glycosylation of the Map 19 kDa protein inhibits innate immune response, such as release of TNF alpha, IL-6 and IL-10 from macrophages, but does not affect antibody binding. Huntley and his groups (2005) demonstrated that this 19 kDa protein can be used to assess cellular immune responses in subclinically infected cattle as well as humoral immune responses in cattle with clinical Johne's disease. A lipoarabinomanan (LAM) antigen which was extracted from the lymph node and milk of paratuberculosis infected sheep and goat, can be used to raise antibody and thus can be useful for serodiagnostic tests (Munjal et al., 2004). An exported 22 kDa putative lipoprotein was identified in an alkaline phosphatase gene fusion library of Mycobacterium avium paratuberculosis and expressed in Mycobacterium smegmatis to produce a C-terminal polyhistidin-tagged protein, which can elicit interferon gamma secretion in blood of vaccinated animals (Rigden et al., 2006). Recently Bannantine et al has characterised a set of Mycobacterium avium subspecies paratuberculosis recombinant proteins viz. MAP0107c, MAP3169c and MAP3640 which provides a powerful tool for proteome- and genome-scale research (Bannantine et al 2010).

With complete genome sequencing of Map K10, post genomic application will play a central role for detection of Map specific antigens. Results of comparative genomics and proteomics assay of Map culture extracts identified 25 Map diagnostic antigens (Bannantine and Paustian, 2006). In silico comparison of Map genome with other mycobacterial genomes discovered some Map specific protein antigens which reacted with sera from infected animals specifically (Cho and Collins, 2006). Genome sequencing of Map K10 had identified 3 Map unique IS elements viz. IS Map 2, Map 02 and Map 04. Comparative genomics based study also identified 17 Map unique large sequence polymorphism (LSPs) (Stevenson et al., 2002). Recently it has been reported that various PPE family proteins of Map are ideal diagnostic candidate genes for the detection of para tuberculosis infection (Huntley et al., 2005, Deb et al., 2010).

**CONCLUSION**

Conventional techniques to detect Map infection are either not precisely specific or lack an optimum degree of sensitivity which cannot be overlooked when screening of a herd is concerned. With the critical need for improved diagnostic tests to detect paratuberculosis infection, effort need to be concentrated on the development of simple, rapid, noninvasive tests that can perform without expensive laboratory equipment. In this context recombinant antigen based diagnostic techniques are very promising but to make it a successful tool search for a novel antigen candidate suitable for specific and sensitive diagnosis and/or vaccination appears to be a well justified approach and need for the hour.

**REFERENCES**

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