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REVIEW

Mycobacterium avium subspecies *paratuberculosis*: An Emerging Bacterial Disease of Global Public Health Significance

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ABSTRACT

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the etiological agent of chronic enteric disease of ruminant known as paratuberculosis (Johne's disease). The disease causes considerable economic losses worldwide due to reduced milk production and eventually, diarrhoea, weight loss and death. Johne's disease (JD) has some pathological similarities with Crohn's disease (CD)in humans, and the role of MAP in the causation of CD has been under investigation for last 100 years. Animals infected with JD shed viable MAP in the blood, and tissues.Consequently, transmission to humans may occur via consumption of animal derived foods. In developing countries, limited information is available on the occurrence of MAP infection in animals and humans. MAP infection has been established in animals and humans may get the MAP exposure through food chain or contaminated environment. Presently, MAP is of great public health significance because it is speculated to be involved in Crohn's disease in humans. The present review summarizes the information primarily on the nature of MAP in animals and humans, economic losses and morbidity and mortality due to JD and CD at global level. Current concept on the possible relationship between MAP and Crohn's disease has also been reviewed.

Key Words: Crohn's disease, Johne's disease,*Mycobacterium avium* subspecies *paratuberculosis*, Public Health, Zoonoses

Introduction

The genus *Mycobacterium* is primarily well represented by *M. bovis*, *M. tuberculosis*, *M. avium* ssp. *avium*, and *M. avium* ssp. *paratuberculosis* that are shared by different animal species including primates(Pal,2007; Wang *et al*., 2014). These pathogens resist adverse environmental conditions (Verma, 2013). *M. tuberculosis* is one of the most notorious human pathogens, infecting nearly one third of human population, and currently number one cause of deaths due to single infectious agent in the world (Verma, 2013; Pal *et al*., 2014). Recently, *M.avium* subspecies *paratuberculosis* (MAP) has emerged as a major and successful animal pathogen with significant zoonotic, and public health concerns since first observed by Johne and Frothingham in 1895 (Pal,2007; OIE,2008).*MAP* is an intracellular pathogen that causes Johne's disease(JD), a chronic (2 to 5 years) intestinal inflammation in cattle, sheep, goats, and wild deer etc. species including primates (Pal,2007;Wang *et al.,* 2014). JD is a chronic granulomatous bowel inflammatory condition in animals provoked by progressive weight loss resulting death.The World Organization for Animal Health (OIE) considers JD as a disease of major global importance. In addition, JD has been categorizes as a reportable disease, considered to be of socioeconomic and /or public health importance within countries and significant in the trade of animals and animal products (OIE, 2008). JD is prevalent worldwideand its prevalence is more frequently being reported in dairy cattle (Mendes *et al.,* 2004).

For every clinical case of JD it has been estimated that about four to eight other animals at farm level may be found positive for subclinical JDor for asymptomatic carriers. In both symptomatic and asymptomatic carriers, the organism multiplies in the mucosa and frequently shed in feces and milk as seen in clinically infected animals (Wang *et al.*, 2014). Newborns are most susceptible to infection, and generally get infected early in their life (2 months of age) through ingestion of contaminated feed, water or milk from their infected mothers (Wang *et al*., 2014). In-utero infection has also been reported (Cooney *et al.,* 2014). The disease represents a hidden threat for farmers. Although most animals get the infection at an early age, the onset of clinical symptoms usually delayed by several years (typically 2–5 years)due to long incubation period. In the intervening years between infection and clinical manifestation of JD, asymptomatic carrier animals can contaminate the farm environment and infecting other members of the herd (Wang *et al.,* 2014).

Clinical signs of the disease generally appear in adult animals, and appearance time depends on other stress factor (Herthnek, 2006). MAP has wide host range and able to survive outside the host for long time (more than 9 months) and therefore it seems more insidious than any other bacteria to human and animal health (Cooney *et al.,* 2014).

MAP is the established cause of JD in domestic and wild animals and has also been associated with the Crohn's disease in humans. There are evidences suggesting association of MAP with human CD have provided time-to-time (ISU, 2007). While this debate on etiology of CD is going on, MAP is insidiously entering in the human food chain and has been isolated from retailed pasteurized milk supplies, milk and milk products, meat and meat products, and water (Verma, 2013).

Crohn's disease with similar pathology to JD causes non-specific, chronic inflammation of the intestinal wall. It usually manifests itself in the terminal ileum. However, it may affect any part of the gastrointestinal (GI) tract, mouth, larynx, esophagus, stomach, and colon (OIE, 2008).

The clinical and pathological resemblance of CD and JD was first recognized in 1913 (Dalziel, 1913). The two diseases share common symptoms (chronic weight loss and diarrhoea), and common pathology

(ileum most frequently involved and granulomatous inflammation). However, the Opinions in the gastroenterology community remain divided about the role of MAP in CD (Sartor, 2005). The current thinking regarding the etiology of CD is that it is multifactorial with genetic predisposition (Nod2 mutations on human chromosome 16), exogenous factors (perhaps an infectious agent such as MAP) and host factors (e.g. 'leaky gut', vascular supply, hormones, and neuronal activity) acting together to induce a chronic state of dysregulatedmucosal immune function. Several expert groups (Austerman, 2012;Bannantine*et al.,* 2014; Ibrahim *et al.,* 2004)have reviewed the available scientific evidence (cultural, polymerase chain reaction (PCR) and serological) suggesting a link between MAP and Crohn's disease over recent years (Verma, 2013). The consensus opinion of all of these reviews is that the definitive evidence proving a causal relationship between MAP and Crohn's disease is not available. There is certainly evidence of an association (the occurrence at the same time and in the same patient of MAP and Crohn's disease) but not necessarily of causation (the organism has directly initiated the disease in the patient). Recent review concluded that, if MAP is involved in Crohn's disease, it is not mean that acting as a conventional infectious agent (Cooney *et al.,* 2014). The objective of this review is to know the nature of *Mycobacterium avium* subspecies *paratuberculosis* in animals as well as to assess the public health and economic importance of the disease and to clarify the current thoughts on the possible relationship between MAP and CD.

