Review Article

Recent Approaches in Diagnosis and Control of Mycobacterial Infections with Special Reference to *Mycobacterium Avium* Subspecies

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ABSTRACT

In ruminants, *Mycobacterium avium* subspecies *paratuberculosis* (MAP) causes chronic persistent infection in intestine and is also known as paratuberculosis/ Johne's disease (JD). It is increasingly being recognized as a problem that adversely affects animal health leading to significant economic losses to livestock industry. Recent emerging evidences have established a link between MAP and inflammatory bowel disease (IBD)/ Crohn's disease (CD) suggesting its zoonotic importance. There are a range of tests (conventional, serological and molecular) to diagnose MAP infected animals / individuals and/or herds/flocks. However, sensitivity and specificity of these tests vary according to stage of the disease. The correct choice and application of each of these diagnostic tests depends on need and may allow establishing a control program. Many steps have been undertaken to control and eradicate the disease. However, no significant success have been achieved till date by controlling infection through hygienic management as well as culling of infected animals thereby increasing the necessity of vaccination. As MAP cannot be killed by pasteurization, both raw and pasteurized milk are considered as a vehicle of transmission of this bacterium to humans. Present paper comprehensively reviews the diagnostic and control strategies for infections due to mycobacteria specially giving importance to *Mycobacterium avium* subspecies *paratuberculosis*.

INTRODUCTION

Johne's disease (JD) is chronic, progressive, infectious granulomatous enteritis which affects ruminants and a variety of domestic and wild life species. In India disease has been reported from all the domestic ruminants [cattle, buffaloes, goats, sheep (Singh et al., 2010a), wild ruminants [blue bulls, hog deer, bison (Singh et al., 2010b, Singh et al., 2011a), other animals [rabbit (Singh et al., 2012b)], primates (Singh et al., 2011b), human beings (Singh et al., 2008), environment [soil and water resources (Singh et al., 2012a)], milk [raw milk, pasteurized milk and milk products (Shankar et al., 2010).

Johne's disease (JD) or paratuberculosis (Para TB) occurs due to *Mycobacterium avium* subspecies *paratuberculosis* (MAP). In 1895 the bacteria was isolated for the first time by Dr. Heinrich Albert Johne that was unable to multiply outside the animals in nature, but in contaminated soil or water, it survived probably more than a year as it could withstand heat; cold and drying. Two most common sign

of disease are progressive loss of weight along with diarrhea (mainly in cattle and buffaloes). Wasting is common in sick animals inspite of healthy appetite. Chronic diarrhea may lead to loss of protein in blood stream which may ultimately result in soft swelling near jaw (bottle jaw). Animals reaching this stage die quickly within a few weeks. Clinical signs until two or more years of infection may rarely be seen either during pregnancy or during parturition.

Clinically ill and asymptomatic animals are the primary source of infection. Transmission is primarily oral, through the colostrum and milk of infected animals, and through pasture and drinking water contaminated with feces. Several independent studies have established vertical transmission through placenta and semen (Lambeth et al., 2004, Singh et al., 2012a). The high density of animals and the poor hygienic conditions facilitate the spread of infection. Ubiquitous presence and high resistance of MAP to adverse environmental factors constitutes a risk for the spread of the disease. Paratuberculosis is a production

disease leading to heavy economic losses to farmers. The approximate economic loss due to paratuberculosis per sheep/farmer/year is around Rs 1,840 (US$ 38.33) in India (Vinodhkumar et al., 2013). There are evidences to relate MAP with human diseases like Crohn's disease (Greenstein, 2003) and type I diabetes (Naser et al., 2013, Sechi et al., 2008). As MAP is not killed by pasteurization (Grant et al., 2005), milk is considered as vehicle of transmission of MAP to humans. Control of JD is therefore critically important to safeguard productivity and human health. Control measures for paratuberculosis depend on diagnosis of animals. Present paper comprehensively reviews the diagnostic and control strategies applied to MAP.

Wild non–ruminant animals that are free–ranging in nature are the known potential vectors that transmit MAP to ruminants that are domesticated (Hutchings et al., 2002). It has been reported that there is frequent inter–species sharing of MAP (Buergelt and Ginn, 2000; Pavlik et al., 2000). The presence of PPE family of proteins in the organism leads to antigenic variation which may facilitate to invade the immune system. Clinical signs in endemic herds are developed by only few animals whereas majority of the animals remain either asymptomatic or remain as sub–clinical shedders. This results in economic losses and reduction in per animal productivity (Mohan et al., 2009, Deb and Goswami, 2010, 2011).

