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Survival mechanisms of *Mycobacterium avium* subspecies *paratuberculosis* within host species and in the environment—A review

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ABSTRACT

***Mycobacterium avium* subsp. *paratuberculosis* causes chronic inflammation of the intestine known as Johne's disease (JD) in domestic and wild ruminants including primates. MAP has also been associated with inflammatory bowel disease (IBD) so called Crohn's disease (CD) of human beings, which is incurable even after surgery. By virtue of the pasteurization resistant power, high endemicity of the infection in animals continues to be the permanent source of infection to human population. High bio-burden of MAP in wide range of biotic (animal hosts including human beings) and abiotic environment in each and every country where it has been investigated, serves a reminder about the survival abilities of the MAP in diverse range of environmental conditions. Ability of the MAP to evade immune system of the host and the temporal events during infection of the macrophages, is an area of major concern and research activities as the pattern of distribution are quiet different from those of other pathogenic intracellular organisms. Moreover, the organism can survive over a wide range of environmental conditions such as high and low environmental temperatures, pasteurization, low pH, and high salt concentration etc. This superior survival efficiency from environmental degradation and dormancy within host allows the pathogen to be available for causing disease and pathogenicity in animals and human beings, when conditions are favorable. Perusal of literature reveals that, despite the availability of whole genome sequence of MAP, a very little is known about the replication, persistence and survival mechanisms of this pathogen. Therefore, this review tries to address the survival mechanisms of *Mycobacterium avium* subspecies *paratuberculosis* in the different host species and adverse environmental conditions in order to allow designing of more rational diagnostic and control procedures.**

Keywords: *Paratuberculosis*; Johne's Disease; Reservoir; Disease; Survival; MAP; Animal

1. INTRODUCTION

German scientists, H. A. Johne and L. Frothingham, isolated an acid-fast bacterium from animals with a wasting disease characterized by chronic granulomatous inflammation that principally affected the ileum, around 100 years ago. The animal disease became known as Johne's disease or *paratuberculosis*. *Mycobacterium avium* subsp *paratuberculosis* (MAP) is a sharp veterinary pathogen [1] and unambiguously responsible for causing

paratuberculosis or Johne's disease (JD), a chronic granulomatous gastroenteritis in domestic and wild ruminants, worldwide [2-9]. The disease has been reported on every continent [10-12] and causes serious economic losses to dairy farmers because of loss of milk production and early culling of cows [13,14]. Studies on the disease, epidemiology, incidence, prevalence, economic losses, diagnosis, pathology, management and control have been mainly carried-out in the developed countries of the world and information is limited on MAP infection and disease in developing and poor countries [15-17]. This is mainly due to the complexity of the MAP infection and problems in the diagnosis of the disease. However, studies in India showed the high prevalence of disease in domestic (goat, sheep, cattle and buffaloes) and wild (hog deer, blue-bulls, bison, etc.) ruminants, other animal species (camel, rabbit, etc.) and primates [2,9,18-20]. MAP has also been reported from environment (Pasteurized milk, soil, river water, etc.) sources [21,22]. Biotyping of the MAP showed that it is a unique bio-type, ("Indian Bison type"), which has been reported first time in the world [23-25]. This biotype hast very broad host range and diagnostics and vaccine have been developed for the control of disease in livestock population [26,27]. Similar information is not available from major parts of the world. Studies also showed MAP has also been implicated as the causative agent in some and possibly all cases of Crohn's disease (CD), a gastrointestinal disease of humans having similar histo-pathological findings as found in JD [28-31]. Similarities between the clinical symptoms and gross pathology of Johne's and Crohn's disease were first noted over 80 years ago. Recently, the question of the role for MAP in Crohn's disease has aroused considerable controversy within the scientific and medical communities and MAP is still not been unequivocably established as the cause of human disease, however, evidences show some kind of association though not necessarily causal, between MAP and at least in cases of Crohn's disease [20,32]. Recent studies in India reported high presence of MAP in the CD patients, persons suffering with Inflammatory Bowel disease (IBD), colitis and apparently healthy persons, animal keepers, animal attendants, non-animal keepers etc. Both direct and indirect tests (blood PCR, ELISA and blood culture) recorded high presence of MAP in their stool samples (culture, microscopic examination, PCR) [26,33,34]. For many years, it was thought that the insertion element IS900 was specific to MAP. Therefore, this sequence was used to design primers for polymerase chain reaction (PCR) tests used to detect MAP in veterinary, clinical and food samples or to confirm the acid-fast isolates as MAP was the basis of molecular typing methods such as restriction-fragment length polymerphism [23,35]. However, nowadays, reports of IS900-like sequences in other mycobacteria [36-38] casts doubt on the specificity of IS900 PCR for MAP. This has prompted researchers to look for more specific targets and several alternative MAP-specific targets *i.e.*, IS-*Mav*2 [39] HspX protein [40] and F57 [41] have been reported. Recent analysis of the genome sequence of MAP uncovered two new sequences with no homologues amongst other mycobacteria; IS_MAP02 (six copies) and IS_MAP04 (four copies) [42]. A nested-PCR method targeting IS_MAP02 has been developed [43] and the utility of this new method for diagnostic purposes requires investigation.