The Organism

Mycobacterium avium subspecies *paratuberculosis*is a member of *Mycobacterium avium* complex of *Mycobacteriaceae* family (Radostits *et al*., 2006). It is aerobic, short slender rod of 1-2 mm long and 0.5 mm wide, non-motile, Gram-positive,acid fast bacteria. MAP is extremely fastidious, and requires more than 8 weeks of incubation to grow in the laboratory (Bannantine and Stable, 2002). Since MAP fail to synthesize soluble iron chelating compound (mycobactin), cultivation media must be supplemented with mycobactin (Radostits *et al*., 2006). MAP like other mycobacteria have thick waxy cell wall containing 60% lipid layer, which confers it properties of acid fastness, hydrophobicity (Bannantine and Stable, 2002), resistance to chemicals e.g. chlorine and physical processes e.g. pasteurization (Bannantine and Stable, 2002). MAP shares some antigenic determinant and has similar characteristic with phylogenetically related *Mycobacterium avium* subspp.*avium*. The whole genome sequence of MAP strain K-10 (Genbank accession number AE016958) has been published by Cousins and co-investigators(2002). Genomic analysis showed that MAP K-10 has a single circular sequence of 4,829,781 bp with 69.3% G + C content and encodes 4350 predicted ORFs, 45tRNAs and one rRNA operon (Cousins *et al.,* 2002). Prior to genome sequencing only 3 IS elements were identified in MAP; IS900, IS131 and ISMav2 (Cousins *et al*., 2002). MAP K10 genome contains 17 copies of the previously described IS900, seven copies of IS1311, and three copies of ISMav2. Basic molecular typing technique (IS1311 PCR-REA) targets a point mutation in IS1311 sequences at 223rd (C/T polymorphism) and is able to divide of MAP isolates into 3 groups; 'Cattle type' (Type II), 'Sheep type' (Type I) and 'Bison type' (B type) (Cousins *et al*., 2002). JD in cattle, goats, deer, camelids is mainly caused by C type, whereas sheep are usually infected by S type. However, in India the Indian Bison type genotype is the major genotype infecting domestic and wild ruminants in different agro-climatic region of the country. The occurrence of the 'Indian Bison Type' genotype of MAP has also been reported in patients of Crohn's disease and animal health care workers (suspected for Crohn's disease) from North India (Momotani *et al.,* 2012).

History of MAP

Paratuberculosis was first described in Germany in 1895 by Johne and Fronthingham (Pal,2007). Leonard Pearson (1868-1909) was first to describe the occurrence of JD in the U.S. in 1908. The causal agent was initially named as *Mycobacterium enteriditis* in 1910, and Johne, and later known as *Mycobacterium avium* subspecies *paratuberculosis* (Singh *et al.,* 2006).

Johne's disease was first confirmed in Australia in 1980. It is presumed that infection originated from sheep imported from New Zealand in the 1970s. Johne's disease is not known in 1980 to exist in Western Australia (Singh *et al.,* 2006). A requirement that imports be derived from flocks, which had negative surveillance tests to Johne's disease provides the necessary protection for the sheep industry in Western Australia. In South Africa, ovine paratuberculosis was unknown until an infected Merino ram was imported in 1967.During the 1990s the disease spread among sheep farms in the Western Cape and Eastern Cape provinces.

Host Range and Susceptible Animal Groups

MAP is a multi-host pathogen. Although, most information from clinical and experimental infections concerns domestic animal species, presumably all domestic and wild ruminants are susceptible. Reports exist of infection in water buffalo, white-tailed deer, red deer, roe deer, elk, bison, bighorn sheep, Rocky Mountain goat, aoudad, mouflon sheep, camel, mountain goat, reindeer, antelopes, yak, and moose. Thellama, and alpaca can also be affected by this disease (Stevenson *et al.,* 2009). However, monogastric animals can be receptive. Horses and pigs can be infected experimentally with development of intestinal lesions (OIE, 2009). Rabbits, mice, hamsters, and guinea pigs are used as laboratory animals (Okuni, 2013). Wild rabbits in Scotland have been shown to harbour these mycobacteria in their lymph nodes and intestines (Radostits *et al*., 2006). Other non-ruminant wildlife species infected in Scotland include small carnivorous mammals as well as carrion-eating birds and rodents. Also,two reports of infection in nonhuman primates (mandrill and stumptail macaque) exist (Stringer, 2010).MAP has also been isolated from primates (Singh *et al.,* 2006). Risk of transmission of MAP from animal to human is higher with domestic ruminants than wild life animals for obvious reasons.

JD (MAP) Infection in Animals

Johne's disease has become the most important disease of domestic livestock (Verma, 2013). JD is listed as reportable disease of OIE, disease free certification is necessary for the export of animals and their products (OIE, 2008). JD is chronic, granulomatous, enteritis of ruminants. MAP may persist in intestine and other tissues of animals for years without causing clinical disease (Manning and Collins, 2001). However, subclinical animals develop clinical disease, if stressed (environmental, nutritional, pregnancy, parturition, lactation or any other concurrent disease) (Radostits *et al*., 2006). Clinically sick animals show chronic diarrhea, debility, progressive weight loss and emaciation. Decreased serum concentrations of calcium, total protein and albumin have been also reported in cattle and sheep with clinical JD (Mohammed and Mohammed, 2012). MAP is frequently excreted in feces, milk and semen of clinical or sub-clinical infected animals thus increasing environmental load of MAP (Verma, 2013). Newborn animals acquire infection from infected mothers or environment, early in life but symptoms usually develop later at 2-6 years of age in cattle. Venereal and transplacental transmission from Laos reported (Bannatine*et al.,* 2003 (Pribylova *et al*., 2011). Pathology of disease varies indifferent animal species and between different organs within animals. Affected animals are unable to absorb digested nutrients and fluid from intestinal tract and if do not recover may die but appetite may remain normal until death (Pribylova *et al*., 2011).

The available diagnostic measures for JD suffer from poor sensitivity and specificity and fail to detect the infection in the early stage of disease (Manning *et al.,* 2008).Culture is considered the 'Gold standard' (Zapata *et al*., 2010). Mostly the diagnosis is made at postmortem examination. There is no treatment of JD (Sockett, 2000). Though a variety of antimicrobials have been tried for satisfactory treatment and is not considered a long-term option due to high cost

associated with treating entire herd for prolong periods (Taylor, 2002). In poor and resource constrained countries, status of JD with respect to national prevalence, production losses, genotypes, diagnosis, control measures, prevalence in other animals etc., is not known (Bastida and Juste, 2011).

