



Review Article

Survival tactics of *Mycobacterium avium* subspecies *paratuberculosis*

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Abstract: Pathogenic mycobacteria have evolved the mechanisms to subvert host immune response in the favor of longtime persistence and proliferation in the intercellular environment of the host, with resulting in functional dysregulation and disease in the host. Among the genus mycobacteria, *Mycobacterium avium* subspecies *paratuberculosis* is a robust pathogen, have a remarkable capacity to persist in the host and adverse environmental conditions (pasteurization temperature, high pH) and recently, emerged as a major concern of public health significance. *Mycobacterium avium* subspecies *paratuberculosis* is the causative agent of Johne's disease in animals and also has been incriminated as the causative agent of Crohn's disease in human beings. Therefore, understanding the factors that contribute to the longevity of this pathogen is essential to restrict the clinical outcomes of infection and design the control strategies. The present review summarizes our understanding of factors that contribute to the survival of MAP within the host and different environmental sources.

Keywords: *Mycobacterium avium* subspecies *paratuberculosis*; Survival mechanism; Host pathogen interaction; Macrophage; Environment.

1. Introduction

Infection is a result of battle between host and pathogen. It commences with the invasion of pathogen and proceeds to the attachment, multiplication and colonization in the host tissues or cells. For a successful pathogen, it is necessary to persist for a long time in the host and able to circumvent the host's immune system as per their favor. Pathogenic mycobacteria have been found to be able to escape the organization of the host immune system and multiply and persistent within macrophage using super survival mechanisms [52-53]. Recently, World Health Organization (2010) has estimated that nearly one third human population of the world is infected and nearly 1.7 million people die each year due to tuberculosis, a chronic infectious disease caused by *Mycobacterium tuberculosis*; one of the most successful human pathogen [15]. In case of animals, *Mycobacterium avium* subspecies *paratuberculosis* (MAP) has recently emerged as major animal pathogen

with significant economic losses and zoonotic concerns [33].

Mycobacterium avium subspecies *paratuberculosis* infection in animals causes *paratuberculosis*, an incurable, chronic wasting and debilitating infectious disease, characterized by progressive weight loss and intermittent or severe diarrhea, leading to protein-losing enteropathy and ends with the death of affected animals [84]. Subclinical and clinically infected animals frequently excrete abundant amount of MAP bacilli in their feces and milk, and increase the risk of infection to newborns and also increase the bioburden (environmental load) of MAP bacilli. MAP shows high resistance towards environmental stress and able to survive greater than 1 year in soil and on pasture [22, 86]. It is not reproducibly killed by suboptimal pasteurization standards [33] and the presence of MAP in pasteurized milk and milk products posed the risk of MAP exposure to human beings [64].

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MAP has also been found to be associated with the etiology of Sarcoidosis, diabetes type 1 [59], Blau syndrome [27] and Crohn's disease [33, 37] in human beings. However, there is a continued debate in the scientific literature to prove or disprove the role of MAP in human infections [7-8, 51]. There are many references and excellent reviews in the literature covering the zoonotic potential of MAP [36, 57], however, information about the mechanisms of MAP survival within the host and different environmental sources is limited. Present review describes and highlights the survival of MAP, its persistence within the host and different environmental sources (Soil, pasture and land; surface water, biofilm and environmental protozoa) and factors contributing to super survival of MAP.

2. Survival of MAP within host

2.1 Site and mode of MAP entry (Bacterial uptake)

Inhalation of bacilli into the lower respiratory tract is the major and the preferred portal of entry for mycobacterial pathogens like tuberculosis and leprosy bacilli. However, in case of MAP, animals receive the bacterial load at an early age through in utero transmission or by ingestion of contaminated milk or colostrum and lead to clinical disease in the late phase of life [71-72]. After ingestion, MAP transcytosed to the intestinal wall of the host through microfold epithelial cells (M cells) overlying Dome of Peyer's patches in gut-associated lymphoid tissue [34, 54]. Mechanism of MAP adherence, internalization and regulation in M cell is not fully known. However, recent *in-vitro* studies showed that attachment and internalization of *Mycobacterium* species by epithelial cells is dependent on the interaction between fibronectin attachment proteins (FAPs) and fibronectin (FN) [48, 62]. Unlike other intestinal epithelial cells, M cells display $\beta 1$ integrins (receptor for FN-opsonized mycobacteria) on their luminal faces in high density, therefore serve as a major portal of entry by various *mycobacterium* species [14, 21, 48]. MAP has receptors to bind soluble FN, which in turn binds to integrins on M cells and mediate internalization in the cell [63].