JD is a unique animal disease having the distinction of being reported from all the countries where ever investigated. It has a devastating effect on the livestock productivity (early culling and reduced milk production) and livestock industry incurs huge economic losses [13,44]. MAP is transmitted in herds both directly through semen, milk, colostrum and in-utero and indirectly by oral route through contaminated feed, fodder, pasture, waters etc., (fecal oral route) [45]. Programs for the control of MAP infection in domestic livestock at the National level are in progress in many developed countries [46]. To reduce the prevalence or to eliminate MAP infection, the measures used are tested and culling of infected animals or depopulation of herds/flocks of infected animals. MAP is described taxonomically as obligate pathogen and parasite of animals and theoretically it can be eradicated by removal of all infected animals from herds. However, MAP can survive for prolonged periods in the environment, particularly grazing land, water bodies and enabling it to withstand periodic absence of suitable hosts. Present review addressed the survival mechanisms of MAP in host species and in the environment.

2. SURVIVAL MECHANISM OF MAP BACILLI IN HOST SPECIES

Researches on pathogenesis and immunology of MAP infection are necessary to allow design of more rational diagnostic and control procedures. Few of the specialized micro-organisms can survive inside macrophages designed specifically to kill bacteria. These include *Mycobacteria*, *Salmonella*, *Listeria*, *Coxiella* and *Corynebacteria.* However, hallmark of MAP pathogenesis is their ability to survive and replicate within macrophages. Different mechanisms are employed as survival strategies and mycobacteria are exceptional in the duration and persistence of this interaction. Survival of pathogenic mycobacteria is attributed to the fact that the mycobacterial phagosome does not fuse with lysosomes [47,48].

As far as the organ-specific cell specificity is concerned, MAP target the lymphoid tissue of the intestinal mucosa, and past evidences suggested that MAP enters the host system by M cells at the Peyer's patches. It is able to invade intestinal macrophages and is capable of

resisting host defense and multiplies intra-cellularly to reach very high numbers. This is mainly due to the MAP capacity to inhibit activation of macrophages, inhibition of phagosome acidification and attenuate presentation of antigens to the immune cells [49]. MAP is able to modulate the ruminant innate immune response for their survival [50].

2.1. Entry of MAP Bacilli in the Phagocytes

After ingestion, MAP bacilli enter intestinal tissues through M cells in the Peyer's patches of small intestine. MAP expresses fibronectin proteins that mediate uptake of MAP through binding on M cells and [51]. After crossing intestinal epithelial layer, sub-epithelial macrophages phagocytose MAP. Macrophages are known to have several receptors that are involved in the uptake of mycobacteria [52]. These receptors are immunoglobulin receptors (FcR), complement receptors viz., CR1, CR3, and CR4, scavenger receptors and mannose receptors. CR3 is the major receptor involved in the uptake of mycobacteria including MAP [53]. Therefore, MAP takes advantage of phagosome acidification and IL-1β processing for its efficient transverse through the epithelium and enters the macrophage [54].