Epidemiology

The disease occurs worldwide most commonly in ruminant like cattle, and to a lesser extent in sheep and goats (Singh *et al.,* 2006). The disease is wide spread in cattle in Europe and has been spread to many countries by the export of infected clinically normal purebred stock (Radostits *et al*., 2006). It is of major importance in cattle , and sheep in temperate climates , and some humid, tropical areas. The incidence is greatest in animals kept intensively under climatic and husbandry conditions, which are conducive to the spread of infection. The Icelandic story of paratuberculosis is an example of the risk of importing animals into a country without accurate tests or testing. In 1933, 20 stud rams of the Karakul breed were imported from Germany into Iceland to improve the quality of the skin of Icelandic sheep. The imported sheep appeared healthy and had certificates of good health control. After two months of quarantine they were distributed to 14 farms in the main sheep farming areas. The first clinical case of paratuberculosis in sheep was diagnosed in 1938 or 5 years after the arrival of the sheep. Gradually, infection spread from these five original farms during the next few years, and over the next 18 years, 20-30% of the farms in the sheep breeding areas were infected (Singh *et al.,* 2006). Within 16 years paratuberculosis together with other diseases (maedivisna and jaagsiekte) almost ruined the sheep industry, the main agricultural industry in Iceland. The first clinical case of Johne's disease in Iceland occurred on these sheep farms in 1944. The annual morbidity of sheep during the epidemic averaged 8-9 % in affected areas and was up to 40% on individual farms (Radostits *et al*., 2006).Non-ruminant species (omnivores, carnivores) are also infrequently infected, and few of these infections have been shown to progress to either clinical illness or systemic pathology.

Prevalence of infection

The prevalence of infection in a region is difficult to estimate because of the uncertainty of the diagnosis and the failure to report cases unless a specific survey or eradication program is undertaken. It appears that the prevalence of infection has been increasing in the last 10 years and there are large variations in the estimates of prevalence, Because of the insidious nature of Johne's disease, it is considered to be potentially a hidden threat to the livestock industry (Radostits *et al*., 2006).

Method of transmission

Exposure to the organism can occur by a variety of routes. The most common is through nursing from an infected dam (via contaminated teats or direct shedding of the organism into the colostrum/milk) or ingestion of fecal contaminated solid feed and water. Infected cows and other species excrete MAP directly into the milk during at least the late disseminated stage of the infection. Up to 45% of clinically affected cows may excrete the organism in milk (Singh *et al.,* 2006).

The organism can be found in the colostrum of subclinical cases; the organism was found in 36% of colostrum samples from heavy shedders and 9% of samples from light shedders, nearly three times as often as it is found in mill (Radostits *et al*., 2006). Thus colostrum of infected cows, if fed to calves, could serve as a potential source of infection. Some herds have attempted to avoid this route of infection by pasteurizing colostrum and raw milk. There is firm evidence of the presence of MAP in commercially pasteurized cow's milk manufactured for retail sale (Radostits*et al*., 2006). The potential public health aspect is an issue given that an association with Crohn's disease in humans is possible but uncertain. However, the presence of the organism in pasteurized milk is undesirable. The organism has been found in raw goat's milk in Norway Conditions in cheese production have been shown to have little effect on the viability of MAP and viable bacteria have been found in hard and semi- hard cheese 12 days after production (Nielsen *et al.,* 2008). Therefore consumption of cheese manufactured from raw goat milk in Norway might lead to transmission of MAP to humans (Nielsen*et al.,* 2008). Because of the normally long incubation period, infected animals may excrete organisms in the feaces for upto15-18 months before the appearance of the clinical signs. The organism has been isolated from the genitalia and the semen of infected bulls, survives antimicrobial addition and freezing, and as a result intrauterine infection may occur (Sevilla *et al.,* 2005). However, small numbers of the organism experimentally inoculated into the uterus of cattle at the time of insemination are destroyed and do not result in systemic infection of the dam or persistent hypersensitivity (Collins, 2004). It is possible to isolate the organisms from the uterine flush fluids of clinically infected cows and experimentally the organism may adhere to the ova in spite of a 10-step wash procedure to insure the removal of potential pathogens from embryos (Grewal*et al.,* 2005). Isolation of MAP from the semen of bulls and rams is unusual and represented by single case reports (Radostits *et al*., 2006). Serologically positive animals, which are moderate shedders of MAP, might be used as donors of embryos without transmitting the organism. The data indicate that transmission of the organism from moderate shedders via the trophoblast is unlikely before the stage of development of cotyledons. However, this does not exclude the possibility of transmission of the organism at a later stage in pregnancy (over 60 days) . It is hypothesized that the epitheliochorial placenta is impermeable to the organism from 42 to 49 days post insemination but that this could change after 60 days (Dhand *et al*., 2009).

All of these modes of transmission are most significant in animals in relatively advanced stages of the disease and provide the passage of the organism from one generation of hosts to the next, and are the reason for recommending artificial rearing of calves and culling of progeny from infected cows during control and eradication programs (Bastida and Juste, 2011). Spread of the organism from farm to farm is usually due to trading of livestock which are unknown infected carriers and shedders of the organism but lateral spread of feaces across boundary fences also occurs. Tracer sheep have been used to detect the sheep strains of MAP on pasture , Lambs, weaners, and adult ewes are introduced to pasture with varying amounts of MAP contamination and monitored using (Hruska *et al*., 2011). skin tests, gamma interferon assay, fecal culture, and serial necropsy of small groups for up to 15 months after first exposure. Culture from tissues was the most sensitive method for detecting early infection in sheep and serologic tests had low sensitivity during the early stages of naturally acquired infection (Jayakao *et al*., 2004). Experimental infection of weaner sheep at 12-16 weeks of age with the strain of MAP, and detection of infection was accomplished within the first few months post-exposure. Field studies have shown that the nymphs of the Oriental cockroach (*Blattiaorientalis*) may serve as a passive vector of MAP. Also, earth worms and adult Dipteramay be vectors of the organism on cattle farms with paratuberculosis. Ovine trichostrongylid larvae (*Haemonchus contortus*, *Ostertagia circumcincta*, *Trichostongylus colubriformis*) may become contaminated with MAP and may play a role in the transmission of the organism. The survival of MAP in amitrazbaseddip fluid for at least 2 weeks suggests that dips could play some role in the trans-mission of Johne's disease in cattle. The main risk is to calves suckled by cows that have just been dipped and whose udders are covered in dip fluid. Fetuses from clinical cases in ewes were

infected. From sub-clinically infected ewes, only 1.6% of fetuses were infected , and no fetuses were infected from a sample of uninfected ewes (ISU, 2007).