JD is spectral in nature presenting bacteriological as well as immunological and pathological spectra that leads to variation in efficacy of the methods of diagnosis at various points of time during the process of infection. The most specific though time consuming test is to culture the organism from faeces and tissues especially lymph nodes at the ileo–caecal junction and small intestinal tissues (Sockett et al., 1992; Cocito et al., 1994). A rapid diagnostic test is PCR that detects MAP in faeces as well as blood and tissues and the sensitivity can be compared with bacterial culture (Tripathi et al., 2002). In order to clear the infection, during early stage of infection, the host mounts a strong cell mediated immune response (CMI) and in the later stages humoral immune response (Clarke, 1997; Perez et al., 1999). Tests based on CMI response viz., Johnin purified protein derivative (PPD)–based cutaneous testing; gamma interferon assay along with lymphocyte stimulation test are found to be sensitive though not commonly used to detect MAP infection at the early stage (Molina et al., 1996; Storset et al., 2001; Kurade et al., 2004). The assays based on antibody include: complement fixation test (CFT); agar gel immunodiffusion (AGID) as well as enzyme linked immune sorbent assay (ELISA). There is thus necessity of evaluating the efficacy of the diagnostic tests that are commonly used to detect infection caused by MAP.

Stages of Johne's disease In Animals (Mainly Cattle):
Disease has following three stages.

Stage I
State I is a sub–clinical infection that is silent and cannot be clinically detected. It is seen in calves; heifers and young stock that are below two years of age and in animals that are adult having exposure to MAP with limited doses. At this early stage the infected animals are even difficult to detect with the diagnostic tests based on culture and serology. Progress of stage I to Stage II occurs slowly taking over many months or even up to years, depending upon nutritional status of animals and other stress factors (hygiene, over–crowding, pregnancy, parturition, lactation, other infections etc.).

Stage II
Stage II is also sub–clinical in nature occurring typically in heifers (old) or adult. At this stage the animals appear healthy but shed bacilli at adequate numbers in the manure that are detectable in fecal culture. At this stage some animals may be detected by culturing the organism from blood. Fecal culture may be used to confirm the animals that are positive in blood test (ELISA). Animals under this stage serve as a major source of infection (hidden threat) to the in contact susceptible animals.

Stage III
Stage III is advanced clinical stage of the disease. Stress, like parturition may often initiate the onset of the clinical disease. At this stage animals usually shed the watery pea–soup dung. There is progressive loss of weight with gradual drop in production of milk without any effect on appetite. Transient recovery may occur in few animals with relapse on exposure to another stress. Animals shed the bacilli in large amount (in billions) and hence most of the animals are culturally positive (fecal) at this stage. Most of the animals under this stage of infection can also be detected by serological tests like ELISA and AGID. Clinical signs persist for several months which may eventually result in death. Animals are usually slaughtered due prolonged emaciation before death. Emaciation with fluidic diarrhea is common in the terminal stage along with development of 'bottle jaw'. In the later part of stage III the carcass may fail to pass meat inspection for consumption by human.

A significant hazard to maintain and spread infection is animals that are sub–clinically infected. On introduction of a sub–clinically infected animal (that looks apparently healthy) may result in transmission of the disease in the naïve animals. MAP can spread quickly via feces after introduction of infected animal in herd. Bacilli are sheded in large number in feces that leads to feed and water contamination. Udder, milk, water and feed, when contaminated with manure, facilitate transmission of MAP via ingestion. Colostrum and milk serve as potential source of infection to young animals. Vertical (In–utero) transmission may also takes place.

DIAGNOSTIC METHODS
For detection of MAP, bacterial culture is considered as gold standard. However, because of the lack of shedding at early stage of infection this test does not allow accurate identification of truly infected animals. Tests for the diagnosis of paratuberculosis primarily depend on (i) direct detection of bacilli (MAP) by fecal microscopy, culture of the organism and immunofluorescence (infected tissues) (ii) detection of its genome in feces/tissue/blood by PCR. Indirectly, it can be detected by measurement of MAP–specific antibodies (ELISA) and T cells (lymphoproliferation assay). Additionally histopathology may be performed to visualize the degenerative changes in the infected tissues. Each of these tests has a variable sensitivity and specificity and hence it is necessary to carefully analyze the findings of each test independently.

Direct detection of MAP

Microscopy

Ziehl–Neelsen (ZN) or acid fast staining is based on the resistance of the Mycobacteria to decolorization by acid alcohol treatment. This method has the advantage of being simple, fast and inexpensive, but has the disadvantage of having low sensitivity and specificity. In cases of severe diarrhea, MAP concentration decreases relative to the amount of feces, thus increasing the likelihood of false negative results. Concentration of bacilli in feces by centrifugation and subsequent decontamination using decontaminant Hexa–decyl pyridinium chloride (HPC) improves the sensitivity of this test (Eamens et al., 1998). A similar condition occurs in animals with sub–clinical paratuberculosis (ParaTB), where there is low rate of fecal excretion. In smears or sections of tissues (intestines near ileo–ceccal valve or in the intestinal lymph nodes with gross lesions) visualization of groups of brightly pink colored bacilli within the resident macrophages in the lesions is highly suggestive of ParaTB.