2.2. Intracellular Survival of MAP

Activated macrophages are the main effector cells involved in the killing of mycobacteria. IFN-γ and other pro-inflammatory cytokines are important in activation of resting macrophages. Activated macrophages produce reactive oxygen intermediates (ROIs) or reactive nitrogen intermediates (RNIs) to kill invading microbes. However, mycobacterial products, including sulfatides, LAM and enzymes like super oxide dismutase (SOD), catalases, etc., are able to scavenge ROIs [55]. In addition, mycobaceria encodes for four protein NADH-dependent peroxidase and peroxynitrite reductase system. This system has alkyl hydro-peroxide reductase C (AhpC) that catalyzes the NADH-dependent reduction of peroxynitrite and hydro-peroxide. Oxidized AhpC is reduced by AhpD, regenerated by dihydrolipoamide acyltransferase (DlaT). Finally, dihydrolipoamide dehydrogenase (Lpd) mediates reduction of Dla and completes the cycle [56]. However, free fatty acids (FFA) have strong antimycobacterial activity. It has been shown that RNIs in combination with FFA plays a crucial role in killing of mycobacteria [57]. In addition, defensins (cytotoxic peptides) are important host defense mechanisms against mycobacteria [58]. Also granulysin (antimicrobial protein produced by cytolytic T lymphocytes and NK cells) is able to kill mycobacteria [58].

Mycobacterial inhibition of phagosomal maturation is well documented and numerous studies have described mechanisms credited for this inhibition including reduced activity of a macrophage enzyme, sphingosine kinase [59]; secretion of lipid phosphatase that inhibits phosphatidylinositol 3-phosphate production, thereby disallowing the acquisition of lysosomal constituents by phagosomes [60] thus disrupting the phagosome acidification [31] interaction of mycobacterial mannose capped lipoarabinomannan (ManLAM) with mannose receptors (MRs) on macrophage resulting in limited phagolysosome fusion [61,62]; and attenuate presentation of antigen to immune system (Weiss and Souza, 2008). Though these mechanisms have not been fully elucidated for MAP, however, survival of MAP in murine and human macrophages mimics that of *M. tuberculosis* [63]. Phagosome-lysosome fusion has been found to be poor in monocytes that ingested viable MAP than monocytes that ingested killed MAP and Ca^{2+}/CaM and phosphatedylino 3 kinase-dependent pathways are required for optimal monocyte anti-MAP activity (modified) [64].

Recently, it has been shown that MAP uses the JNK/SAPK pathway to regulate cytokine expression in bovine monocytes since addition of SP600125 (specific chemical inhibitor of JNK/SAPK) failed to alter phagosome acidification and enhanced the capacity of monocytes to kill MAP bacilli [65]. Gene expression analysis of bovine macrophages infected with MAP demonstrated a decrease in ATPase expression that correlated with lack of phagosome acidification [66]. Experiments with other mycobacteria had shown that ATP treatment increases anti-mycobacterial activity of macrophages through cytosolic phospholipase A2 (cPLA2)-dependent generation of arachidonic acid [67].

A major signaling receptor incriminated in susceptibility to MAP infection is TLR2, which activates the MAPK-p38 pathway and may activate the NF-kB pathway [68]. Both pathways initiate IL-10 transcription. This early production of IL-10 inhibits the pro-inflamematory cytokines, chemokines, IL-12, and other major histo-compatability (MHC) factor class-II expression [69]. Therefore, MAP LAM-induced TLR2-MAPK-p38 signaling with resultant excessive IL-10 expression has emerged as one of the mechanisms by which MAP organisms survive within host mononuclear phagocytes. Phagocytosis of MAP results in a marked and persistent decrease in expression of MHC class-I and class-II molecules within 12 to 24 hours after phagocytosis. The MHC class-I and class-II expression by macrophages remained low even after addition of IFN-γ [66]. Additionally, microarray studies indicated that MHC class-II expression was down regulated in MAP-infected macrophages [66]. Irreversible down regulation of MHC expression could contribute to the paucity of T-cell infiltrates and tubercle formation observed in Johne's disease lesions [3]. *M. avium* subsp. *paratuberculosis* infection

alters the responses of bovine monocytes to TLR9 stimulation thus able to generate the environment within host that favors the infection [70]. MAP also inhibits gamma interferon based signaling of bovine monocytes [71]. MAP can regulate the cytokine expression in bovine monocytes by activation of Jun N-terminal kinase/ stress-activated protein kinase (JNK/SAPK) pathway, which helps in its survival and to change the immune and inflammatory responses [65,72].