Pathogenesis

Following oral ingestion, the organism localizes in the mucosa of the small intestine, its associated lymph nodes and, to a lesser extent, in the tonsils and supra-pharyngeal lymph nodes (Mohammed and Mohammed, 2012; Radostits *et al.,* 2006). The primary site of bacterial multiplication is the terminal part of the small intestine and the large intestine. At least three different groups of animals can occur depending on the host bacteria relationship that becomes established(Mohammed and Mohammed, 2012). In the first group, animals develop resistance quickly, control the infection and do not become shedders (infected resistant) . In the second group, the infection is not completely controlled; some animals will partially control the infection and will shed the organism intermittently, others will become intermediate cases which are incubating the disease and will be heavy shedders of the organism. In the third group, the organism persists in the intestinal mucosa and from among these animals the clinical cases develop (Mohammed and Mohammed, 2012). The organism is phagocytized by macrophages which in turn proliferate in large numbers and infiltrate the intestinal submucosa which results in decreased absorption, chronic diarrhea, and resulting malabsorption. There is a reduction in protein absorption and leakage of protein into the lumenof the jejunum. In cattle, the loss of protein results in muscle wasting, hypoproteinemia, and edema (Manning and Collins, 2001). In sheep, a compensatory increase in protein production in the liver masks the protein loss and clinical signs appear only when this compensatory mechanism fails. Within the macrophages, the bacteria remain viable and protected from humoral factors. In vitro studies indicate that blood derived macrophages from clinically normal cows or cows infected with *M. paratuberculosis* were incapable of destroying the organism (Radostits *et al.,* 2006).

Clinical Findings

According to Radostitsand others (2006),four stages of paratuberculosis in cattle have been described:-

Stage one

Silent infection: Calves, heifers, and young cattle up to 2 years of age. There are no clinical signs and no effects on body weight gain or body condition but these animals may shed the organism. Clinicopathologic tests cannot detect the infection but culture of the feces or demonstration of the organism in tissues may be possible.

Stage two

Subclinical disease: Carrier adults: no clinical signs but may be affected by other abnormalities such as mastitis or infertility. Most of these animals will be negative on fecal culture but 15-25 % may be positive on fecal culture. These are also negative to most serological tests and if not culled will move onto stage 3.

Stage three

Clinical disease: Clinical disease is the tip of the iceberg in terms of the total number of infected animals in the herd. The 'Iceberg concept' states that for every animal with clinical signs born in the herd, another 15-20 animals are infected but less than half of whom will be detected by a sensitive fecal culture. Clinical signs do not appearbe fore 2 years of age and are commonest in the 2 to 6-year age group. Cases occur only sporadically because of the slow rate of spread of the disease. Gradual loss of body weight despite a normal appetite, During a period of several weeks, concurrent with the weight loss, diarrhea develops. Milk production declines and the temperature, heart rate, and respirations are within normal limits. The fall in milk yield is often apparent in the lactation before diarrhea commences. The animal eats well throughout but thirst is excessive. The feces are soft and thin, like thick pea soup, homogeneous, and without offensive odor. There is marked absence of blood, epithelial debris, and mucus. Diarrhea may be continuous or intermittent with a marked tendency to improve in late pregnancy only to reappear in a severe form soon after parturition. A temporary improvement may also occur when animals are taken off pasture and placed on dry feed.

Stage four

Advanced clinical disease: As the disease worsens, emaciation is the most obvious sign and is usually accompanied by intermandibular edema which has a tendency to disappear as diarrhea develops. The diarrhea is characterized by a fluid 'water hose' or 'pipe stream' passage of feces. The course of the disease varies from weeks to months but always terminates in severe dehydration, emaciation, and weakness necessitating destruction.

Morbidity and mortality

MAP has been responsible for increased mortality, reduced reproductive performance and higher susceptibility to MAP organism in dairy herds. Due to the subclinical infections and lack of data on prevalence, morbidity and mortality caused by MAP, JD has not received the priority in developing countries (Verdugo, 2013). In Australia, average mortality rate of ovine JD (OJD) was estimated in 12 farms as 6.2% (range 2.1 to 17.5%) in 2002 and 7.8% (range 1.8 to 14.6%) in 2003 (Sulficar *et al.,* 2009). In India, information on mortality, and morbidity losses due to JD in large ruminant, but some information on goat and sheep are available. In study 18.7% morbidity and 14 % mortality was estimated in JD suspected in Jumanapari goats of Mathura region, North India (Dow, 2011). In Rajasthan, a 7 years (1990-1997) study showed high morbidity (27.1 75.1%) and mortality (8.1- 19.1%) due to JD in Multan Carpet wool than Chokla breed (Morbidity- 29.0 –61.7%, and Mortality 4.6 – 24.8%) of sheep (Mee and Richardson, 2008). In most countries, there is no proper documentation of morbidity and mortality due to JD and sometimes misdiagnosed with weakness and other alimentary diseases.

Necropsy Findings

Lesions are confined to the posterior part of the alimentary tract andits associated lymph nodes (Radostits*et al.,* 2006).The terminal part of the small intestine, the cecum, and the first part of the colon are usually affected. In advanced cases the lesions may extend from the rectum to the duodenum. Typically, the intestinal wall is three or four times normal thickness, with a corrugated mucosa and prominent thickened serosallymphatics. The ileocecal valve is always involved, the lesion varying from reddening of the lips of the valve in the early stages to edema with gross thickening and corrugation later. A high incidence of arteriosclerosis has been observed in advanced cases of JD, with a distinct correlation between the vascular lesions and macroscopic changes in the intestine. The mesenteric and ileocecal lymph nodes are enlarged andedematous, but unlike tuberculosis, foci of necrosis and mineralization are rarely visible. The characteristic microscopic findings include large numbers of epithelioidmacrophages and multinucleate giant cells within the lamina propria and submucosa of affected gut segments and within the paracortical areas of draining lymph nodes. A granulomatous lymphangitis is often visible (Radostits*et al.,* 2006).

