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Case Study

MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS INFECTION IN CATTLE: PATHOGENESIS AND DIAGNOSIS

Sushil Kumar, *Ran Vir Singh, Anuj Chauhan, Subodh Kumar & Arvind Sonwane
Division of Animal Genetics, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India 243122
*Corresponding author's email: drranvirsinghivri@gmail.com

ABSTRACT
Paratuberculosis or Johne's disease caused by *Mycobacterium avium subsp. Paratuberculosis* (MAP) is a chronic granulomatous enteric disease that affects all categories of ruminants including cattle, goats, camels and buffaloes. The infection is typically characterized by a long incubation period followed by loss of condition, a reduction in milk yield, oedema, anaemia, weight loss, chronic progressive and diarrhoea leading to cahexia. Currently here is no treatment and it invariably leads to the death of the affected animal. MAP is mostly acquired orally in cattle and other ruminants and often in young calves although intra-uterine transmission of MAP occurs. Control of the disease in ruminants is dependent on the early detection and culling of infected animals. The principal diagnostic tests for MAP infection in animals are individual or pooled faecal culture, ELISA and IFN- release from activated white cells in response to MAP antigens. Lately, PCR diagnostics have also been introduced. It has been suggested that this microorganism may be associated with Crohn's disease (CD) in humans. To eliminate paratuberculosis infection from cattle herds, early identification and elimination of MAP-shedders is a must. Rapid and cost-effective diagnosis methods of MAP remain a high priority task not only for animal breeders but also for the food production industry and for public health concern.

KEY WORDS: *Mycobacterium avium* subspecies *paratuberculosis,* diagnosis, pathogenesis, cattle.

NTRODUCTION
Animal husbandry is an integral part of India's agricultural economy as it contributes 25.6% of the agricultural GDP and 4.1% of the National GDP (BAHS, 2014). This scenario suggest that livestock is likely to emerge as an engine of agricultural growth trends in the coming decades and the importance of livestock goes beyond its food production function. Livestock sector creates a continuous stream of income and employment and reduces seasonality in livelihood patterns particularly of the rural poor (Birthal and Ali, 2005). Diseases have a negative influence on the livestock production system including productivity losses, loss of well-being of human beings (food safety and quality), prevention or control costs (production costs, public expenditure) and suboptimal use of production potential. Many methods have been employed to control the spread of animal diseases and their negative effect. *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the etiological agent of Johne's disease (JD) is a highly pathogenic mycobacteria affecting dairy cattle and other domestic ruminants globally (Boelaert *et al*., 2000; Gasteiner *et al*., 1999; Singh *et al*., 2008; Singh *et al*., 2013a). It is chronic progressive granulomatous infection of high yielding cows leading to increased culling (Benedictus *et al*., 1987; Tiwari *et al*., 2005) decreased milk yield (Benedictus *et al*., 1987; Tiwari *et al*., 2007), higher death rates (Kreeger, 1991) and increased susceptibility to other infections (Tiwari *et al*., 2009). Clinical and asymptomatic cows shed large number of bacilli in feces and milk (Sweeney *et al*., 1992; Streeter *et*

al., 1995; Slana *et al*., 2009; Singh *et al*., 2014a). Colostrum and milk is important source of transmission of MAP to new born calves and human population (Grant, 2003; Ayele *et al*., 2001; Slana *et al*., 2008). Calves become infected soon after birth due to higher susceptibility to MAP during first year of life, though clinical symptoms appear at later age (2–6 years). Loss due to reduced milk yields alone in case of *Mycobacterium avium* subspecies paratuberculosis (MAP) infected cows were reported to be Rs 54,442.5 /cow/lactation in India5. It was estimated that MAP costs the US dairy industry $200 to $250 million annually due to increased cow replacement costs and reduction in milk production and also decreased fertility in high-shedding animals. Role of MAP in public health as the causative agent of Crohn's disease has also long been discussed. Due to the histopathological features of Crohn's disease closely resembling those found in animals with the paucibacillary form of Johne's disease, it has been suggested that the two diseases shared the same aetiology. Specific and sensitive diagnostic methodologies as well as a better understanding of the pathogenesis of JD are prerequisite to develop control programs to eradicate the disease. Research in past few decades has focused to develop protocols for the detection of MAP in feces, milk, tissue and environmental samples using various protocols. Diagnosis of *M. avium* subsp. paratuberculosis infection is difficult because of the pathogen's slow growth and the lack of diagnostic tests sensitive enough to detect most sub clinically infected

cattle, many of which intermittently shed the pathogen, thus serving as sources of infection of susceptible cattle (Wells *et al*., 2006).