Apoptosis constitutes major mechanism to limit pathogens by preventing dissemination [73]. TNF alpha is required for induction of apoptosis in response to infection. Interestingly, pathogenic mycobacteria release neutralizing reagents for TNF alpha receptors [74]. Also release of TNF alpha receptors in turn is regulated by IL-10 production [74]. Pathogenic mycobacteria may selectively induce IL-10, leading to decreased TNF alpha activity and reduced apoptosis. LAM also prevents apoptosis of mycobacteria-infected cells in a Ca^{2+}-dependent mechanism [75]. LAM antagonizes apoptosis by preventing an increase in cytosolic calcium concentration. Cytosolic calcium facilitates apoptosis by increasing the mitochondrial membrane permeability, promoting the release of pro-apoptotic products viz., cytochrome C [76]. LAM also stimulates phosphorylation of pro-apoptotic protein *i.e.* Bad, whose phosphorylation prevents the molecule from binding to the antiapoptotic proteins Bcl-XL and Bcl-2, which prevent the release of cytochrome C from mitochondria [76]. Studies have shown that high levels of TRAF1 and IL-1a mRNA were found in lesions of ileal tissues associated with MAP-infected cattle [75] and macrophages within these lesions are responsible for these high levels, and it has been proposed that enhanced expression of TRAF1 would increase resistance to externally triggered apoptosis and lead to failure to properly activate following engagement of CD40 on macrophages by T cells expression CD40 ligand [77].

2.3. Temporal Events during Map Infection in Macrophages

Growth and survival of MAP within macrophages has been an area of intensive study because of its implications in pathogenicity. For example, *M. bovis* has been shown to grow within macrophages whereas BCG strains do not [78]. This observation is controversial since there are reports of immuno-compromised patients with disseminated BCG. Although multi-species studies are complex and multi-factorial, MAP appears to be able to survive in secondary lysosomes better than does *M. tuberculosis*. In macrophages co-infected with *Coxiella burnetii,* an intracellular pathogen known to inhabit and replicate within secondary lysosomes [79], MAP growth was not impaired, but in contrast, *M. tuberculosis* bacilli that co-localizes with *C. burnetii* containing vacuoles, do show reduced growth [80]. The growth of MAP can be measured at early stages during infection of non-activated macrophages by bacterial cell counts following serial dilutions on HEYM slants, which shows a slow decline in *M. paratuberculosis* viability. After infection, an initial growth phase occurs until 24 hours post-infection where mycobacterial counts begin to decline. In majority of the cases, an increase in bacterial counts occur after 70 hours post-infection up until 95 hours where a second decline in viable MAP occurs. It suggests that while MAP survives much longer in macrophages than some other pathogens [81,82] including other species of mycobacteria [83], there remains a significant decrease in viability over time. Thus, it is of interest to examine the progression of MAP infection by immuno-electron microscopy.

A reliable method to label intracellular mycobacteria needs to be developed first, for which affinity purified rabbit antibodies against a whole cell sonicated lysate of MAP are usually used that label the outer periphery of Middlebrook 7H9 cultured MAP. This purified antibody preparation can then be used to label the organism within infected macrophages. The purified antibody is highly specific as all gold particles are associated with the mycobacteria and no labeling of the surrounding background or macrophages is observed. MAP-infected macrophages fixed in glutaraldehyde show vacuoles harboring the organism which are tightly arranged not spaciously and remain in very close contact with each other [80,83]. The size, number, and morphology of the organism appear to remain relatively constant throughout the observed time period. At all times post-infection, MAPs are mostly found as groups inside vacuoles. Occasionally, single bacilli are observed within a tight vacuole. An increase in the percentage of degraded MAP is observed with time and interestingly, the organisms remain morphologically unrecognizable. When labelled with immunogold, the presence of MAP antigen is demonstrable. These findings indicate that macrophages can kill and degrade a percentage of mycobacterial cells in a given infection. Several intracellular pathogens like *Chlamydia* and *Coxiella burnetti* undergo readily distinguishable morphological changes during infection of host cells [84,85]. This is clearly not the case for MAP as they remain morphologically similar at least up until 4 days post-infection. However, an increased percentage of degraded mycobacterial forms can be observed over time. These degraded forms appeared by 24 hours post-infection and are hardly recognizable morphologically, but labelling with immunogold particles indicate the presence of mycobacterial antigen [86,87]. Such type of *in-vitro* assays can provide an useful insight into the host-pathogen relationship.