Differential Diagnosis

The characteristic features of clinical Johne's disease include chronic diarrhea which does not respond to therapy, progressive weight loss, andemaciation ina single animal. The definitive etiological diagnosis can be obtained by using a combination of serological tests, fecal

culture, and biopsy of intestine. In cattle, the clinical disease must be differentiated from diseases, which cause chronic diarrheain adult cattle. The chronic nature of Johne's disease is usually sufficient to differentiate it from the other common enteritis of cattle. Salmonellosis, coccidiosis, and gastrointestinal helminthiasis are usually acute and the latter two occur principally in younger animals, and are distinguishable on fecal examination for oocysts, and helminth eggs. Secondary copper deficiency (chronic molybdenum poisoning) is likely to be confused with JD in cattle, but is usually an area problem affecting large numbers of animals and responds well to the administration of copper(Radostits *et al.,* 2006). Other debilitating diseases in which diarrhea is not an important clinical finding are malnutrition, chronic reticuloperitonitis, hepatic abscess, pyelonephritis, lymphosarcoma, and amyloidosis. Idiopathic eosinophilic enteritisin cattleischaracterized clinically by chronic diarrhea and weight loss, and recoverymay occur following treatment with dexamethasone (Radostits *et al.,* 2006).

Diagnostic methods of MAP

Several methods have been used to diagnose Johne's disease, but many of them suffer from inferior specificity and sensitivity (Sulfica*et al*., 2009). In fact, because of the pathobiology of paratuberculosis the slow progress of infection and the inappropriate immune response, it is impossible for any method to perform well during all stages of the disease (Slana *et al.,* 2008). During the early cell-mediated immunity (CMI), detection of cytokines, such as IFN-γ, a product of T lymphocytes, can be done in vitro by enzyme-linked immune sorbent assay (ELISA) (Jayakao*et al*., 2004; Bannantine*et al*., 2014). In vivo skin testing with the antigen Johnin has also been employed (Pavlas, 2005; Naser*et al*., 2009). Neither of these tests is specific for MAP, and it is known that *Mycobacterium avium* subsp. *avium* can cause false positive results (Manning *et al.,* 2008).

In stage II and III, when the concentration of antibodies increases, serological tests can be useful in some circumstances. Complement Fixation (CF), however, lacks in both sensitivity and specificity. The sensitivity performance of agar gel immunodiffusion (AGID) is better, but not as good as that of antibody ELISA, which, however, still suffers from insufficient specificity (Radostits*et al.,* 2006). Greater specificity is achieved when the presence of the aetiological agent, MAP, can be demonstrated and confirmed with bacteriological or molecular methods, if the animal sheds bacteria in faeces, milk or occasionally in semen. From post mortem or slaughtered animals, gross lesions of corrugated ileum, as well as typical histopathological lesions and acidfast bacilli, is suggestive of paratuberculosis, but for specific diagnosis, culture and/or PCR is needed. Likewise, characteristic acid-fast bacilli in fecal samples are suggestive of paratuberculosis, but culture and/or PCR is necessary for a reliable diagnosis (Radostits *et al.,* 2006).

Bacteriology

MAP is a fastidious organism and has a generation time of 1.5 to 4 days when cultured in liquid media (Scanu*et al.,* 2007). As mentioned above, culture of clinical material, usually faeces, may sometimes take more than 16 weeks to yield visible colonies on solid media, such as Herrolds Egg Yolk medium supplemented with mycobactin. Optimal sensitivity is difficult to achieve, as chemicals used for decontamination of the faster growing sample microflora also kill some of the MAP, or decrease their viability (Zapata *et al.,* 2010). If the animal is an intermittent or low shedder, false negatives may therefore occur. Although culture is problematic, isolation of the bacteria makes the method 100% specific, as the colony material can be further tested for confirmation of the result with molecular methods or with the classical methods to judge growth and morphological characteristics (Naser*et al.,* 2009). Colonies should be small, raised and white or pale yellow. Lack of growth in a control tube, without mycobactin, guarantees that the organism is mycobactin dependent (Sockett, 2000). Because of the specificity and the high sensitivity, compared to other methods, culture is since long considered the "gold standard" for diagnosis of paratuberculosis. However, for subclinical infection, sensitivity of culture and other agent detection methods on faecal samples is very poor, and culture could,therefore, only be regarded as "gold standard" on faecal samples from clinical cases that nearly always are shedding MAP. To be regarded as a general "gold standard" for paratuberculosis, it has to be applied on suitable lymph nodes and intestinal samples (preferably, last part of ileum and adjacent lymph node) (Radostits *et al.,* 2006).

Polymerase chain reaction (PCR)

The most widely used target gene for detection of MAP is IS900, first described in 1989 (Green, *et al*., 1989), and presently considered specific for MAP. The MAP genome is reported to have 15 to 20 copies of the insertion element, and the sequenced strain K-10 has 17 copies (Li *et al*., 2005). This high target copy number gives an increased sensitivity compared to systems targeting single copy genes, which makes it popular in molecular diagnostic methods for paratuberculosis. However, successful detection of IS900 is not necessarily definitive for identification of MAP, as previously presumed. It has many similarities with genes of other mycobacteria, which means that detection with PCR systems located in a conserved area may cause false positives in some strains, as previously reported (Bannantine *et al*., 2014). This is especially true with the equivalent gene instrain 2333, with 94% identity to suggest measures to increase PCR specificity for IS900 include the use of annealing temperatures higher than 60 ˚C (Bannantine*et al*., 2014). A temperature increase will, however, reduce false positives in an arbitrary manner and compromise the sensitivity. Hence, there is a need for confirmative methods for PCR positives. Sometimes, when PCR is performed in parallel with culture, growth characteristics (as described above) can confirm the PCR result, but when fast results are needed or when culture fails because of contamination or bad viability, molecular methods should be used (Ibrahim *et al*., 2004).

One of the challenges with molecular detection of MAP is to get the genomic DNA out of the bacteria, which is protected by its thick and waxy cell wall (Bannantine*et al*., 2014).

Table 1. Summary of diagnostic test accuracy (subclinical infection)

Test	Sens	Spec	Speed	Cost ($US)
Fecal Culture	55%	100%	8-16 weeks	$10 – 15
DNA Probe	33.5%	100%	1-2 days	$25 – 35
ELISA	45-55%	99%	1-2 days	$4 – 6
CF	25%	99%	1-2 days	$4 – 6
AGID	25%	100%	2-3 days	$5 -10

Source: Sockett, 2000

Treatment

Currently, no antimicrobials are approved for the treatment of JD. *M. paratuberculosis* is more resistant to chemotherapeutic agents in vitro than *M. tuberculosis* so that prospects for suitable treatments poor. Because of this lack of efficacy and the failure of any of the antimicrobials to provide a bacteriological cure, treatment is not recommended. The antimicrobials which have been used are summarized here: Streptomycin has most activity against the organism but treatment of affected cattle with daily doses of 50 mg/kg BW 1M causes only a transient improvement in clinical signs

(Taylor, 2002).