Bacterial Culture: Liquid Media and Solid Media

Identification of viable MAP by bacterial culture is considered as reference diagnostic test or the 'Gold Standard Test'. The most appropriate specimens for bacterial culture are feces, milk and tissues (intestine near Ileo–Caecal Junction and mesenteric lymph nodes) (OIE, 2000). Due to the intermittent excretion of MAP, it is advisable to take serial samples over time (Pinedo et al., 2008). Bacterial culture requires at least 100 CFU/g of feces (minimum detection limit) (Merkal, 1970). Since this amount is exceeded by animals with clinical ParaTB, but not by sub–clinical low and / or moderate fecal shedders, only 15–25% of them can be detected by bacterial culture (Whitlock and Buergelt, 1996). Bacterial culture of milk from animals at this stage is difficult because bacterial excretion rate is 2–8 CFU/50 ml (McKenna et al., 2005). Culture from tissue specimens provides a sensitivity of 70% and a specificity of 95% (Tiwari et al., 2006).

In vitro cultures of MAP require supplementation of medium with mycobactin J. For cultures on solid media, Herrold egg yolk medium (HEYM) with mycobactin J, Löwenstein–Jensen, or synthetic media, such as Middlebrook (7H10 and 7H11) are commonly used. The colonies formed are small, hemispherical, about 1 mm in diameter, smooth and shiny with central nipple and spreading surrounding. While culturing on HEY medium, dis–colouration of the medium from green to yellow takes place before the actual appearance of MAP colonies. Primary isolation of MAP is very difficult and takes long time (atleast 6 to 8 weeks) despite supplementation with Mycobactin J. MAP bacilli initially depends on external source of mycobactin J but old cultures of MAP start producing Mycobactin. Many times the incubation period may take longer time if mycobactin J (the growth promoter) is old, though supplying companies never put expiry dates on it. The cultural criteria to identify MAP are the slow growth rate, the morphology of the colonies, acid fastness in ZN staining and dependence on mycobactin J (mainly in primary culture). Comparisons of culture media suggests that Middlebrook 7H10 medium is the best suited medium

to recover MAP in primary cultures (Sohal and L'Homme, 2012).

A preliminary MAP liquid medium (Middlebrook 7H9, 7H12) supplemented with oleic acid, bovine albumin, dextrose and catalase (OADC) may be used to accelerate the detection of MAP and the activation of MAP prior to the culture on solid medium. Automated systems for the detection of bacterial growth in a liquid medium have been developed. An example of this is the radiometric BACTEC 460 system (Becton Dickinson Inc.), which contains a precursor radiolabeled with C^{14} that detects changes in CO_2 concentration due to bacterial respiration. Other automated systems use fluorometric, barometric and colorimetric methods (Schwartz et al., 2000, Stich et al., 2004). Different MAP strains have different nutritional requirements, hence, it is recommended to use multiple media for primary isolation of MAP; HEYM, Lowenstein–Jensen, and Middlebrook (Sohal and L'Homme, 2012). The use of these three media allows to detect 100% of type I/III strains and 98 % of type II strains (de Juan et al., 2006). It is known that different bio–types of MAP (Cattle type, sheep type, bison type, human type) have preference for different culture medium and among MAP biotypes, 'sheep type' bio–types are most difficult to isolate in primary culture and so are the human strains. Author has the experience of Cell Wall Deficient (CWD) colonies when medium gets dry. These CWD colonies look dew drop like and cannot be captured in camera. These CWD colonies appear late than usual colonies and on further incubation grow translucent and opaque like usual MAP colonies (Manning and Collins, 2001).

The advantages of bacterial culture are the accurate diagnosis by isolation of MAP and also allow classification of the animals according to their level of excretion. The disadvantages are the high cost and the long incubation period that causes a significant delay in taking control measures. The use of automated systems such as BACTEC MGIT 960 shortens the time of detection (4 to 7 weeks) and can detect as low as 10 CFU/ml (Shin et al., 2007). Automated systems require special equipment, specialized personnel and antibiotic combinations, all of which increases the costs.

Concentration of MAP and Diagnosis

Concentration of microorganisms in the sample is an important variable for isolation methods. Several bacterial concentration techniques, such as centrifugation, sedimentation, filtration and immune–magnetic separation (IMS) have been described. According to studies by Grant et al. (2000), centrifugation at 2500 x g for 15 minutes yields the highest concentration of MAP in the sediment (69.4%), 17.6% remaining in the whey portion and 13% in the fat. Filtration is based on the tendency of MAP to form clumps larger than other bacteria and fungi, which allows a good differential concentration. IMS is based on the use of magnetic nano–particles (beads) bound to anti–MAP antibodies (Ab) that interact with surface antigens, allowing the separation and concentration. This technique has been used especially in milk samples where MAP concentration is relatively low (Gilardoni et al., 2009). Magnetic separation has also been developed based on a characterized peptide aMptD of MAP, which, bind to magnetic bead (Stratmann et al., 2006). Recently, Foddai et

al. (2011), used beads coated with the specific peptides biotinylated aMp3 and biotinylated aMptD in spiked milk. With this technique they showed a sensitivity that reached 85 % to 100% in capturing MAP.

Fluorescent Microscopy

Fluorescent antibody test (FAT) of fluorescent microscopy is based on the use of a fluorigenic compound, which, by enzymatic action in viable cells, transformed into carboxy–fluorescein. This method has been used to detect very low (-10^2 CFU/ml) number of organisms present in the pasteurized milk with a sensitivity of 73% (D'Haese et al., 2005). The FAT may also be useful for confirmation of the MAP in tissues specimens.