2.2 Mechanisms of intercellular survival (macrophage-mycobacterium interactions)

Macrophage cells are the targeted sites for the long-term persistence and survival of mycobacteria within the host [10]. The initial interaction of bacilli with the macrophage takes place through cellular receptors as complement receptors (CR1, CR3, and CR4), immunoglobulin receptors (FcR), mannose receptor, and scavenger receptors [2, 30, 66]. However, it is not known whether bacteria interact with one or more of these receptors during *in vivo* infection, *in vitro* studies indicated that the response of macrophages is receptor dependent and interaction of receptors decide the fate of ingested bacilli in the cell. For example, interaction with CR1 receptor does not stimulate the

production of superoxide anion [24, 38]. Interaction with CR3 prevents the production of reactive oxygen and blocks the maturation of phagosomes harboring the bacteria, thus preventing fusion with lysosomes [69]. On the other hand, interaction with Fc receptors increases the production of reactive oxygen intermediates and allows the fusion of the bacteria-containing phagosomes with lysosomes [5].

Activated macrophages are the main effector cells involved in the killing of mycobacteria. IFN- γ and other pro-inflammatory cytokines are important in the activation of resting macrophages [76]. 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 superoxide dismutase (SOD), catalases, etc., are able to scavenge ROIs [16]. In addition, mycobacteria encode for four protein NADH-dependent peroxidase and peroxynitrite reductase system. This system has alkyl hydroperoxide reductase C (AhpC) that catalyzes the NADH-dependent reduction of hydroperoxide and peroxynitrite. Oxidized AhpC is reduced by AhpD, which is regenerated by dihydrolipoamide acyltransferase (DlaT). Finally, dihydrolipoamide dehydrogenase (Lpd) mediates reduction of Dla and completes the cycle [65]. However, free fatty acids (FFA) have strong antimycobacterial activity [4, 61]. It has been shown that RNIs in combination with FFA plays a crucial role in killing of mycobacteria (Akaki *et al.*, 2000). In addition, defensins (cytotoxic peptides) are important host defense mechanisms against mycobacteria [47]. Also, granulysin (antimicrobial proteins produced by cytolytic T lymphocytes and NK cells) is able to kill mycobacteria [47].

Mycobacterial inhibition of phagosomal maturation is well documented and numerous studies have described mechanisms credited for this inhibition including reducing [49]; secretion of lipid phosphatase that inhibits phosphatidylinositol 3-phosphate production, thereby disallowing the acquisition of lysosomal constituents by phagosomes [78]; and interaction of mycobacterial mannose-capped lipoarabinomannan (ManLAM) with mannose receptors (MRs) on macrophage resulting in limited phagolysosome fusion [41]. Though these mechanisms have not been fully elucidated for MAP, however, survival of MAP in murine and human macrophages mimics that of *M. tuberculosis* [60]. A study showed that phagosome-lysosome fusion was poor in monocytes that ingested viable MAP, while monocytes that ingested killed MAP [87]. The same study also showed that Ca^{2+}/CaM and phosphatidylinositol 3-kinase-dependent pathways are required for optimal monocyte anti-MAP activity [87]. Recently, it has been shown that MAP uses the JNK/SAPK pathway to regulate cytokine expression in bovine monocytes since the addition of SP600125 (specific chemical inhibitor of JNK/SAPK) failed to alter phagosome acidification and enhanced the capacity of monocytes to kill MAP

bacilli [67]. Gene expression analysis of bovine macrophages infected with MAP demonstrated a decrease in ATPase expression that correlated with lack of phagosome acidification [79]. Experiments with other mycobacteria had shown that ATP treatment increases antimycobacterial activity of macrophages through cytosolic phospholipase A2 (cPLA2)-dependent generation of arachidonic acid [77].