As we know, all intracellular bacterial pathogens enter host cells surrounded by a membrane bound vacuole [88,89]. Some pathogens in heavily infected cells collect into a single vacuole within the same cell [90]. It has been found that J774 macrophages infected at a ratio of 5:1 show separate MAP—containing vacuoles within the same macrophage even after 48 hours post-infection. Likewise, *M. tuberculosis* and *M. avium* appear to remain in distinct phagosomes that do not harbor more than one bacillus per vacuole. However, in MAP-infected macrophages, one of the first phenotypic alterations following activation with cytokines is the coalescence of individual MAP containing vacuoles into communal vacuoles with many bacilli [91]. The significance of separate MAP-containing vacuoles is still unclear. Thus, it can be inferred that there appears to be a tenuous relationship to gain control between MAP and the macrophage with survival at stake. The macrophage can control growth and even kill MAP. However, the organisms are cytotoxic to macrophages or induce apoptosis at high doze.

3. SURVIVAL MECHANISM OF MAP IN ENVIRONMENT

3.1. Soil, Pasture, Faeces and Environment

MAP has a robust ability to survive in the environment [92]. MAP tends to move slowly through soils and remain on grass and upper layers of pasture soil, posing as hazard of infection for grazing animals and may have the potential to move through soil and/or water as a result of heavy rainfall or irrigation [92,93]. MAP is able to survive in the environment up to 152 to 246 days depending on specific conditions. Time that is required to eradicate the organism from the environment needs verifications. It has been presumed that at least 6 months to a year is required to render pastures safe after grazing by infected cattle [94], however its DNA can be detected from the samples of soil and plants collected from the field 2 years after destocking of infected animals [95]. Previous studies from England, France and US has reported that soil types and soil composition; soil and/or water physiochemistry are connected with the incidence of *paratuberculosis* in livestock herds [45,96-99]. Longevity of MAP may be reduced by drying of soil, exposure to sunlight, changes in ambient temperature, pH below 7.0, high ammonia level and low iron contents [100]. MAP survives for longer duration in water, sediment behind dams [101] and domestic water reservoirs [102]. So, it can be concluded that water reservoirs may play significant role in MAP infection on farms. There is increased risk of this disease, if the animals were raised on rich organic soil, probably due to adsorption of MAP to clay content [98].

Studies have been conducted on survival of MAP in cattle slurry (pH 8.5, dry matter 7%), swine slurry (pH 8.3, dry matter 8.3%), and a mixture of the two (pH 8.4, dry matter 7.7%) at 5˚C or 15˚C and it has been reported that the survival time is 252 days in all three kinds of slurry at 5˚C, and at 15˚C it is 182 days in swine slurry, 98 days in cattle slurry, and 168 days in mixed slurry [103]. The viable count of MAP bacilli has been reduced of 90% to 99%, if the organism in feces becomes mixed with soil. The survival of *Mycobacterium avium* subsp. *Paratuberculosis* has been studied by culture of fecal material sampled at intervals for up to 117 weeks from soil and grass in pasture plots and boxes. Survival for up to 55 weeks has been observed in a dry fully shaded environment, with much shorter survival times in unshaded locations. Moisture and application of lime to soil did not affect survival. UV radiation was an unlikely factor, but infrared wavelengths leading to diurnal temperature flux may be the significant detrimental component that is correlated with lack of shade. The organism survives for up to 24 weeks on grass that germinated through infected fecal material applied to the soil surface in completely shaded boxes and for up to 9 weeks on grass in 70% shade. The observed patterns of recovery in three of four experiments and changes in viable counts were indicative of dormancy, a hitherto unreported property of this taxon. Adps-like genetic element and relA, which are involved in dormancy responses in other mycobacteria, are present in the *M. avium* subsp. *Paratuberculosis* genome sequence, providing indirect evidence for the existence of physiological mechanisms enabling dormancy. However, survival of *M. avium* subsp. *Paratuberculosis* in the environment is finite, consistent with its taxonomic description as an obligate parasite of animals [100].

