# continuing education

# Update on paratuberculosis:
# 1. Epidemiology of Johne's disease and the biology of *Mycobacterium paratuberculosis*

When Liam Aylward, the Minister of State at the Department of Agriculture and Food, announced a review of the ways in which it has dealt with Johne's disease in cattle, he stated that "success in tackling the problem is achievable only on the basis of a sustained commitment by all the key players." Therefore,it is opportune to review current knowledge of the disease. This is the first in a series of papers adapted from the Johne's Information Centre website (http://johnes.org), updated by MICHAEL T. COLLINS, President, International Association for Paratuberculosis.
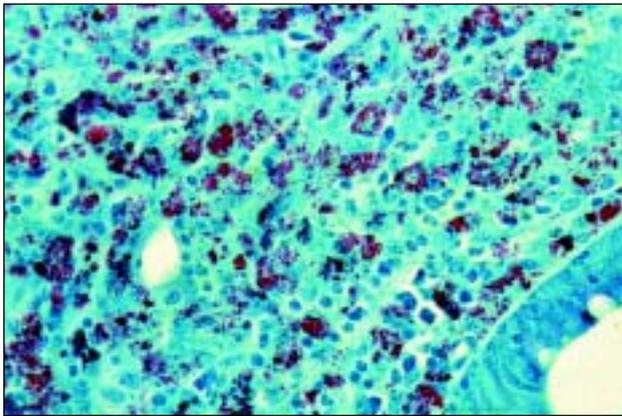


**FIGURE 1:** Section of ileum of a cow showing clumps of acid-fast bacteria.

## Epidemiology of Johne's disease
### Prevalence

Johne's disease, a granulomatous enteritis of ruminants caused by *Mycobacterium paratuberculosis* (Figure 1) and manifest clinically by chronic diarrhoea and progressive emaciation, has been reported on every continent. Sweden and some states in Australia are the only regions of the world that can claim freedom from Johne's disease based upon a reliable disease reporting system and extensive surveys using laboratory tests. The reported prevalence of infected animals by country is at least partially a reflection of the diligence with which veterinarians and animal owners look for the disease. Johne's disease is more common in dairy cattle than in other ruminants; this is likely a function of animal husbandry. In many countries

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- A wide range of animal species are susceptible to infection with *Mycobacterium paratuberculosis*.
- The infection appears to be spreading both among and across species and is becoming more common.
- The infection begins in very young animals but signs of illness do not appear until they are adults.
- Infected adults pass the infection to neonates via faeces and milk contaminated with the organism.

Johne's disease is fairly common in small ruminants like sheep and goats but precise survey data have not been published. In the early 1990s, Johne's disease was found in relatively few sheep flocks in Australia, prompting efforts to eradicate the disease. Since then, with improved diagnostics and surveillance efforts, the disease has been found to be fairly widespread, particularly in the state of New South Wales, and control strategies that include vaccination are being considered.

## Sources of infection

*M. paratuberculosis* is an obligate pathogenic parasite of animals; the only place it can multiply in nature is in a susceptible host, within a macrophage (Figure 2). When *M. paratuberculosis* leaves an animal, for example in the faeces, it can survive for a long time in soil and water, but it cannot multiply outside the animal host. Consequently, the primary source of infection is an infected animal. When an infected animal is introduced to a herd, the opportunity for transmission of *M. paratuberculosis* to other animals increases the longer that animal remains in the herd. As the infection progresses in the animal, the frequency and number of the bacteria being excreted increases.
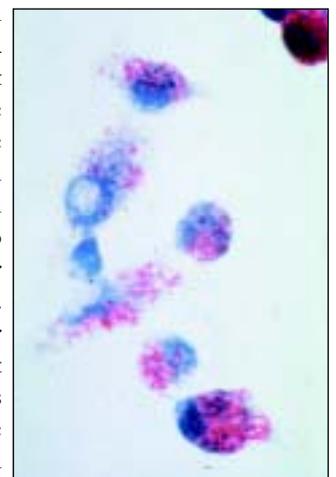