A major signaling receptor incriminated in susceptibility to MAP infection is TLR2 [45]. TLR2 activates the MAPK-p38 pathway and may activate the NF- κ B pathway [82]. Both pathways initiate IL-10 transcription. This pathway induces transcription of interleukin (IL)-10. Early production of IL-10 suppresses proinflammatory cytokines, chemokines, IL-12, and major histocompatibility factor class-II expression [44]. 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 [13, 44]. Phagocytosis of MAP results in a marked and persistent decreased expression of MHC class-I and class-II molecules within 12 to 24 hours. The MHC class-I and class-II expression by macrophages remained low even after addition of IFN- γ [80]. Additionally, microarray studies indicated that MHC class-II expression was down regulated in MAP-infected macrophages [79]. Irreversible down regulation of MHC expression could contribute to the paucity of T-cell infiltrates and tubercle formation in the lesions of JD [21].

Apoptosis constitutes a major mechanism to limit pathogens by preventing the dissemination of *M. tuberculosis* [43]. TNF- α is required for induction of apoptosis in response to infection. Interestingly, pathogenic mycobacteria release neutralizing reagents for TNF- α receptors [6]. Also, release of TNF- α receptors, in turn, is regulated by the IL-10 production [6]. Pathogenic mycobacteria may selectively induce IL-10 [81], leading to decreased TNF- α activity and reduced apoptosis. LAM also prevents apoptosis of mycobacteria-infected cells in a Ca²⁺-dependent mechanism [3]. LAM antagonizes apoptosis by preventing an increase in cytosolic calcium concentration. Cytosolic calcium facilitates apoptosis by increasing the permeability of mitochondrial membranes [73]. This promotes release of pro-apoptotic products such as cytochrome c [46]. LAM also stimulates phosphorylation of Bad a pro-apoptotic protein. Phosphorylation of Bad prevents the molecule from binding to the antiapoptotic proteins Bcl-2 and Bcl-XL. Free Bcl-2 prevents the release of cytochrome c from mitochondria [46]. Studies have shown that high levels of TRAF1 and IL-1 α mRNA were found in lesions of ileal tissues associated with MAP-infected cattle [3], and macrophages within these lesions were responsible for these high levels [17]. 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 cell expression CD40 ligand [17].

Dormancy of *mycobacterium* is considered to be an important strategy in their survival in the host. Several studies showed the important gene in MAP, however, the exact role of the molecule have not clarified yet [42]. The essential role of vitamin B6 biosynthesis for survival and virulence of *Mycobacterium tuberculosis* was reported [26]. In *Salmonella*, a key gene in dry-resistance was reported [74], however similar study have not carried out in MAP. Further studies on these topics reported in other pathogenic *Mycobacterium* should be applied to research in MAP.

3. Survival of MAP in the environment

MAP is known to have survival and long-time persistence abilities for various environmental sources (Table 1) and have the potential of transmission to new hosts by ingestion, inhalation and inoculation of the bacilli [56, 85]. Knowledge about the survival and persistence of MAP in different environmental sources is necessary to better understand the epidemiology and formulate control strategies for MAP infection.

3.1 Soil, Pasture and Feces

Many *Mycobacterium* species have been recovered from soil and pasture land [18, 40, 89]. MAP is able to survive in the environment up to 152 to 246 days depending on specific conditions. The 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 MAP infected pastures safe after grazing by MAP-infected cattle [19]. Previous studies from England, France, and U.S. has reported that soil types and soil composition are connected with the incidence of *Paratuberculosis* in livestock herds [11]. The 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 [86]. In an investigation, the 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 was studied. The study reported that at 5°C the survival time was 252 days in all three kinds of slurry, and at 15°C, it was 182 days in swine slurry, 98 days in cattle slurry, and 168 days in mixed slurry [39]. The viable count of MAP bacilli has been reduced from 90 to 99% if the organism in feces becomes mixed with soil [86]. Cattle urine is also hostile to MAP survival and increasing concentrations of urine (2-10%) at pH 6.3 to 6.6 caused decreased survival rates of the organism. Anaerobic digestion of slurry in biogas plants was studied. The slurry was spiked by MAP (3.3×10^3 to 2.7×10^4 bacilli/gm) and held at mesophilic conditions (moderate temperatures; 35°C or 95°F) or thermophilic conditions (high temperatures; 53-55°C or 127-131°F). At mesophilic conditions, MAP was survived at 7, 14, and 21 but not

at 28 days. At thermophilic conditions, viable MAP could not be detected in as short as 3 hours. Like other mycobacteria, the cell wall of MAP bacilli plays an important role in the survival of MAP in the environment and adverse conditions [55].