MAP infection in Cattle

The organism, MAP is an first observed by Johne & Frothingham in 1895. *MAP* causes paratuberculosis or Johne's disease, an intestinal granulomatous infection (Thorel *et al*., 1990). Paratuberculosis is found most often among domestic ruminants (cattle, sheep, goats, camelids and buffaloes) as well as wild ruminants (cervids) and has a global distribution. The disease has also been reported in horses, pigs, rabbits, stoat, fox and weasel (Greig*et al*., 1999). Under natural conditions, the disease in cattle spreads by ingestion of *MAP* from the contaminated environment. The disease persists after the introduction of infected animals. Infection can be spread vertically to the fetus (Larson & Kopecky, 1970) and semen can be infected with the organism (Sweeney *et al*., 1995). The primary source of infection in calves is milk from infected cows or milk that is contaminated with the faeces of diseased cattle.The diagnosis of subclinical paratuberculosis is difficult. Although not considered a zoonotic agent, *M. paratuberculosis* has been identified in intestinal biopsy tissue from patients with Crohn's disease, inflammatory enteritis in humans.

Etiology

Family Mycobacteriaceae to which MAP belongs also includes the tuberculosis and leprosy-causing species *Mycobacterium tuberculosis* and *Mycobacterium leprae* respectively **(**Wayne and Kubica 1986). In common with other mycobacteria their cell walls contain mycolic acids and are acid-fast, resisting decolourization with acidified alcohol because of the presence of this waxy material, and making the cells particularly difficult to disrupt to release DNA for PCR purposes. Under the microscope *MAP* cells appear as plump rods $1–2\,\mu m$ in length which typically occur as clumps of up to several hundred bacterial cells. *MAP* is the slowest growing of the cultivable mycobacteria and primary culture from veterinary/clinical or food specimens can take 3–4 months or longer. However, once established, a *MAP* isolate will produce colonies within 3–6 weeks upon subculture at their optimum growth temperature of 37°C under aerobic conditions. Colonies are small (1–2 mm), white and domed with an entire margin; rough colonies are rarely seen. The organism has a requirement for the incorporation of the iron-chelating compound mycobactin J into any complex medium used for its cultivation (Merkal and McCullough 1982). This mycobactin dependency represents a characteristic unique to *MAP*. Another unique identifier is the insertion element IS900 that occurs as 14–18 copies within the genome of *MAP* (McFadden *et al.* 1987; Green *et al.* 1989). This IS900 element forms the basis of molecular detection methods for *MAP* such as PCR assays.

Pathogenesis

Cattle become infected as calves by ingestion of faeces, contaminated milk, feed and water (Merkal *et al*., 1984).Following oral ingestion, M. paratuberculosis localizes in the mucosa of the small intestine, its associated lymph nodes and to a lesser extent in the tonsil and pharyngeal lymph nodes. The primary site of bacterial multiplication is the terminal part of the small intestine and the large intestine. *M. paratuberculosis* is phagocytised by macrophages which in turn proliferate in large numbers and infiltrate the intestinal submucosa resulting in decreased absorption and chronic diarrhoea and malabsorption (Hole, 1953; Gilmour, 1976). Thickening of the wall of the intestine and corrugation of the intestinal epithelium is also prominent (Seitz *et al*., 1989). Unlike *M. tuberculosis, M. paratuberculosis* is highly resistant *in vivo* to most standard anti-tuberculosis drugs. The organism cannot be reliably detected by culture in the laboratory, particularly when present in low abundance or in spheroplast form without a bacillary cell wall (Hope *et al*., 1996). Different strains of *M. paratuberculosis* from different preferred hosts (Pavlík *et al*., 1999a), range from very slow growing to uncultivable, although methods are improving.