Cattle urine is also hostile to MAP survival and increasing concentrations of urine (2% - 10%) at pH 6.3 to 6.6 caused decreasing survival rates of the organism. During a study conducted to detect the anaerobic digestion caused by MAP in bio-gas plants, slurry has been spiked (3.3 × 103 to 2.7 × 104 bacilli/gm) and held at mesophilic conditions (moderate temperatures; 35˚C or 95˚F) or thermophillic conditions (high temperatures; 53˚C - 55˚C or 127˚F - 131˚F) [104]. At mesophillic conditions MAP was survived at 7, 14, and 21 but not at 28 days. At thermophillic conditions viable MAP could not be detected in as short as 3 hours. The cell wall of MAP bacilli plays an important role in the survival of MAP in the environment and adverse conditions. However, exact survival mechanisms are not known. According to one hypothesis, mycobacteria are able to enter into a state of non-replication or dormancy (spore forming) during stress like nutrient deficiency or hypoxia and may persist for longtime in host, soil and environment [49].

3.2. Surface Water

Survival of MAP has been reported for about 6 to 18

months in tap or pond water in sealed bottles and for about 15 months in distilled water [29]. In neutral water (pH 7.0) MAP was survived up to 517 days (17 months) while for pH 5.0 and pH 8.5 water the longevity of MAP was reduced up to 14 months. Interestingly, MAP can remain culturable in lake water microcosms for 632 days and persisted to 841 days [102]. The presence of fatty acids, lipids and waxes in the cell wall of MAP is responsible in part for the extreme hydrophobicity and play an important role in the survival of MAP in water [105]. Cell wall of MAP leads to adsorption to air: water interfaces, surfaces (e.g. pipes), and to phagocytosis by macrophages and protozoa [106]. The survival of MAP in water is potentially as significant for disease transmission as survival of the organism on soil and pasture [107, 108].

3.3. Biofilms

MAP has not previously been isolated from biofilms in water distribution systems, although many other mycobacteria have been [109]. The closely related *M. avium* appears to have found a particular niche in hot water systems, so biofilm formation by MAP would not be entirely unexpected, although mycobactin-dependence of MAP may be a complicating factor. *Mycobacterium avium* subsp *paratuberculosis* is capable for rapid and sustained biofilm formation on livestock watering trough construction materials and this biofilm plays crucial role in their pathogenesis too [44,110,111]. The adhesion capability of organisms depends on the surface properties as well as on material surface being colonized [112,113]. Bolster *et al.* (2008) [105] investigated processes to controlling the transport of MAP through aquifer materials and reported that cell wall of MAP has a strong negative charge and is highly hydrophobic. The hydrophobic nature of cell wall may predispose these organisms to adherence [114]. Lower adhesion of MAP to stainless steel and plastic surfaces may be due to weaker interactions between the hydrobphobic cell wall of MAP and the inert surfaces of those materials. As like other bacteria, MAP in biofilms is more resistant to chemical stress than bacteria suspended free in water. The fact that bacteria are more resistant to chemical stresses when present in biofilms could also potentially contribute to MAP's survival in the environment. Hence, the importance of biofilms in MAP survival is certainly an area worthy of study.