- Isoniazid: For cattle at 20 mg/kg BW orally daily for up to 100 days.
- Clofazimine: For cattle at a dose of 600 mg daily for 10 months
- Monensin: For cattle at a rate of 147.5 mg/kg in the feed daily for 120 days.

Economic importance

The economic losses associated with Johne's disease causes considerable and huge economic losses in developed and developing countries. Most of the losses occur due to subclinical stage of disease, in the form of progressive weight loss, reduced milk production, lower slaughter value, and premature culling, and reduced fertility (Mohammed and Mohammed, 2012).

Economic losses due to bovine Johne's disease have been estimated in New England – $15.4 million, Wisconsin –$52.3 million, Pennsylvania–$5.4 million, US- $200-250 million, Australia- $2.1million per annum. Direct production and treatment losses was studied annually in an average (7%) JD infected cow herd and found highest losses occur due to, JD than Bovine viral diarrhoea, neosporosis, and enzootic bovine leukosis (Hughes *et al.*,2007)in dairy cattle can be sub-stantial and occur across all herd sizes and regions. Lost milk production and higher net cow replacement costs contribute to decreased value of production per cow inventory in affected herds. In a 1996 study of the National Animal Health Monitoring System in USA, affected herds experienced an economic loss of about $US100 per cow compared to Johne's free herds. In herds with at least 10% of their culls cows with clinical signs of the disease, economic losses were over $US200. These high prevalence herds experienced reduced milk production of over 700 kg per cow per year, culled more cows but had lower cull-cow revenues, and had greater cow mortality than negative herds. In dairy herds in the Canadian Maritimes, total annual costs for an average Johne's disease-infected, 50-cow herd were $US2472. Losses at herd level The economic losses due to subclinical Johne's disease include: reduced feed efficiency, decreased milk production, decreased milk fat and protein, reduced slaughter weight at culling, decreased fertility, premature culling and increased incidence of mastitis (Hughes *et al*., 2007).

Control

Johne's disease can be controlled and even eliminated from infected herds, however, it takes a comprehensive understanding of thedisease by owners of the animal, consultation with a veterinarian in addition to the requirement of one or more of the available diagnostic tests. In ruminants the control of JD is challenging because of the ubiquitous nature of the organism, the long incubation period, most cases are subclinical, and the laboratory tests available lack sufficient sensitivity to identify infected animals which allows the infection to spread within and between herds. Although there are large gaps in the understanding of various aspects of the epidemiology of MAP, and the diagnostic tests are not reliable in the early stages of infection, enough is known about the essentials of control programs for dairy herds which were proposed 50 years ago (Bastida and Juste, 2011).

Two vaccines exist for JD; one is made from killed MAP and the other from live attenuated vaccine. Vaccines do not stop animals from becoming infected or from shedding the bacteria. They can reduce signs and delay their onset but are not useful in controlling Johne's disease on farm (Kalis *et al.,* 2001).Thereis little difference from currently recommended control strategies. The implementation of herd or flock level control programs, establishment of test negative or low-risk herds or flocks, and reduction of environment and feed contamination with MAP are attainable goals. Because of the inaccuracy of the diagnostic tests available, it is impossible to eradicate the disease, other than by complete depopulation of the herd and restocking with non-infected animals. Eradication strategies are usually not practical for economic reasons and the impossibility of acquiring non-infected animals. The next best option is control of the disease ata very low level of prevalence. Veterinarians usually are uncertain how to begin a control program in a herd once clinical disease has been encountered. Generic control recommendations often fail because they do not account for uncertainties inherent in control recommendations or for the unique circumstances of an individual herd. The literature on the control of MAP infection in agricultural species on a worldwide basis has been reviewed (Sockett, 2000).

According to Hashemi *et al.*(2013) a complete comprehensive control program consists of understanding three major aspects which should be explained to the producer before planning a control program:

- ✓ Issues that impede efforts to control JD
- ✓ Characteristics of tests and alternative testing strategies that influence control programs
- ✓ Developing farm-specific control programs.

MAP infection in human (Crohn's disease)

Crohn's disease drives its name from the description of 8 cases of regional ileitis described by Crohn, Ginsburg and Oppenheimer in 1932 at the Mount Sinai hospital in New York (Singh *et al.*, 2006). However, the first clear description of the disease was made by Dalziel in 1913 at the western infirmary in Glasgow (Singh *et al.*, 2006; Pal, 2007). The earlier term of regional ileitis was replaced by regional enteritis in 1960 when it was recognized by Lockhart-Mummery and Morson that the disease was not confined to the ileum when they described primary CD of the colon. This entity was subsequently termed granulomatous colitis by American clinicians, together with ulcerative colitis and unclassified chronic colitis: CD belongs to a spectrum of diseases more generally designated as" chronic bowel disease''. Chiodini*et al.* (1989) isolated -first time- *Mycobacteriumavium*subspecies *paratuberculosis*(spheroblast form) from biopsy of the CD patient and subsequently by other workers. Meanwhile, Naser et al. (2000) isolated MAP for the first time from milk of lactating mother suffering with CD. Presence of MAP was also confirmed from blood samples (systemic infections) in humans, like in animals (Naser *et al.*, 2004).

CD is a systemic disorder manifested as chronic inflammation of intestine. It is characterized by weight loss, abdomen pain, diarrhoea and general malaise (Pal, 2007; Verma, 2013). Its peak onset is in late teenages to early adult life, with a secondary peak in the elderly (Scanu*et al.*, 2007). Clinical presentation depends on the site of inflammation. Pain is a common feature particularly those with small bowel involvement. Diarrhea, often with bleeding, occurs in colonic CD. Patients with ileal disease may present with an illness very similar to acute appendicitis (Goswami *et al.*, 2000). Fistulae are characteristic of CD(Singh *et al.*, 2006). Approximately 25% of patients with CD develop perianal complications, in the form of fistures (Momotani*et al.*, 2012). Bowel obstruction is a known complication, which may require surgical resection. It has been reported that up to 80% patients with CD will require surgery at some stage of their illness (Verma, 2013), and approximately 50% of surgical cases require additional surgery within five years and eventually patient develop short bowel syndrome which makes it extremely difficult to digest food. Treatment with immunosuppressive drugs and surgical removal of the affected portion of intestine are used to control symptoms of CD, but at present,

there is no cure for this disease. Generally, the patients live with chronic pain throughout their lives (Verma, 2013).