Clinical signs

Calves younger than four months of age are highly susceptible to infection; however clinical signs are not manifested until 2 or more years of age. But unlike calves wild ruminants infected via milk, commonly manifest clinical signs at 8 to 12 months of age (Manning *et al*., 1998). Factors such as poor nutrition, concurrent parasitic, viral or bacterial infection, heavy milk production, or transportation stress may influence the rate of development of clinical disease following infection (St-Jean and Jeringan, 1991). The pH of the soil may influence the severity of the clinical signs. Cattle raised on alkaline soils, especially in limestone rich areas, may have a high incidence of infection but little clinical disease. A high prevalence of infection is recorded in the United States of America on acidic soils in contrast to alkaline soils (Kopecky, 1977). In cattle the disease is characterised by chronic and intermittent diarrhoea that is not responsive to treatment, oedema of the throat and abdomen, loss of coat colour, emaciation and eventual death. The chronic nature of the disease entails the late clinical manifestation of paratuberculosis as late as 3 to 5 years after infection (Riemann and Abbas, 1983).

Whitlock and Buergelt (1996), divide infected cattle on the basis of the severity of the clinical signs in to four stages:

1. **Silent stage:** This stage represents young animals (calves, heifers) to the age of 2 years without any clinical symptom of the disease. At this early stage of the infection animals shed the organism in undetectable level, thus M. paratuberculosis could be detected only by tissue cultures or histology examination of the intestine or lymph nodes.

2. **Subclinical stage** (adult animals without visible clinical signs of paratuberculosis): At this stage, antibodies and cell mediated immune responses (CMI) against M.paratuberculosis may be detected. Only 15–25% cases of infected animals are detectable by faecal culture. Most of the animals in this group are often culled due to cases other than paratuberculosis.

3. **Clinical paratuberculosis:** In several weeks of clinical manifestation of the disease animals loose weight and suffer intermittent diarrhoea. Some animals may recover to the second stage, while the majority progress to the fourth stage with persistent diarrhoea. Faecal culture and serologic examinations of these animals are positive.

4. **Advanced stage of clinical paratuberculosis:** Oedema of the throat, cachexia and persistent diarrhoea are characteristics of this stage. Most of these animals are send to emergency slaughter or die of dehydration and cachexia.

Epidemiology of MAP in organs
As the disease advances the infection is disseminated in organs distant from the gastrointestinal system via the blood and lymphatic vessels. MAP can be found within macrophages in the *lamina propria* of the intestine, mesenteric lymph nodes, foetus, mammary gland, and uterus (Merkal, 1984). Phagocytes containing intracellular mycobacteria disseminate infection to other parts of the body and also probably migrate back onto the mucosal surface to shed bacilli (Lugton, 1999). The bacteria are carried by macrophages to other sites particularly the uterus, the foetus, the mammary gland, the testes and semen of bulls. *M. paratuberculosis* was detected in blood, cow's milk, semen of bulls, lymph nodes, different parenchymatous organs like liver, kidney, spleen, lung, uterus, mammary gland, testes, epididymis and bulbourethral gland of infected animals. Isolation of *M. paratuberculosis* from udder tissue (Taylor *et al*., 1981), supramammary lymph nodes (Doyle, 1954) and milk (Alexejeff-Goleff, 1929) of cows with clinical signs of paratuberculosis has been reported.

Prenatal infection
Although the widely known infection of new-born animals occurs by oral ingestion of the pathogen, calves may acquire infection in utero (Sweeney, 1996). Many studies have been carried out to solve the issue of whether *M. paratuberculosis* could be acquired in the womb of dam. Isolation of *M. paratuberculosis* from the uterine mucosa and tissue of the foetus was reported earlier. The reproductive organs of cows are reported to be included in the many sites where *M. paratuberculosis* has been isolated. Congenital infection by paratuberculosis was first reported by Alexejeff-Goleff (1929). Similarly, Hole (1953) reported isolation of *M. paratuberculosis* from cotyledons of a cow with paratuberculosis. Pearson and McClleland (1955) have examined the foetuses of two cows with paratuberculosis and isolated the organism from both the foetus and the uterine mucosa.