**FIGURE 2:** M. paratuberculosis inside cultured macrophages.

*M. paratuberculosis* infects the intestine and is excreted in faeces: thus, ingestion of faecal-contaminated feed or water is the most common way animals become infected. In manure, *M. paratuberculosis* can remain alive for over a year, depending on environmental conditions. Milk from infected female animals is another important source of infection. The likelihood of *M. paratuberculosis* being excreted into milk increases with time as the infection progresses. The probability of young animals becoming infected by drinking milk from infected dams is a direct function of the time spent with the mother and/or how often they are fed colostrum or milk from infected females. The chances for transmission of the infection from mother to offspring is greatest where husbandry practices allow young animals to remain with the dams and nurse naturally. The bacteria may be excreted directly into the mother's milk and/or the surface of the teats might be contaminated with infected manure.

Pond water contaminated with manure carrying *M. paratuberculosis* is another potential source of infection. A less likely, but possible, source of infection is pasture contaminated with the bacteria.

Johne's disease has been reported in free-ranging wildlife, but their role in the ecology of *M. paratuberculosis* is not known. Similarly, it is not known if wild birds can become infected or transfer the bacteria between farms.

### Transmission of infection

Risk factors affecting the probability of transmission of *M. paratuberculosis* to other animals have not been measured. Consequently, only general statements can be made about circumstances that promote transmission of infection. Age of animal is perhaps the most well-recognised factor affecting transmission. In cattle, there is an age-dependent increase in resistance to *M. paratuberculosis* infection. This means it takes a larger dose of the bacterium to infect an adult (over two-years-old) than it does to infect a young animal (up to six-months-old). This may be true for small ruminants also, but it is not as well studied and clinical reports suggest there is a greater susceptibility of sheep, goats and deer to this infection, even as adults. The extent and duration of exposure to contaminated manure and milk from infected adult animals directly affects the risk of transmission. Clean, dry birthing environments and housing of young animals away from the adult herd or flock diminishes the likelihood of transmission. Conversely, dirty maternity pens or faecal contamination of feed and water supplies will promote spread of the infection. Provision of milk from animals free of the infection or pasteurisation of milk for feeding young animals is essential to prevent new cases of Johne's disease.

There is evidence that *M.paratuberculosis* has been disseminated within the cattle population in the Republic of Ireland by means of pooled colostrum, fractions of which had come from infected cows imported from the continent of Europe.

## Biology of *Mycobacterium paratuberculosis*
### Relationship to other mycobacteria, taxonomy and nomenclature

On a genetic basis, *M. paratuberculosis* is virtually identical to *Mycobacterium avium*; however, the phenotypic characteristics of the two organisms are different. *M. paratuberculosis* grows much more slowly, requires an iron-transport chemical known as mycobactin for *in vitro* growth, forms rough colonies on solid agar media, and infects mammals rather than birds. Consequently, the most appropriate taxonomic classification and proper name for *M. paratuberculosis* has been under debate (Thorel *et al.*, 1990). An opinion supported by the International Association for Paratuberculosis is that *M. paratuberculosis* should be reclassified as a subspecies of *M. avium* and thus renamed *M. avium* subspecies *paratuberculosis* (abbreviated *M. avium* subsp. *paratuberculosis*). This subspecies designation appears in many recent publications concerning the organism. For simplicity, the name *M. paratuberculosis* is used in this paper. Similarities and differences between *M. paratuberculosis* and *M. avium* will be discussed throughout this section.

While sharing many genetic similarities with *M. avium*, *M. paratuberculosis* is less closely linked genetically to *Mycobacterium tuberculosis*, the cause of tuberculosis. Also, it is not closely related to the cause of leprosy in humans, *Mycobacterium leprae*. However, it does share certain biological characteristics with these mycobacterial pathogens. Scientists often draw parallels among these mycobacterial organisms to try to understand the basic mechanisms by which they cause disease.