The etiology of CD is mysterious and still controversial, although it is suggested that patients with CD are a different group and the etiology may not be same for all patients (Wang *et al.*, 2014). There is a genetic predisposition to develop CD, recently 3 independent studies suggested that mutation in a gene on chromosome 16, known as NOD2/CARD 15 are associated with CD (Verdugo, 2013: Verma,2013).

Infectious cause for CD has been sought since the disease was first described in 1932 and number of microorganism has been suggested as the cause of CD (Nielsen, 2013). But, MAP has the attention for the causative agent of CD due to the clinical signs and pathological similarity of JD with CD initially and later frequently isolation of MAP from tissues of CD patients (Verdugo, 2013). Early studies did not detect MAP in tissues from patients with CD by conventional staining and culture techniques (Naser *et al.*, 2004). Failures in detection may be due to unusual nature of cell wall deficient MAP in patients with CD and/or challenges in culturing MAP with its fastidious and slow growing characteristic (Sevilla *et al.*, 2005). Problems in culture were overcome by use ofMAP specific IS900 insertion sequences for proxy diagnosis of MAP infection (Kreeger, 1991). Identification of this insertion sequence from bacterial culture, feces and tissues by PCR technique have made rapid and sensitive detection with accuracy possible (Verma, 2013). However, PCR fails to differentiate the live MAP or MAP DNA (Slana *et al.*, 2008). RT-PCR was used for amplification and confirmed the presence of MAPRNA in the test samples (Naser*et al*., 2009). Using in-situ hybridization by IS900 specific probe, the presence of MAP in biopsy samples of CD was confirmed and provides evidence for role of MAP in the etiology of CD (Zapata *et al.*, 2010).Sero-reactivity of CD has been examined in ELISA using protein antigen from MAP (Pavlas, 2005). Test has shown significant higher proportion of MAP specific antibodies in CD patients than control sera (Verma, 2013). MAP has been recently isolated from breast milk of lactating mothers (Bannantine 2014 and Naser 2000),and from blood of CD patients indicating that MAP infection is systemic like paratuberculosis in animal (Tylor and Zaatagi, 2004).

ETIOLOGICAL THEORIES OF CD AND REALITY

According to Singh et al (2010b) there has been critical debate in scientific literature, concerning the etiology of CD has been heightened recently, and is now focused mainly on the following three postulates.

Autoimmune and genetic predisposing theory

Autoimmune theory assumes that CD results from inappropriate ongoing activation of the mucosal immune system driven by the presence of specific antigenic stimulation from normal luminal flora. Studies on mouse models shows impairment of mucosal epithelial barrier and influence of gut flora have been used to support autoimmune theory. However, critics claim that deregulated immune responses are not the primary disorder but secondary to an underlying infection. The treatment strategies for CD are aimed at suppressing inflammation and immunity. CARD15 genes present on chromosome 16q12 show genetic correlation with CD. Three main mutations in CARD15 gene (R702W, G908R and 007fsinsC/ 3020 ins C) have been identified in CD patients. CARD15 gene encodes a cytoplasmic protein (NOD 2) that is mainly expressed in monocytes, macrophages and granulocytes, but has also been identified in epithelial cells. NOD2 proteins play a role in the reorganization and intercellular killing of bacteria with its function as to initiate an appropriate immune response to kill them. The mutations in CARD 15 have been identified in 20-25% of Caucasian CD patients and only 7-15% of healthy controls. However, no association between CD and CARD15 has been found in Japanese patients. CD occurs in patients without the mutant gene and the mutant gene can be found in people without the disease, suggesting that a subgroup of CD is not related to a dysfunction of CARD15 gene (Singh *et al.*,2010b).

Immune deficiency theory

The immunodeficiency theory hypothesizes that disturbances of innate immunity play a central role in the pathogenesis of CD. Korzenik and Dieckgraefe (2000) suggested dysfunctional neutrophils played central role in inflammatory immune process of CD. Dysfunctional neutrophils allow microbe to successfully infect macrophages and survive long enough, meanwhile a compensatory, excessive Th1 immune response is activated resulting in the CD. Neutrophil dysfunction is hypothesized to result from interplay of genetic factors, environmental factors, or possibly exotoxins associated with subsets of gut flora. Clinical trials based on this theory showed that Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) stimulates neutrophil activity both qualitatively and quantitatively (Singh *et al.*,2010b). As with most cytokines, GM-CSF is pleiotropic and has multiple effects on the immune system. The initial results were very promising and led to further testing. Korzenik *et al.*, (2005) reported that after eight weeks of daily GM-CSF injections 48% of CD patients receiving 100 point improvement on the CD activity index versus only 26% of the control subjects. Forty percent of the treated group went into complete remission versus 19% of controls (Korzenik*et al.*, 2005). The present therapy should be aimed to boost innate immunity rather than suppressing it. This theory suggests that subsets of gut flora may exert an immune suppressive effect on neutrophils presumably through exotoxins.