Postnatal infection
As calves are the most susceptible group in a herd, faecal contamination of teats and the presence of mycobacteria in colostrum and milk expose suckling neonatalanimals to ingest large doses of the organism. Contaminated pasture, water and feed may also be responsible for infection (Chiodini *et al*., 1984a). The risk of infection is prominent in loose housing system or at pasture, where calves are frequently in contact with cows shedding the organism via their faeces or milk. Streeter *et al*. (1995) carried out a study in a herd with high prevalence of paratuberculosis infection and isolated *M. paratuberculosis* from the colostrum of 8 (22.2%) cows and from the milk of 3 (8.3%) cows. They also have pointed out that heavy faecal shedders are also more likely to shed the organism in the colostrum than are light faecal shedders. Calves born from paratuberculosis free dams acquire infection in their early age by ingestion of *M.paratuberculosis* via contaminated feed, water and utensils (Chiodini *et al*., 1984a).

Infection in Adult animals
Only a small dose of organisms may be required to establish infection in a new-born calf, and overwhelming age-related resistance by introduction of a large dose of organisms to an adult cow is probably possible (Collins *et al*., 1994). The outcome of infection in adults is not well understood but some animals exposed for the first time as adults may develop clinical disease while others develop only a sensibility to Johnin (antigen extracted from *M. paratuberculosis* used for skin testing) for short periods although they may become carriers of the organism without manifesting clinical signs (Larsen *et al*., 1975).

Faecal oral route of infection
The primary route of infection in cattle population occurs by oral ingestion of *M. paratuberculosis* from contaminated feed and water. In an intensive farming system, where animals are kept indoor, the most common problem is faecal contamination of feed by use of common equipment for faeces and feed handling or feed bunk designs that allow faecal contamination. Although adults are considered refractory to *M. paratuberculosis* infection, a sufficient dose can probably cause infection and disease. In an extensive farming system, usually at pasture, animals concentrate in areas of water, feed and mineral supplements, where close contact of individuals increases the chance of infection. *M. paratuberculosis* contamination of feed, water, and soil represents the major risk factor for. *M. paratuberculosis* contamination of feed, water, and soil represents the major risk factor for the spread of the disease in farmed deer and wild ruminants in zoological gardens (Boever and Peters, 1974). Free ranging wild ruminants can be infected at pasture, temporarily or previously used by infected cattle (Riemann *et al*., 1979). Greig *et al*.(1999), demonstrated the concept of inter-species transmission of *M. paratuberculosis* between livestock and rabbits running in pasture.

Artificial insemination
Although bulls are the least in number in a given animal population, they can be significant sources of infection. In grazing herds, they may be mated to cows with susceptible unweaned calves. They also have direct contact with breeding cows by natural mating or indirectly by artificial insemination. Amstutz (1984) has pointed out that the prevalence of paratuberculosis is higher in bulls than cows. *M. paratuberculosis* organism may be incorporated in to the cow via semen collected from a shedder bull or semen contaminated during collection (Larsen and Kopecky, 1970). Faecal contamination of semen by *M. paratuberculosis* has been also reported by Edmondson *et al*. (1948).

DIAGNOSIS
The major difficulty encountered in the diagnosis of paratuberculosis is the exact identification of subclinical cases. Infected animals may not show symptoms of the disease for 3 to 5 years after infection and by the time clinical signs are manifested animals have already enough time to contaminate the environment. Moreover, the intracellular and slowly progressive nature of *M. paratuberculosis* complicates the diagnosis process. These are largely responsible for the relatively low sensitivity ofthe currently available tests for Johne's disease. Bearing in mind the four stages of Johne's infection, current tests

generally cannot detect early stage I infection and theyfail to detect many of the subclinically infected animals in Stage II. For this reason, a combination of more specific and sensitive diagnostic methods should improve the accuracy of diagnosis (Whitlock and Buergelt, 1996).