### Virulence factors

Like other mycobacteria, *M. paratuberculosis* has the capacity to grow and multiply within macrophages: it is a facultative intracellular pathogen. No specific mechanisms have yet been found to adequately explain this ability to survive the antibacterial activities of macrophages. In general terms, two properties of mycobacteria contribute to their intracellular survival:

1) the chemically unique mycobacterial cell wall that is resistant to destruction or penetration, and
2) factors produced by mycobacteria that can neutralize the antibacterial chemicals produced inside macrophages.

Survival and multiplication in the host animal is a prerequisite to causing disease. Again, the mechanisms by which mycobacteria cause disease are not well understood. Pathological changes are induced both by the direct action of toxic chemical components of the mycobacterial cell wall and by immune response of the host to the presence of *M. paratuberculosis*.

### Host range

*Mycobacterium paratuberculosis* has a broad host range. The types of animals most commonly infected are ruminants (cattle,

sheep, goats, deer, elk, antelope, bison, etc.) and "pseudo-ruminants" (species that have a three-chambered stomach: camelids such as llamas, guanacos and alpacas). In addition, there have been a few reports of *M. paratuberculosis* infecting pigs, horses, and nonhuman primates. In one report, the same strain of the bacterium was found to infect dairy cattle plus numerous rabbits in the region and a number of different carnivore species (fox, stoat, badger, raven) that may have been preying on the rabbits. Multiple reports in medical literature claim to have detected genetic components of *M. paratuberculosis* in humans with Crohn's disease and some report isolation of the live organism. The significance of these findings has yet to be determined.

## Environmental distribution

*M. paratuberculosis* is not thought to be free-living (able to grow and multiply) in the environment. It is an obligate parasitic pathogen of mammals. This means infected animals are the only place in nature where growth and multiplication of the bacterium can occur. When found in soil or water samples, it can be assumed that *M. paratuberculosis* is simply persisting in those places (not multiplying) after being deposited there through faecal contamination from an infected animal.

The environmental distribution of *M. paratuberculosis* is markedly different from that of *M. avium*, which can grow and multiply outside a host animal because of its ability to produce mycobactin that enables it to acquire iron from the environment. *M. avium* is commonly found in lakes, streams and domestic water supplies. Certain acidic soil types, notably peat bogs, contain higher than average numbers of *M. avium*. A tenuous association between the occurrence of Johne's disease and geographical regions with acidic soils has been reported (see "survival in soil", p572). The strength and the biological basis of this association remain to be determined.

### *M. paratuberculosis* in the laboratory

#### Cellular morphology and chemistry
- *M. paratuberculosis* is a small (0.5 x 1.5 micron) rod-shaped bacterium, that grows in clumps
- Gram-positive
- Acid-fast positive (stains red) with the Ziehl-Neelsen or Kinyoun's stains
- Cell wall composed of a thick waxy mixture of lipids and polysaccharides.*

   *While most strains of *M. avium* produce a surface glycolipid that allows strains to be serotyped (i.e., distinguished using antibodies specific for each glycolipid subtype), *M. paratuberculosis* strains lack such glycolipid antigens on their surface.



**FIGURE 3:** Smooth M. paratuberculosis colonies on Middlebrook 7H11 agar without Tween 80.

#### Colonial morphology
The size, colour, and texture of a colony of *M. paratuberculosis* is dependent in part on the type of bacteriological medium on which it is cultivated. On Herrold's egg yolk agar medium, one of the most commonly used culture media in veterinary diagnostic laboratories, the colonies appear small, somewhat rough and off-white to yellow in colour. Pigmented (yellow) strains have been reported in sheep. On Middlebrook agar medium without Tween 80 (a detergent that improves the growth rate), the colonies are very rough in appearance and resemble those of *M. tuberculosis*. With addition of Tween 80, the growth rate of *M. paratuberculosis* increases and its colonial morphology becomes smooth and domed resembling that of *M. avium* (Figure 3).