Bioluminescence

Oxidation of protein luciferin is catalyzed by the enzyme luciferase, resulting in oxyluciferin. This reaction requires ATP, which is provided only by viable bacteria. Luriferase based method has been proposed for rapid detection of MAP (Sasahara et al., 2004).

Molecular Techniques

Detection of MAP–Specific Gene Segment

The characterization of the IS900 insertion sequence which has 1,451 base pairs and is present with 15 to 20 copies in the MAP genome has enabled the specific identification of the bacterial DNA by the polymerase chain reaction (PCR). Although some studies have described elements similar to IS900 (IS900–like sequences) in other bacterial genome but they can be differentiated through the characterization of the amplified segment by sequencing or genotyping by methylation–restriction (Mundo, 2005). PCR may be performed in the specimens like feces, milk, blood and tissues (ileum, or jejunum, or jejunal near ileocecal valve, or ileocecal lymph nodes). Care should be taken to avoid contamination with PCR inhibitors which could cause false negative results.

PCR has become popular alternative to culture; however, sensitivity of the conventional PCR is hampered by factors like inhibitory substances, low load of MAP in clinical samples (feces, stool, milk, tissues, semen, vaginal discharges), handling clinical samples (feces, milk, tissues) which are highly contaminated with other microbes and materials etc. Hence variants to conventional PCR have been proposed like; i) nested PCR, which involves two rounds of amplification of the same sequence with different primer pairs each and thus allows to increase the sensitivity of the reaction; ii) multiplex PCR, which uses several pairs of primers in the same reaction to amplify multiple target sequences of the bacterial genome simultaneously; iii) real–time or quantitative PCR (RT–PCR), which uses a fluorochrome–labeled probe complementary to an intermediate fragment of the target sequence that is amplified. The quantification of fluorescence emitted during each PCR cycle is proportional to the amount of DNA. Other variants are the PCR amplification system called loop–mediated isothermal amplification (LAMP), which does not require the use of a thermocycler (Enosawa et al., 2003), and the triple real–time PCR (TRT–PCR), designed by Irenge et al. (2009).

The advantage of PCR is the timely detection of MAP, without the need of viable bacteria in the sample. The advantage of RT–PCR is that it allows the immediate observation of the target amplification, quantification and has greater sensitivity than bacterial culture (Bogli–Stuber et al., 2005). The use of IS900 in this type of PCR is sensitive to detect very low numbers of MAP, but is inadequate for accurate quantification of CFU in the sample, since it is present in many copies within the bacterial genome. LAMP, on the other hand, has high sensitivity and specificity, is not laborious, and does not require special equipment, all of which make it an inexpensive diagnostic tool. The disadvantage of PCRs is their high cost. To avoid any possibility of false positive (contamination) and / or of false negative (inhibitory components of the polymerase), appropriate negative and positive controls should run along with each batch.

In PCR, isolation of DNA from clinical samples is very crucial and for this purpose each laboratory has to standardize its own protocols (Fernandez–Silva et al., 2011; Kaur et al., 2011; Gupta et al., 2012). Since number of MAP bacilli are usually low in clinical samples (blood, milk, feces, vaginal discharge, and semen). The advantage of MAP is that the DNA can be also be isolated from decontaminated sample (by freezing and thawing) for direct PCR (Singh et al., 2007a). Similarly there is concept of free DNA in culture medium (liquid and / or solid) which may be exploited to investigate presence of MAP DNA in biological fluids. Isolation of DNA by scratch method can have added advantage of isolating DNA directly from slides.

In– Situ PCR

In situ PCR may be used to detect MAP–specific sequences in formalin fixed tissue samples. This method may also be useful for the detection of spheroplasts and for the detection of the DNA inside Mycobacteria infected tissue (Delgado et al., 2011).

PCR–Restriction Enzyme Analysis (REA)

In order to identify MAP sources and the pattern of transmission for improving JD control programme molecular technique like PCR–REA is a powerful tool. It has been implemented in the recent past for improving as well as for simplifying traditional biochemical as well as molecular methods. The discriminatory power of single nucleotide polymorphism (SNP) has been particularly used in PCR–REA. Two particular gyrB gene fragments that carry polymorphism that is type–specific in nature have been amplified specifically (Whittington et al., 2000; Castellanos et al., 2007, 2010).

Multiplex PCR

The Luminex technology has got the capacity to test unto hundred analytes. On the basis of IS900 and IS901; IS1245 as well as dnaJ gene a multiplex PCR has been developed allowing the detection of certain other species of Mycobacterium along with MAP viz., Mycobacterium hominissuis; M. silvaticum etc. This multiplex PCR has got a sensitivity of 10^3 colony forming units (CFU) for each of the strain of bacteria in a single reaction of PCR. This has also enabled this test's use for DNA isolation directly from the tissues of the sheep that are heavily infected (Gastaldelli et al., 2010, Moravkova et al., 2008).