Detection of the immune response

As paratuberculosis first triggers the immune response of the host animal in different stage of the disease, various cellular and humoral responses are observed in the course of the clinical development.

Cell mediated immunity (CMI)

Though various methods for the detection of CMI exist, such as intradermal test (Körmendy, 1988), the lymphocyte transformation test (Buergelt*et al*., 1977), the migration inhibition test (Bendixen, 1977), and assays for interferon-gamma (IFN-) production test (Wood *et al*., 1989), only two of them will be stated here.

Skin testing

It tests for the delayed hypersensitivity (DTH), commonly referred to as skin tests have been used for many years for diagnosing bovine tuberculosis. For paratuberculosis this test is performed similarly to tuberculin test by intradermal inoculation of an extract of *M. paratuberculosis*. An increase in the thickness of the skin on the site of injection >4 mm within 24 to 72 hours is considered as positive. Nevertheless, this test is not recommended because of lack of specificity and poor correlation with the infectious status of the animal (Chiodini, 1984a). The sensitivity of the Johne's skin test is about 54%; specificity, about 79% (Hermel, 1998).

Interferon-gamma detection (IFN-)

This method is similar to that of skin test except that IFN-test is performed in vitro. IFN- is released by lymphocytes after their exposure to antigens. Animals that are, or have been infected with *M. paratuberculosis* have cells circulating in their blood that have been "trained" to recognise the antigens of this bacterium and respond by releasing significant amounts of IFN- . Two assays known as a bioassay (Wood *et al*., 1989) and sandwich enzyme immunoassay (EIA) have been evaluated (Rothel*et al*., 1990). Results indicated that the IFN- preferred to bioassay. Studies have been performed to attain diagnosis of paratuberculosis in young animals by the detection of IFN- (Collins and Zhao 1995). However, these results indicated that non specific reactions and uncertain interpretation of assay limited the use of IFN- EIA in young animals.

Humoral immune response

In the initial stages of infection, *M. paratuberculosis* induces a cell-mediated response, which keeps the infection confined to the intestinal wall. It doesn't, however, produce antibodies in the bloodstream that serology tests could detect. At stage the animal isn't shedding bacteria, so even a faecal culture wouldn't detect an infected animal. As the infection progresses to clinical disease, that cell-mediated response drops off and a humoral response, which produces antibodies, predominates (Hermel, 1998). Humoralimmunity emerges 10 to 17 months after infection (Lepper*et al*., 1989) thus testing before this age should not be recommended. Three serological tests to detect serum antibodies of cattle infected with *M. paratuberculosis* are being used in most

diagnostic laboratories. The AGID (Agar Gel Immunodiffusion), ELISA (Enzyme-Linked Immuno sorbent Assay) and CFT (Complement Fixation Test) are easy to perform though lacking sensitivity.

AGID

The AGID test has a high specificity (> 90%) in cattle with clinical signs compatible with Johne's disease (in late stages III and IV). Infected cattle without clinical signs are less often positive on AGID. The sensitivity is estimated to be 30% in pre-Stage IV infections (Hermel, 1998). The AGID test was among the first serologicaltests developed for the diagnosis of paratuberculosis. In the first half of the 1990s this test was used as a supplementary method where all animals older than 18 months tested positive for AGID were subject to faecal culture and consequent culling from the farm (Pavlík *et al*., 2000c). However this test is considered less sensitive than both the ELISA and the CFT (Nielsen *et al*., 2001).

CFT

It detects complement-fixing antibodies to *M.paratuberculosis* in the blood serum. The specificity of the CFT is considered to be lower than that of both theAGID and ELISA. Moreover, this test is reported to detect antibodies 1 to 5 months later than the ELISA (Ridge *et al*., 1991). The CFT, which is required by many countries for export or import, is intermediate in sensitivity and specificity to AGID and ELISA. With many false positives and false negatives, the CFT is not recommended for routine diagnostic use. Antigens used in the assays in different countries vary in composition depending on the method of preparation (Hermel, 1998).