#### Biochemical characteristics
Biochemical tests used to distinguish among other species of mycobacteria are not used to identify *M. paratuberculosis*. The tests are taxing to perform, due to the extremely slow growth rate of the organism, and test results vary among strains of the organism.

#### Molecular genetics
The DNA of *M. paratuberculosis* is >99% identical with that of *M. avium*. This is the reason that many characteristics of the two bacteria are similar. The genetic feature that distinguishes one from the other is the presence of multiple copies of a short DNA element called an insertion sequence (IS) that is unique to *M. paratuberculosis* and is named IS900. Genetic probes used for detection of *M. paratuberculosis* in clinical specimens or in cultures are based on detection of IS900. A second insertion sequence, named IS901, that is approximately 60% similar in DNA sequence to IS900, was recently found in some strains of *M. avium*. How these insertion elements affect the biology and pathogenic capacity of *M. paratuberculosis* or *M. avium* is not understood. There is evidence that they play a major role.

## Resistance to heat and cold

The thermal tolerance of *M. paratuberculosis*, specifically the capacity to survive pasteurization, is the subject of considerable interest. Some published reports suggest that the organism can survive standard commercial pasteurization, while others suggest it can not. Thermal tolerance curves indicate that *M. paratuberculosis* is comparable in heat resistance to *M. avium* and far more heat resistant than *Listeria*, another facultative intracellular bacterium that is found in raw milk.

Concerning cold, Richards and Thoen (1977) showed that the number of living organisms in faecal samples from cattle naturally and experimentally infected with *M. paratuberculosis* was significantly decreased after freezing at -70°C for three weeks. Continued refrigeration up to 15 weeks did not result in further decline in the number of *M. paratuberculosis*.

Studies on suspensions of *M. paratuberculosis* ($10^6$/ml) in broth culture media held at refrigerator temperatures (4°C) gave similar results. Counts of living bacteria declined precipitously with greater than 1 log decrease within five days. Then, a residual population of apparently cold-tolerant *M. paratuberculosis* cells (roughly 1% of the starting number) persisted to the end of the experiment at 25 days (Collins, unpublished data). For comparison, when *Mycobacterium bovis* ($10^7$ *M. bovis*/ml) were suspended in phosphate buffer (pH 7.2) and held at refrigerator temperatures (2 to 4°C), 50% survived to 21 days and 2% survived for one year.

## Resistance to ultraviolet light (UV) and gamma irradiation

UV doses required for bacterial and viral inactivation are relatively low, typically in the range of 2 to 6mWs/cm$^2$ for 1 log inactivation. (Since water characteristics such as pH, hardness, turbulence, turbidity, and biological oxygen demand dramatically affect disinfection efficiency of UV, any generalization of these doses to other water treatment protocols would be ill-advised.) When $10^5$ to $10^6$ *M. paratuberculosis* were suspended in sterile deionized water, 4mWs/cm$^2$ was sufficient to achieve a 1 log reduction in viable counts and, at UV doses greater than 15 mWs/cm$^2$ complete disinfection was achieved (Manning, unpublished data).

Older literature concerning the effects of natural sunlight on mycobacteria in the environment indicated that sunlight (presumably UV radiation) decreases the survival rate and that *M. paratuberculosis* is more resistant to adverse effects of sunlight than is *M. bovis*. However, recent work in Australia indicated that UV light had minimal effect on the viability of *M. paratuberculosis* in soil spiked with the bacteria.