In– situ RT– PCR

In–situ RT–PCR is having high specificity enabling detection of transcripts in the cells that originally lead to their synthesis and capable to detect mRNA expression inside

mycobacteria infected tissue. *In–situ* method is also popular and frequently used for the research purpose by other workers in mycobacteria infected tissue. (Chen and Fuggle 1993; Nicol et al., 2008; Rocca et al., 2010).

Cell Concentration and PCR

The use of IMS prior to PCR in milk samples has a sensitivity of 100.0%, while the sensitivity of PCR alone is of 23.0% (Gilardoni et al., 2009). The advantage of this technique is increased specificity in the concentration of the microorganism, allowing high repeatability of the assays, and the elimination of potential polymerase inhibitors in the samples.

In– Situ Hybridization (ISH)

ISH is a molecular technique that uses a labeled probe to specifically DNA or RNA on a histologically processed tissue section, allowing their tissue localization. ISH in MAP diagnosis uses a specific DNA probe. Small probe easily penetrates tissues and reaches the target sequence, but may induce no specific reactions or weak staining that may impair the reading of the assay. In contrast, a larger probe may have difficulty in penetrating the tissue and finding the target sequence. ISH is a technique that has been used to detect spheroplasts in animal samples and samples from Crohn's disease infected patients (Secchi et al., 2001).

Histological Diagnosis

Histopathological Diagnosis

Tissue samples can be obtained from distal portions of the ileum, ileocecal valve, mesenteric lymph nodes and biopsy or scraping of the rectal mucosa. Microscopically, the characteristic lesion is chronic diffuse catarrhal enteritis characterized by hyperplasia of macrophages, lymphocytes, plasma cells, epitheloid and giant Langhans cells (multinucleated) in the lamina propria, intestinal sub–mucosa and para–cortical region of regional lymph nodes, atrophy and fusion of intestinal villi with thickening of the mucosa. In some cases, granulomatous lymphangitis can also be observed. In the lymph nodes, the sub capsular and peritrabecular cortical sinuses contain numerous macrophages. Microscopic observations after ZN show acid–fast bacilli, in clumps or within macrophages. The advantage of the histopathological diagnosis is that it allows to identify animals with focal lesions associated with sub–clinical stages, whose fecal and / or milk excretion is insufficient for bacterial culture or PCR (Waller, 2000). However, its disadvantage is that it requires trained personnel for sample study.

Immuno–Histochemistry (IHC)

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

Indirect Diagnosis: Host Immune Response

The indirect diagnosis depends on the stage of disease. Sub–clinical stage 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. The three phases of disease depend on many factors. Some of them are:

Cellular Immune Response

Diagnostic tests that evaluate this response are the intradermal reaction (*in vivo*) and the detection of gamma interferon production (*in vitro*). The discriminatory power of both tests is low due to their cross–reaction with other environmental mycobacteria.

Intradermal Reaction (IDR)

The test is performed by intradermal inoculation of PPD–J (Johnin). The skin thickness is measured with a caliper before and 72 hours after inoculation. An increase in skin thickness greater than 2–3 mm is considered positive. IDR has an estimated sensitivity of 54.0% and a specificity of 79.0% (Kalis et al., 2003). The advantages are that it is easy to perform in the field, and that there is a chance of early detection of infected animals. The disadvantages are its low sensitivity and its low specificity (due to probable cross–reactions). Modification of Johnin and Tuberculin tests (Double intra–dermal Tuberculin / Johnin) and intra–venous Johnin have also been used for the diagnosis of JD in domestic livestock. However, there are complications regarding the interpretation of the results of skin test. The effect of various cut–off values while conducting skin test have not been recorded. The test performance moreover may be affected significantly by subtle difference in antigen occurring in several batches of antigen. For this reason research is further required for increasing the skin test's value (Whittington and Sergeant, 2001; http://www.scahls.org.au/asdt.htm).

Interferon Gamma (IFN–γ)

This test evaluates the specific production of cytokine IFN–γ by T lymphocytes after stimulation with PPD. In animals with the sub–clinical stage, the sensitivity of this test is higher than that of the serological tests, but low in absolute terms (41.0%) (Gwozdz et al., 2000a). The advantage of the IFN–γ test is the significant secretion of IFN–γ during the early stages and may be used to detect animals in the sub–clinical stage. However, it has several disadvantages: i) the possible cross–reactions, ii) the need to process the sample quickly since cells must be alive, iii) its high cost and iv) its low sensitivity (Stabel and Whitlock, 2001).

Lymphocyte Transformation Test (LTT)

Peripheral blood mononuclear cells' (PBMCs') ability in recognizing and responding to MAP antigen can be investigated at different days post vaccination when pulsed with protoplasmic antigen. The Simulative Index value of vaccinated goats was significantly higher at 30 DPV & onwards (p<0.05) at 95% confidence interval. There is considerable variation in the lymphocyte transformation response in case of animals that are heavily infected when antigenic stimuli are given and consistent unresponsiveness is shown by certain animals. In animals that are uninfected, the number of positive reactions recorded depends on the population. The greater proportion is found in herd that has got a proven history of paratuberculosis (Stabel and Joff, 2004; Singh et al., 2010a).