Mycobacterial theory

This theory states Mycobacterial spp. are the causeof CD. This theory embraces all what was described about immune dysregulation, predisposing genetic factors, immune deficiency and the role of gut flora. Dalziel (1913) reported the histopathologicand clinical similarities of JD, intestinal tuberculosis, and human chronic granulomatous enteritis and suggested *Mycobacteria*spp. As the possible cause of CD,due to frequent isolation and similarity of CD with paratuberculosis, MAP fined more attention than other *Mycobacterium* spp. Mycobacterial molecules dysregulate immune signaling pathways as one of its evolved survival strategies. MAPsurvival in murine macrophages and human PMNCs suggested the ability of this microorganism to resist phagolysosome fusion, by maintaining association with the early endosomes. There are some other considerable evidences that support mycobacterial theory in CD. Though genome of many mycobacterial species has been sequenced but comprehensive knowledge of the pathogenic mechanism of mycobacterial infections is not fully known, and little is known about specific disease causing abilities of MAP. However, increased gut permeability and monocyte dysfunctions results impaired immune regulation are well known in CD. Epidemiological evidences suggest that the increased gut permeability in CD is determined by exposure to environmental factors. MAP present in human as cell wall deficient form, escape host immune response and reside in macrophage cells. Zurand others (2003)showed that MAP infected macrophages were able to stimulate production of IL-2 and IFN-γ in CD4+ cells. Cytokine production and initiation of cellular immune response by host causes appearance of intestinal granulama and cellular response is initiated in the nearby lymph nodes in an attempt to clear the infection. This inflammatory process results in corrugated intestinal epithelium. In addition, it also causes damage to enteric glial cells and enteric neurones of gut. Through ligands such as HupB protein, which participates with specific terminal trisaccharides in mediating initial Schwann cell adhesion by the leprosy bacillus, MAP shares some of the neuropathic properties of *M. leprae*. It would also participate in establishing the entericneuritis and neuronal changes well known in CD, and clearly demonstrated in gut of MAP-infected animals. Damage to enteric glial cells in a transgenic mouse model has been

shown to impair gut mucosal as well as vascular integrity, and result in inflammatory disease of the small *Mycobacterium avium* subspecies *paratuberculosis* as possible cause of CD: Abnormalities affecting enteric glial cells and enteric neurons are clearly involved in pathophysiology of CD, and it is probable that these are caused by MAP.

PUBLIC HEALTH ISSUES

MAP has a strong zoonotic potential while existing as a sharp veterinary pathogen. Infected animals secrete huge amounts of MAP bacilli in their faeces and milk (Pal, 2007). MAP is able to survive pasteurization temperature and is resistant for chlorine treatment. Hence, the opportunity exists for humans to become infected with this organism via milk, milk product, beef and water supply (Verma, 2013).

Milk and dairy products: Clinical, subclinical, and apparently healthy animals excrete MAP in their milk (Grant, 2005a). Moreover, it is more heat resistant than other *Mycobacterium* spp.and *Coxiella burnetti*(the current target of pasteurization) and is able to survive at the current pasteurization standards (Mihajlovic *et al.*, 2011). The first study to confirm the presence of MAP in commercially pasteurized milk was carried out in UK, 1.8% pasteurized milk samples tested positive for MAP culture (Ryan and Campbell, 2006). MAP has been frequently isolate from pasteurized milk from Republic of Ireland, Canada, Australia, Czech Republic and India (Verma, 2013). In India, first time, the presence of live cultivable MAP was investigated in un-pasteurized milk and commercial brands of pasteurized milk and milk products, marketed in 3 major cities of North India for human consumption. A very high contamination of MAP 43.7 and 55.5% in unpasteurized and pasteurized milk respectively were reported (Singh *et al*., 2010a). Viable MAPhas been also found in milk of human with CD, which increases the risk of MAP exposure to newborn. Presence of viable MAP in dairy products other than liquid milk has not been studied extensively. The presence of MAP DNA in 49.0% infant milk powders samples from 10 manufacturers in 7 European countries by PCR and viableMAP in one samples was reported (Verma, 2013). In a study, 55.5% milk products were positive in culture (Beaudeau*et al*., 2007). Above evidences suggest that consumption of un-pasteurized and pasteurized milk and milk product is not safe and may be preferred route of transmission of MAP to human population (Verma, 2013).

Meat and meat products: - MAP causes systemic infection in advance stage of JD and has been frequently isolated from intestine and mesenteric lymph node of clinical, sub-clinical and apparently healthy animals. Besides target tissues MAP was distributed to udder, uterus and testis (Austerman, 2012). In a study MAP from large intestine (36.0%) and mesenteric lymph node (34.0%) tissues of slaughtered buffaloes was reported from India (Verma, 2013). However, MAP was isolated from 43.6% intestine and 40.0% MLN of slaughtered kids. It was suggested that meat from old dairy cows, used to make ground beef for human consumption may represent a source of MAP infection for consumers (Austerman, 2012). Studies indicate that meat obtained from sub-clinical and apparently healthy animals can be contaminated with MAP (Grant, 2005b; Nielson *et al*.,2008; Wynne *et al.*, 2011). Meat could also be contaminated with fecal material during slaughtering and processing procedures (Nacy and Buckley, 2007). No information is available regarding the destruction of MAP at cooking and meat processing methods. It is possible beef consumption can be the cause of human exposure to this pathogen (Verma, 2013).

Water: MAP is excreted in feces of symptomatic and asymptomatic JD infected animals, and is able to survive in the environment for long periods (Dow, 2011). Water derived from underground sources is unlikely to be affected by pollution from JD infected animals and further low level of contamination can take place through agriculture run-off in catchments area, used for supplying water for domestic use as well as to estuarine the environment. MAP is able to survive digestion by protozoa that are usually bacteriovores. Indeed this, it has been suggested, allow MAP to acquire a phenotype more pathogenic to human beings (Mihajilovic*et al*., 2011). Such internalization may also afford MAP added protection against agents such as chlorine that are used in water treatment operations. MAP located intracellularly was significantly more resistant than free MAP. This work showed that water may be a vehicle for spread of MAP from animal to human. There is need to determine the efficacy of water treatment operations to remove or inactivate MAP. Non-processing or proper disposal of animals and human excreta also help to contaminate the water resources and environment of MAP. Due to high resistance of MAP to environmental degradation help to increase the load of bacilli in the environment, which in-turn gain access to human and animal food chain, and thus maintain the cycle of MAP in environment (Mihajilovic *et al*., 2011; Verma, 2013).

Conclusion

Rising incidences of JD, presence of MAP in food chain are alarming signs for public health. Due to high economic losses, long incubation period, difficult diagnosis, strong survival potential, existence in human food chain and possible link to CD, MAP is an emerging pathogen of concern worldwide. There is anurgent need to design the strategies to control the spread of MAP in. The growing evidences showed that MAPmay be a strong candidate for causation of CD in humans. However, some systemic studies are required, to correlate exposure to MAP to incidence of CD, specific evaluation of exposure of patients with CD and MAP. The clinical and pathological similarities of CD with other mycobacterial disease and partial fulfillment of Koch postulate indicate that the presence of MAP in the environment and food chain supply is not safe. Hence,there are urgent need to restrict the spread of the MAP infection in animals and prevent its transmission to human from animals in areas here the disease is present.

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