ELISA

The ELISA has been most widely used for screening herds. Detection of infection by ELISA techniques appears to be dependent upon the disease stage of the animal tested. ELISA sensitivity for clinical cases has been reported to be 85%, while the sensitivity is about 15% for subclinical cases (Hermel, 1998). Absorption of serum samples using *M. phlei* is done to remove most non-specific antibodies to related bacteria such as other mycobacteria, Nocardiaasteroides and other closely related bacteria (Nielsen *et al*., 2001). ELISA has been most widely used for screening purpose of herds. Most experts on paratuberculosis recommend any animal testing positive forJohne's based on ELISA be confirmed by faecal culture.

Detection of *M. paratuberculosis*

This method implies to the direct detection of the bacterium that causes the infection.

Conventional culture

Faecal and tissue culture is the most widely used diagnostic test for *M. paratuberculosis* (Nielsen *et al*., 2001). Standard bacteriological method has been used for almost100 years and is based on the culture of *M. paratuberculosis* on a media containing a growth factor Mycobactin: HEYM (Herrold's Egg Yolk Media) (Whipple *et al*., 1991) or modified Löwenstein-Jensen medium are the preferred media used in many diagnostic laboratories (Jørgensen, 1982). Isolation of the organism on solid growth media is recommended by Whitlock and Rosenberger (1990). The problem associated with this test is that the strain of *M. paratuberculosis* isolated from

sheep frequently fails to grow on standard culture media, a long incubation period (5–16 weeks) and moderately expensive cost. The advantage of this method over serological methods (CFT and AGID) is its high specificity (100%). Merkal (1970), reported that culture will detect infected animals shedding more than 100 CFU/g of faeces, and the reported diagnostic sensitivity of faecal culture is roughly 50% (Shin, 1989)

Radiometric culture (BACTEC)
This method is a radioactive-based detection method adapted from the one used to isolate *M. tuberculosis* in humans. Collins *et al*. (1990b) demonstrated that the BACTEC system, if modified, could also be used to diagnose paratuberculosis. The culture media is commercially available but requires supplementation with additional nutrients to enable the grow of *M. paratuberculosis*. The main advantage of this method over the standard one is that it can detect low numbers of *M. paratuberculosis* andcan detect the bacterium faster (in 7 weeks) than standard culture methods. The other advantage is the BACTEC method can grow *M. paratuberculosis* from a wide variety of animal species, including sheep. Disadvantages are that the BACTEC method is more expensive, requires an instrument to read the culture vials, and involves handling of radioisotopes (Sockett *et al*., 1992).

DNA probe or PCR based (non-culture detection)
The application of molecular biology methods as diagnostic tool for identification of paratuberculosis in cattle is currently under development and evaluation. The insertion sequence IS900, discovered in the late 1980s, is the only genetic marker so far used for specific detection of *M. paratuberculosis* (Collins *et al*., 1989). About 15 to 20 copies of this sequence are integrated into the genome of *M. paratuberculosis* (Green *et al*., 1989). DNA test based on the 5'-region of IS900, can specifically distinguish *M. paratuberculosis* from other mycobacteria, including members of the *M. avium* species: *M.a. silvaticum* and *M. a. avium*. (Moss *et al*., 1991). DNA probes enable detection of *M. paratuberculosis* without having to grow the bacterium, hence are faster (in less than three days). The main disadvantage of the DNA probe is cost. Moreover, the presence of PCR (Polymerase Chain Reaction) inhibitions in clinical specimens (esp. in faecal samples) limited the successful routine use of this diagnostic method on clinical samples.

CONCLUSION
Paratuberculosis is an untreatable chronic inflammatory infection of the intestines, which affects domestics and wild ruminants. Disease causes huge economic losses to dairy sector due to decreased milk production and premature culling of the animal. To control the spread of MAP, test-based culling is typically recommended. Faecal culture remains the gold standard and its performance has been improved by the commercial availability of BACTEC and MGIT media and the application of IS900 PCR to the culture. PCR based tests have the potential to provide rapid detection of MAP infection at a cost comparable to that of conventional culture. Estimation of the sensitivity and specificity of these assays is necessary,

however, before they are implemented routinely for JD diagnosis.

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