Humoral Immune Response

Later in the disease process high concentration of antibodies against MAP can be detected.

Complement Fixation
This test can identify animals with clinical signs, but not specific enough to be used in control programs.

Agar Gel Immuno–Diffusion
AGID is based on the precipitation of immune complexes formed by the antibodies of infected animals with a soluble antigen from a protoplasmic extract of MAP in a gel matrix of agar. It is a simple, fast and relatively inexpensive method, but has low sensitivity in the early stage of the disease (Ferreira et al., 2002).

Enzyme–Linked Immune–Sorbent Assay
ELISA is the diagnostic test most commonly used for serological diagnosis of JD. It can be applied in the specimens from blood, serum and milk. When ELISA yields positive results in apparently healthy or low–prevalence herds, a bacterial culture should be carried out to confirm the stage of infection.

In order to avoid false negative results, the animals should be re–examined in 6–12 months. The ELISA results are categorized as negative, positive or suspicious according to their optical density (Kalis et al., 2002). Semi–purified PPA harvested from native strain (S 5) of MAP of goat origin has since been compared with commercially purified PPA of cattle origin (Allied Monitor Inc., USA). Purified PPA showed significantly lower (›11.0%) sensitivity as compared to 'indigenous ELISA' but specificity was comparable (Singh et al., 2007b, Singh et al., 2009). Low sensitivity of commercial PPA may be due to the fact that it was of 'cattle' origin from USA. Indigenous (India) ELISA kit has also been compared with commercial ELISA kits (ID Vet and Pourquier ELISA kits, France). Similarly, these commercial ELISA kits had low sensitivity but specificity was high (94.7%). This was due to absorption of test serum with *Mycobacterium phlei* in commercial ELISA kits. The pre–absorption of sera with *M. phlei* improves the specificity (Singh et al., 2009). The sensitivity of ELISA in serum of animals is 7.0% in the silent stage, 15% in the subclinical stage, and 85.0% to 98.0% in the clinical stage (Gilardoni et al., 2012). ELISA test has several advantages, such as easy automation, repeatability and possibility to evaluate multiple samples together. It has very good sensitivity and specificity in clinical stages and is relatively inexpensive. The disadvantage is that the antigenic variability in different ELISA tests of serum and the different ages of the animals

tested can lead to errors in sensitivity and specificity (Collins, 2002). It has been found that there is high chance of higher agreement of serum and milk ELISA test as compared with serum ELISA and faecal PCR test. Due to the differences in days after parturition less correlation has been found in certain cases between serum and milk ELISA. In order to detect anti–MAP antibodies, both milk as well as serum ELISA have been found effective (Klausen et al., 2003; Hendrick et al., 2005; Salgado et al., 2005; Duthie et al., 2007).

Flow Cytometry
This technique allows detection of subclinical infection. 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 expensive and requires sophisticated equipment.

Control of Paratuberculosis
Controlling paratuberculosis is a major challenge as the organism is ubiquoteously present and is able to survive for extended period of time even under extreme environmental conditions as well as its ability to infect multiple hosts species (Pickup et al, 2006). The inability of the diagnostic tests to detect the animals with subclinical infection also possesses a major challenge. Currently most of the practices to control JD rely on management interventions designed for limiting the introduction as well as transmission of the pathogen. The factors at the herd level include: strength of herd and their replacement; common source of water and manger; common housing; manure contamination of feed and water as well as calves, washing of udder before milking and type of housing (Ott et al., 1999; Pence et al., 2003).

Tuberculosis in bovine (in animals most important mycobacterial infection) even though has been controlled with success from almost all over the developed world (Bastida and Ramon, 2011), but inspite of large efforts to control, in several countries, paratuberculosis (the other important one) remains a problem unsolved. Many initiatives have been undertaken to control and eradicate paratuberculosis and there are 3 approaches followed: 1) restriction of transmission of MAP through change in management, 2) test and cull practices for eliminating infection source and, 3) vaccinating replacers for increasing their resistance to infection. The advantages and disadvantages of such strategies are discussed.

Sr. No.	Name/ Kind of Vaccine	Country	Species	Reduction (%)	Reference
1	Laboratory Scale (Live)	USA	Cattle	81.47%	Larsen et al., 1974
2	Fromm (Killed)	USA	Cattle	99.1%	Hurley et al., 1983
3	Laboratory Scale (Live)	Denmark	Cattle	92.9%	Jorgensen, 1983
4	Laboratory Scale (Live)	France	Cattle	81.6%	Argente, 1992
5	Phylaxia (Killed)	Hungary	Cattle	94.7%	Kormendy, 1994
6	Neoparasec (Live)	Germany	Cattle	86.8%	Klawonn et al., 2002
7	Lio–Johne (Live)	Spain	Sheep	80.8%	Aduriz, 1993
8	Laboratory Scale (Live)	Greece	Sheep	93.2%	Dimareli–Malli et al., 1997
9	Gudair (Gudair)	Australia	Sheep	90.0%	Eppleston et al., 2004
10	Laboratory Scale (Killed)	India	Goat	82.1%	Singh et al., 2007c