3.2 Surface water

MAP has the ability to attach with a wide range of soil particles near the surface where it can transmit to grazing animals through ingestion or be released during rainfall and/or runoff events [25]. Direct contact of susceptible animals with surface water or runoff from animal housing poses significant risk of MAP transmission in herds [58]. 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 [23]. MAP survives for longer duration in water and sediment behind dams [85]. 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. It was reported the survival of MAP in distilled water (pH 7.2) up to 455 days (Strain Dominic) [70]. The presence of fatty acids, lipids and waxes in the cell wall of MAP are responsible in part for the extreme hydrophobicity and play an important role in the survival of MAP in water [12]. The cell wall of MAP leads to adsorption to air: water interfaces, surfaces (e.g. Pipes), and to phagocytosis by macrophages and protozoa [68]. Protozoa also act as a reservoir for the MAP. MAP is able to multiply within the vacuoles of protozoa and increase in the number [20]. The survival of MAP in water is potentially as significant for disease transmission as survival of the organism on soil and pasture. MAP was recovered from domestic water reservoirs. So, it can be concluded that water reservoirs may play significant role in MAP infection on farms [56].

Table 1. Previous reports on survival duration of MAP in different environmental conditions /substrates.

Contaminated material	Source of bacilli	Conditions	Duration of survival	Reference
Bovine Feces	Naturally infected	Temperature: -70°C	> 15 weeks	Richards and Thoen, 1977; Richards, 1981
	Cultured bacilli	Uncertain	11 months	Vishnevskii <i>et al.</i> , 1940
Bovine feces in open bowl	Naturally infected feces	Ambient, -2 to 23°C; exposed	< 5 months	Lovell <i>et al.</i> , 1944
Caprine feces in open bowl	Naturally infected feces	Ambient, -2 to 23°C; exposed	> 67 days	Lovell <i>et al.</i> , 1944
Bovine urine and feces	Cultured bacilli	Temperature: 38°C; dark	< 30 days	Larsen <i>et al.</i> , 1956
Bovine urine	Cultured bacilli	Temperature: 38°C; dark	< 30 days	Larsen <i>et al.</i> , 1956
Bovine slurry (mixture of feces, urine, straw, and water)	Cultured bacilli	Anaerobic, Temperature: 5°C	> 252, < 287 days	Jorgensen, 1977
		Anaerobic, Temperature: 15°C	> 98, < 112 days	Jorgensen, 1977
		Anaerobic, Temperature: 35°C	> 21, < 28 days	Olsen <i>et al.</i> , 1985
		Anaerobic, Temperature: 53-55°C	< 1 day	Olsen <i>et al.</i> , 1985
Tap water	Cultured bacilli	pH 7.0, Temperature: 38°C; Dark	> 17, < 19 months	Larsen <i>et al.</i> , 1956
	Cultured bacilli	pH 5-8.5, Temperature: 38°C; Dark	> 14, < 17 months	Larsen <i>et al.</i> , 1956
Tap water in a sealed bottle	Cultured bacilli	pH 7.1-8.0, Ambient, Temperature 9 to 26°C	> 9, < 13 months	Lovell <i>et al.</i> , 1944
Distilled water in sealed bottle	Cultured bacilli	Ambient, 9 to 26°C	> 9, < 13 months	Lovell <i>et al.</i> , 1944
Pond water plus mud, in sealed bottle	Cultured bacilli	pH 5.3-5.9, Ambient, Temperature 9-26°C	> 9, < 13 months	Lovell <i>et al.</i> , 1944
River water in open bowl	Bovine intestinal scrapings	Ambient, -7 to 18°C; shade	> 135, < 163 days	Lovell <i>et al.</i> , 1944
	Bovine intestinal scrapings	Ambient, -7 to 18°C; sun	> 163, < 218 days	Lovell <i>et al.</i> , 1944
Dam water	Cultured bacilli	70% Shade environment	36 weeks	Whittington <i>et al.</i> , 2005
		Semi-exposed location	16 weeks	Whittington <i>et al.</i> , 2005
Dam sediment	Cultured bacilli	70% Shade environment	48 weeks	Whittington <i>et al.</i> , 2005
		Semi-exposed location	36 weeks	Whittington <i>et al.</i> , 2005
Soil and pasture	Naturally infected	Without Shade environment, No irrigation, no lime	32 weeks	Whittington <i>et al.</i> , 2004
		70% Shade environment, No irrigation, no lime	32 weeks	Whittington <i>et al.</i> , 2004
	Naturally infected	Without Shade environment, No irrigation, no lime	5 weeks	Whittington <i>et al.</i> , 2004
	Naturally infected	Partial Shade environment, Irrigation at start, no lime	10 weeks	Whittington <i>et al.</i> , 2004
Soil and fecal material	Cultured bacilli	pH 5.8 to 6.1 Temp: 5°C to about 40°C,	12 weeks	Whittington <i>et al.</i> , 2005
Biogas plant supplied with manure from a herd of JD infected cattle	Naturally infected	Anaerobic, Mesospheric temperature around 41 and 42°C	> 2 months	Salna <i>et al.</i> , 2011