A study on irradiation of *M. paratuberculosis* suspended in bovine colostrum found that if frozen bovine colostrum spiked with $10^4$ bacteria/ml was exposed to 10kGy (kilogray) gamma irradiation ($^{60}$Co gamma-beam facility in Dagneux, France), 100% of the organisms were killed. To place this dosage of gamma-irradiation in perspective it should be noted that 7kGy is the maximum allowable dosage for meat treatment in the

U.S., 30kGy is the allowable dose for treatment of dried spices, and 40kGy is used to sterilise foods for the NASA (US) space programme.

## Resistance to chemical factors: antibiotics and disinfectants

Mycobacteria are notorious for their resistance to antibiotics that kill most other bacteria. Only a select few antibiotics can be used to treat mycobacterial infections effectively and in most cases the course of therapy is weeks to months.

*M. paratuberculosis*, like its close relative *M. avium*, is even resistant to antibiotics that normally are efficacious against *M. tuberculosis*, the cause of tuberculosis. Antimicrobial therapy for Johne's disease is not often attempted, as the cost of the drugs for these large animals and the duration of treatment required make it cost-prohibitive for livestock.

*M. paratuberculosis*, like other mycobacteria, is resistant to common disinfectants. However, phenolic and cresylic disinfectants are effective. Commercial disinfectant products labelled "tuberculocidal" should generally be effective against *M. paratuberculosis*. Research in this area was done in the 1950s and there is little current information to substantiate these observations or make more specific recommendations regarding products, concentrations or required contact times. *M. avium* is more resistant to free chlorine (bleach) than are most other bacteria. At a concentration of 1mg/litre the time for a 1 log reduction in viable counts was roughly 50 minutes. By contrast, the time to achieve a 1 log reduction in *E. coli* was 28 seconds. There was variation in chlorine susceptibility by the *M. avium* strains tested. Those strains that were slowest growing were the most resistant. Water-grown *M. avium* cells were 10-fold more chlorine-resistant than those grown in culture medium. These laboratory findings are supported by epidemiological studies. A survey of disinfection practices in the US found that water utilities maintain a median chlorine residual of 1.1mg/ml and a median exposure time of 45 minutes before the point of first use in the distribution system. Despite this practice, in Los Angeles, California, nontuberculous mycobacteria (*M. avium* and others) were isolated from water in 82% of 55 homes, and in water in all of 31 commercial buildings, and in 15 hospitals. Comparable studies for *M. paratuberculosis* have not been reported; however, unpublished findings indicate that *M. paratuberculosis* is as chlorine-resistant as *M. avium*, if not more so (Collins, unpublished data), an observation consistent with the reported relationship between growth rate and chlorine resistance.

## Survival in surface water

Lovell *et al.* (1944) reported that after spiking sterilized pond water (pH 5.5) with 0.1, 1.28 or 3.4mg wet weight of *M. paratuberculosis* per 100ml of water and holding it at room temperature, the organism was recovered in samples tested monthly for up to nine months. Larsen *et al.* (1956) reported on the survival of *M. paratuberculosis* in spiked samples of tap

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## Dependence on mycobactin

Because of its inability to produce mycobactin (a chemical needed to transport iron), unique among members of the mycobacterial family, *M. paratuberculosis* can grow only inside animal cells where it 'steals' iron from its host's cells, most often the macrophages. Thus, it is an obligate parasitic pathogen of animals.

Compensating for the fact it can seemingly only grow in

animals, *M. paratuberculosis* has developed the ability to persist in the environment for long periods of time waiting for the chance to again infect an animal.