Table 1: Effects of vaccination of shedding of bacilli

Management Change Practices
Management Changes focus on avoidance of contact between infected and healthy animals and decrease load of environmental MAP. Such measures include separation of offspring from dams right after birth; feeding of

paratuberculosis free milk replacer to the calves, raising the heifers (replacement stock) separately, avoidance of manure fertilization of grazing lands of replacement heifers, improvement of farm hygiene in general and elimination of practices that can bring foods or materials that are in the

vicinity of the susceptible/ healthy animals. Further, introduction of new animals should not be neglected and diagnosis of animals and the status of paratuberculosis of herd of origin must be taken into consideration. The major setback to these practices is the MAP's ability of surviving in the environment for extended periods (Whittington et al., 2005). Economic constraints of farms may further limit the success of this approach. Such measures moreover did not provide immediate results (Muskens et al., 2003; Ansari–Lari et al., 2009). Therefore, this kind of strategy is unlikely to be successful in Indian management conditions.

Test and Cull Strategy

Low sensitivity of diagnostic methods is the major setback to this strategy and is expensive on farmers. If the culling is based on fecal culture, there is chance of missing many infected animals as false negatives. Culture may take several week to detect positive animals and during this period infected animal will continue to shed MAP in environment. Disease control and its progress is hence sluggish which is often disappointing as positive animals appear regularly over the years even after removal of all the animals showing clinical disease. ELISA is a cost–effective diagnostic test; however its with sensitivity and specificity is also compromised. Therefore, the decision on animals for culling will always suffer with suspicion on false positive. Another problem is that various diagnostic ELISA kits available commercially have variable specificity and sensitivity (Dieguez et al., 2009, Singh et al., 2007b) thus severely affecting control programs severely. PCR detection based culling have also been approached. Improvements in PCR technique for low reagent prices, improved sensitivity and high throughput processing of samples have made this approach a fruitful strategy. Fecal culture has recently been replaced in Netherlands by tests based on PCR for the control of paratuberculosis (Bastida and Ramon, 2011). In the field, PCR and culling in not yet proven like ELISA and culling strategy.

Vaccination

Although cost-effective and considered as most effective way in controlling paratuberculosis (Fridriksdottir et al., 2000, Juste and Perez, 2011, Juste and Casal, 1993, van Schaik et al., 1996, Reddacliff et al., 2006). Further it has been suggested that vaccination may initiate the ending of the huge problem of paratuberculosis throughout the world perhaps marking the difference between doing nothing and making progress towards control globally (Juste et al., 2002). The main disadvantage of vaccination is that the vaccinated animals cannot be differentiated from the infected animals, and, moreover, interfering with serology of tuberculosis (Juste and Perez, 2011) In Spain, development of live vaccine kept ovine paratuberculosis in check (Juste and Perez, 2011). Goat vaccination against paratuberculosis seems currently in use in Spain, the Netherlands, France, Norway, and India (Juste and Perez, 2011).

Vaccination in Norway is interesting because that was the country where the first large–scale field studies on paratuberculosis control in goats was performed. According to a 1985 report, vaccination resulted in a 98% reduction in postmortem finding of lesions, which, during a period of 16 years, reduced incidence from 53% to 1% (Saxegaard and Fodstad, 1985). In the United States, there is a registered vaccine for use in cattle.

It is assumed that vaccination modifies the immunopathologic processes that lead to the persistent progressive regional intestinal inflammation responsible for clinical disease in such a way that immunized individuals are able to arrest the progression of the infection and the ensuing lesions. This results in reduction of the excretion of MAP and significant decrease in the severity of clinical signs and economic losses.

Vaccination also helps in reducing the environmental contamination due to MAP. In the vaccination study of van Schaik et al. (1996) the overall contamination reduction was 66.6%. This was due to decrease in the number of heavy shedders. In 49 trials of vaccination in sheep (different studies), positive effects of vaccination were seen in 94% of cases (Juste and Perez, 2011). In goats, positive effects in 92% of cases were reported in 24 trials (Juste and Perez, 2011). Table 1, 2 and 3 summarizes the effects of vaccination in different trials across world on various parameters in different species.