3.3 Biofilms

Biofilm formation confers selective advantage for the persistence of mycobacteria in different environmental conditions and provides added resistance to antimicrobial agents [88]. MAP is capable for rapid and sustained biofilm formation on livestock watering trough construction materials. The adhesion capability of organisms depends on the surface properties as well as on material surface being colonized [29]. Some researchers investigated processes in controlling the transport of MAP through aquifer materials and reported that the cell wall of MAP has a strong negative charge and is highly hydrophobic [12]. The hydrophobic nature of the cell wall may predispose these organisms to adherence [75]. Lower adhesion of MAP to stainless steel and plastic surfaces may be due to weaker interactions between the hydrophobic cell wall of MAP and the inert surfaces of those materials [75]. As like other bacteria, MAP in biofilms is more resistant to chemical stress than bacteria suspended free in water [28, 31].

3.4 Free-living amoeba

Free-living protozoa are ubiquitous in the environment and may serve as a potential reservoir for the persistence of animal and human pathogens [50, 83]. Various *Mycobacterium* species have been shown to grow within amoebae [1, 9]. The association of the pathogen with amoeba may result in enhancing the entry, growth rate and virulence of pathogen [20, 68]. MAP is able to survive for weeks in protozoa that are usually bacterivores. Inside protozoans MAP may acquire a phenotype, which is more pathogenic to human beings [35]. Survival mechanism of MAP in amoeba should be similar to that shown for *Mycobacterium avium* subspecies *avium* within free living amoebae. *Mycobacterium avium* subspecies *avium* inhibits lysosomal fusion and replicates in vacuoles that are tightly juxtaposed to the bacterial surfaces within amoebae [20]. MAP survives ingestion by *A. castellanii* and *A. polyphaga*, this intracellular location provides protection from the effects of chlorination [83]. Survival of MAP inside the protozoa provides an ecological niche and a location for the organism to multiply outside the host in the environment.

4. Conclusion and Future Perspectives

MAP has 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 have drawn the attention of researchers towards the development of preventive measures to control the spread of MAP bacilli in animal species and the environment. MAP has

tough and fine mechanisms for the successful survival in infected animals, in livestock waste, on farms, and water. 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 runoff. To restrict the spread of MAP in the environment and minimize the risk of human exposure the future research should be focused towards the identification of key receptors in the host, which play a precious role for the interaction of MAP, trigger pro-inflammatory response and promotes bacterial killing, or intracellular persistence and survival under suboptimal conditions. Comparative functional genomics and gene silencing based research will play an increasingly important role for deciphering the mechanisms behind the ability of MAP survival and long-time persistence in host and under adverse environmental conditions. The information generated by such studies will be beneficial to formulate the effective control strategy for MAP infection in animals.

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