3.4. Insects and Free-Living Protozoa

Insects and free-living protists are ubiquitous in the environment and form a potential reservoir for the persistence of animal and human pathogens. Nematode larvae have a comparatively simple life cycle. The nematode eggs hatch in faeces, feed on bacteria and undergo two moults resulting in third stage larvae retained within a sheath. The larvae at this stage stop eating and are both negatively geotrophic and positively phototrophic causing them to migrate out of the faecal mass and, surrounded by a water film, travel up blades of grass or other vegetation, where they might be consumed by ruminants [115]. The fact that symptomatic animals with Johne's disease can excrete 10^8 MAP per gram of faeces means that there is a strong chance that the external surfaces of the larvae will become contaminated with the organism in the agricultural environment [116]. It is interesting to note that the environmental factors known to favour survival of populations of third stage larvae are similar to those that also favour survival of MAP, *i.e.* protection from incident radiation and heat and availability of moisture [117]. The uptake of MAP by larvae of *Haemonchus contortus*, *Ostertagia circumcincta* and *Trichostrongylus colubriformis* present in sheep faeces has been demonstrated [118]. The nematode-infected sheep faeces was inoculated with MAP and the co-cultures left to incubate, under moist conditions, for 7 days at 25˚C after which the larvae were stimulated to migrate from the faecal mass by light. MAP is found to be intimately associated with the larval bodies. Thus, nematode larvae represent a viable means of transmission for MAP infection. However, such larvae also have the ability to penetrate the gastrointestinal mucosa [117], and hence this may provide an additional mechanism for the delivery of MAP to susceptible animal tissues [116]. In this context, it is interesting to note that a 1000-fold lower dose of *Salmonella typhimurium* was required to establish infection in mice when salmonellae were associated with larvae suggesting that the latter provided a more efficient means of initiating infection [119].

Earthworms and insects, such as Diptera and cockroaches, may also represent possible vectors of transmission of MAP [120]. They live on decomposing material ingesting bacteria, most of which pass through and are excreted in their faeces [121]. Mycobacteria, because of their cell wall structure, are resistant to the digestive enzymatic activity of insects and can be excreted in their saliva and faeces [122]. In a field study of earthworms from environments heavily contaminated with MAP. It has been found that only a small proportion of earthworms contaminated with the organism. They also established, using MAP-contaminated faeces, that the residence time of MAP in the earthworms was comparatively short (up to 2 days). Hence, earthworms may only be a minor contributor to the survival and transmission of MAP in the environment. In another study, isolated MAP has been isolated from *Scatophaga* spp. (flies) collected from pasture grazed on by a confirmed Johne's affected herd and from *Calliphora vicina Robineau-Desvoidy*

(blue bottles) and *Lucilia Caesar* Linnaeus (blowflies), which had been ingesting fluids from the cut intestines of slaughtered cows with *paratuberculosis* in a slaughterhouse. These insects, both larvae and adults, must be considered vectors of MAP and potentially important in the spread of Johne's disease within infected herds [123-125].