Studies have shown that vaccination is the best strategy to control paratuberculosis, because it yielded approximately 3 to 4 times better benefit–to–cost ratios than other strategies (Juste and Casal, 1993). Vaccine programs strongly suggested that vaccination has more a therapeutic than a preventive effect, as confirmed by the positive results obtained when vaccinating adults (Juste and Perez, 2011). Indian vaccination trials also confirmed the therapeutic effects of vaccination, however, these did not focused on difference in preventive and therapeutic values (Singh et al., 2010c, Singh et al., 2011b). Clinical cases are prevented by vaccination thereby leading to high production at a higher ratio of benefit–to–cost leading to contamination risk control (Singh et al., 2007c). Thus it is suggested that paratuberculosis control in small ruminants can be achieved easily via vaccinating (Kohler et al., 2001; Juste and Perez, 2011). Some studies on vaccinations in India have also shown promising results of 'Indigenous vaccine' produced from highly virulant 'Indian Bison Type native goat adapted' biotype of *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Vaccination significantly reduced morbidity, mortality and shedding of MAP, reduced clinical signs (diarrhoea) and enhanced flock immunity in the goatherds and sheep flock naturally infected and endemic for JD (Singh *et al.*, 2007c, 2010c, 2011c, 2013, Shroff et al., 2013). Therefore 'Indigenous vaccine' developed against Johne's disease was both 'therapeutic' and 'preventive' and routine use might reduce the overall disease burden. Further, a long term monitoring of the vaccinated animals is suggested to analyze the proper effects of 'Indigenous vaccine'.For getting best results from vaccination managemental practices for controlling the disease should also be followed. While planning a control programme it must be remembered that there may be interference with the diagnostic skin test for paratuberculosis and its outcome (www.oie.int).

Recent advances in diagnostics, prophylaxis/vaccination alternative therapeutic modalities (Paolicchi et al., 2003; Tripathi et al., 2006; Kumar et al., 2007, 2014; Munjal et al., 2007; Deb et al., 2011; 2013; Dhama

Sr. No.	Name/ Kind of Vaccine	Country	Species	Reduction (%)	Reference
1	Weybridge (Live)	UK	Cattle	99.06%	Wilesmith, 1982
2	Lelystad (Killed)	Netherlands	Cattle	91.82%	Kalis et al., 1992
3	Lio–Johne (Live)	Spain	Sheep	78.29%	Aduriz, 1993
4	Gudair (Killed)	Australia	Sheep	87.5%	Windsor et al., 2003
5	Neoparasec (Live)	New Zealand	Sheep	71.43%	Gwozdz et al., 2000
6	Laboratory Scale (Live)	Greece	Goat	82.78%	Xenos et al., 1988

Table 2: Effects of vaccination on production (mortality or clinical cases)

Table 3: Effects of vaccination on histological lesions

Sr. No.	Name/ Kind of Vaccine	Country	Species	Reduction (%)	Reference
1	Laboratory Scale (Killed)	Netherlands	Cattle	58.9%	van Schaik et al., 1996
2	Silirum (Killed)	Spain	Cattle	38.6%	Garcia–Pariente et al., 2005
3	Laboratory Scale (Killed)	Iceland	Sheep	93.5%	Sigurdsson, 1960
4	Lio–Johne (Live)	Spain	Sheep	100%	Aduriz, 1993
5	Mycopar (Killed)	USA	Sheep	75.3%	Thonney and Smith, 2005
6	Gudair (Killed)	Australia	Sheep	72.7%	Reddacliff et al., 2006
7	Gudair (Killed)	New Zealand	Sheep	75.5%	Griffin et al., 2009
8	Laboratory Scale (Live)	Norway	Goat	97.1	Saxegaard and Fodstad, 1985
9	Gudair (Killed)	Spain	Goat	65.8%	Corpa et al., 2000a
10	Laboratory Scale (Killed)	USA	Goat	66.6%	Kathaperumal et al., 2009
11	Laboratory Scale (Killed)	India	Goat	75.0%	Singh et al., 2007c

et al., 2008, 2013a,b,c,d, 2014; Mahima et al., 2012; Tiwari et al., 2013, 2014; Barad et al., 2014) need to be explored fully in the era of emerging antibiotic resistance. Along with these follow up of appropriate/effective preventive and control measures are very crucial for alleviating high financial impacts due to this important pathogen of animals having zoonotic concerns keeping in view One Health, One Medicine approach (Grant, 2005; Tripathi et al., 2002; Momotani, 2012; Sweeney et al., 2012; Dhama et al., 2013e).

Conclusion and Future Perspectives

Johne's disease is an economically significant disease in livestock which is responsible for considerable loss in the milk as well as meat industry. The economic significance of the disease has made the diagnosis as well as prevention and control of the disease important. Various studies have developed a wide array of diagnostic tests from time to time including faecal as well as tissue culture; necropsy; serology and histology; and molecular detection techniques including various forms of PCR and *in situ* hybridization. For determination of the prevalence of infection, various herd tests have been developed for instituting various control measures. It has been found that no single test is 100 per cent sensitive or specific. Therefore disease control by disposing off positive reactors depends on conducting tests repeatedly at half yearly or yearly interval. It is equally fruitful to remove the offsprings from female reactors. But within a herd without change in hygiene in the management of livestock such procedures are not successful in reducing the transmission of infection. Test and cull strategy has been followed but it is found that low sensitivity of various diagnostic methods is the major setback to this strategy as far as prevention of JD is concerned. Various studies have shown that vaccination is the best strategy to control paratuberculosis. Vaccine programs strongly suggest that vaccination has more a therapeutic than a preventive effect.

A long term monitoring of the vaccinated animals is suggested to analyze the proper effects of 'Indigenous vaccine'. Thus special attention is required in this aspect.

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