MAP is able to survive for weeks in protozoa that are usually bacteriovores. In nature, there is interaction of MAP, insects and free living protozoa [44,126,127]. Many of the studies published on the interaction between mycobacteria and protozoa have used *M. avium* as a model. This member of the Mycobacterium genus, which is most closely related to MAP and can survive phagocytosis by *Tetrahymena pyriformis* and *Acanthamoeba* species [128]. In co-culture with *T. pyriformis*, a ciliated environmental protozoan, and it has been observed that phagocytosis of *M. avium* occurred within 10 min, reaching a maximum within 30 min and it has been suggested that the number of *M. avium* cells per protozoa remain constant throughout an extended incubation period (25 days), indicating that mycobacterial numbers may be subjected to regulation upon ingestion [106]. Limited research has been carried out to date on the interaction between protozoa and MAP specifically. Examination of dam water in Australia has revealed the presence of numerous invertebrate and protozoal species to be present, so there is certainly scope for significant interactions between protozoa and MAP to occur in the natural environment [101]. However, there is little or no evidence of acid-fast cells within the protozoa observed, so there is no firm evidence as yet that ingestion of MAP by protozoa actually occurs in the natural environment. However, there is evidence of interaction between MAP and protozoa at ambient laboratory temperature (20˚C - 25˚C) [129]. Further research is needed to confirm whether interaction occurs between protozoa and MAP at lower temperatures that may be more representative of environmental conditions. MAP is able to multiply within the vacuoles of protozoa and increase in the number [130]. Inside protozones, MAP acquire a phenotype, which is more pathogenic to human beings [106]. MAP survives within *Acanthamoeba* spp. (*A. castellanii* and *A. polyphaga*) this intracellular location provides protection from the effects of chlorination of surface water [126]. Ingestion of MAP by *A. castellanii* and *A. polyphaga* occurred readily in co-culture within 180 min at 25˚C, with 13% and 6.6% of the *A. castellanii* and *A. polyphaga* populations being internalized respectively. MAP survived internalization for up to 24 days at 25˚C and appeared to increase in number within the amoebae over time even in the absence of adventitious mycobactin. It has been further demonstrated that *A. polyphaga*-internalized MAP were more resistant to chlorine levels commonly used for drinking water treatment (2 $\mu g \cdot ml^{-1}$ free chlorine) than free MAP, so protozoan engulfment also afforded this organism protection from chemical disinfectants in addition to enhanced survival [131]. In a more comprehensive study published recently, it has been observed that the interactions between three MAP strains (two bovine and one human) and *A. polyphaga* at room temperature are complex. Using real-time PCR these authors quantified MAP cells in amoebae harvested at various time intervals during co-culture. A small proportion (2.5% - 11%) of the MAP populations were initially internalized by the *A. polyphaga*, suggesting that only single MAP cells as opposed to clumps were internalized. Immediately after ingestion MAP numbers declined sharply suggesting that only a proportion of the MAP population are able to adapt and survive internally. However, subsequently, between days 8 and 12, the surviving intracellular MAP replicates and numbers increased 10-fold or more. At 28 days protozoan encystment was associated with another sharp reduction in numbers of intracellular MAP, but this is followed after the 10th week by substantial increases in the number of MAP cells even though trophozoite formation and encystment occurred two more times. Thus, it leads to the decision that MAP has the potential for long-term persistence within environmental amoebae [129]. It has also been found that phagocytosis of *M. avium* by *Acanthamoeba castellanii* afforded the bacterium greater protection against antibiotics such as rifabutin, azithromycin and clarithromycin than when in a planktonic state [128]. Furthermore, macrophage and mouse models of infection have shown that phagocytosis of *M. avium* by *A. castellanai* enhance the entry of the former into epithelial cells leading to enhanced virulence [130]. The increased virulence is not because of selection but induction of a more virulent phenotype.

Survival mechanism of MAP in amoeba is similar to that shown for *Mycobacterium avium* within free living amoebae. Pathogenic amoebae, such as *A. castellanii*, have the ability to traverse the intestinal epithelium, and hence can carry internalized mycobacteria with them The amoebae in this case acts as "Trojan horses" by enabling the mycobacteria to evade initial host defence mechanisms [132]. *Mycobacterium avium* inhibits lysosomal fusion and replicates in vacuoles that are tightly juxtaposed to the bacterial surfaces within amoebae [130]. Interestingly, it has been demonstrated that *M. avium* is able to survive encystment of *Acanthamoeba polyphaga* and use of microscopy has shown that the bacteria had located themselves within the double walls of the infected cysts [133]. As protozoan cysts are easily transported by the wind in aerosol form there may be implications for the transmission of MAP via this route also.

4. CONCLUSION AND FUTURE PROSPECTIVES

MAP has been recently emerged as most successful animal pathogen with significant zoonotic concerns. The economic impact of disease due to production losses or replacement of animals, need of JD free certification for export of livestock or livestock products and public health concerns has drawn the attention of researchers towards the development of preventive measures to control the spread of MAP bacilli in animal species and environment. MAP has strong mechanisms for the successful survival in Johne's-infected animals, in livestock waste, on farms, and water. Best management practices including biofilm free trough surfaces; livestock waste (manure) management are important components of domestic agriculture enterprises. Survival of MAP in the manure and soil increases the opportunities for contact between MAP and animals and enhance the chance for contamination of surface water by rainfall run-off and/or irrigation. Future research should be focused towards revealing further insights to the temporal events during MAP infection of host immune cells like macrophages and to decipher the mechanisms behind the ability of MAP to survive and grow under suboptimal conditions for the control of disease in animal hosts and restrict the spread of bacilli in environment to minimize the risk of human exposure.

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