### Host range
• **Ruminants**: cattle, sheep, goats, deer, elk, antelope, bison,
• **Pseudo-ruminants**: camels, llamas, and alpacas.
Infrequently in non-ruminant species: horses, pigs, chickens, rabbits, fox, non-human primates and people with Crohn's disease.

water of different pH held at 38°C in the dark. In water at neutral pH (7.0), *M. paratuberculosis* was recovered up to 17 months (517 days) post-inoculation, while for pH 5.0 and pH 8.5 water *M. paratuberculosis* was isolated up to 14 months post-inoculation. Consistent with these earlier reports, Sung and Collins (1998) found that when distilled water (pH 7.2) was inoculated with $10^6$ *M. paratuberculosis* cells/ml and viable counts were determined on a monthly basis, the time for a 1 log reduction (D-value) was 68.5 days, and viable *M. paratuberculosis* cells were found up to 455 days (strain

Dominic). Similar findings were reported for *M. avium*. When suspended in tap water and held at 4°C or 20°C , *M. avium* survived beyond 485 days.
For more general references regarding mycobacteria in water the reader is directed to the review article by Collins *et al.* (1984). Subsequent to publication of that review, recognition of the global AIDS epidemic and associated opportunistic *M. avium* infections led to a resurgence of research interest in this mycobacterial pathogen and, in particular, investigations concerning the source of this organism. Most investigations

point to domestic water supplies as the source of human *M. avium* infections due in part to their abundance in the environment and their resistance to chlorination. Under natural environmental conditions, warmer waters with lower pH have been associated with higher levels of *M. avium*. Similar factors could affect survival of *M. paratuberculosis* in water.

## Survival in biofilms

Studies concerning *M. paratuberculosis* in biofilms (complex communities of microbes, and slime secreted by microbes, that cover surfaces of pipes and other surfaces exposed to bacteria-containing fluids) have not been reported. Work on other mycobacteria indicates that biofilms may be an important replication site for mycobacteria found in water (Falkinham et al, 2001). Organic substances like plastics and rubber were found to be more intensely colonized than inorganic substances such as copper and glass. The significance of mycobacteria in biofilms is that all bacteria in such locations are more resistant to chemical stress than are bacteria suspended free in water.

## Survival inside free-living amoeba

Studies on replication of *M. paratuberculosis* in amoeba have not been reported; however, *M. avium* has been shown to be capable of replicating inside free-living amoebae such as *Acanthamoeba*. Moreover, the virulence of the organism was enhanced after growth inside these amoebae. Unpublished data indicate that similar relationships could exist between free living amoebae and *M. paratuberculosis*. If true, this would provide an ecological niche for the organism outside the infected animal and provide a location for the organism to multiply in the environment.

## Survival in soil, faeces and soil-faeces mixtures applied to the surface of fields/pastures

On the subject of environmental survival of *M. paratuberculosis*, the publication by Lovell *et al.* (1944) has become a classic reference. A series of studies using naturally-infected bovine faeces were conducted in which the infected faecal matter was exposed to a variety of natural conditions such as freezing, drying, sunlight, changes in ambient temperature, and rain, with regular attempts to re-isolate the organism. In general, it was found that *M. paratuberculosis* survived in faeces kept outdoors for up to 152 to 246 days, depending on specific conditions. Drying of soil appeared to shorten survival. Given the longevity of *M. paratuberculosis*, the authors recommended that a pasture contaminated by the organism should be considered a potential source of infection for at least one year. It would be interesting to repeat the study using more sensitive methods for the detection of *M. paratuberculosis* than those then available to Lovell and colleagues.

Factors that may shorten the estimated survival time of *M. paratuberculosis* in soil are drying, exposure to sunlight, pH above 7.0 and low iron content. Cattle urine is also hostile to survival of *M. paratuberculosis* and increasing concentrations of cattle urine (2 to 10%) caused decreasing survival rates (at pH 6.3 to 6.6). A 1999 study on survival of *M. paratuberculosis* in spiked soils in Australia found shorter survival in dry alkaline soils and no apparent effect of UV light.

Observations regarding the associations among soil pH, calcium or iron content and the incidence of paratuberculosis have a long history. The review article on Johne's disease by Doyle (1956) covers most of the early observations on association of soil type and the incidence of paratuberculosis and the review by Johnson-Ifearulundu and Kaneene (1997) complements it by covering more recent literature. The fact that this observed association (i.e., not a proven causal link), in particular concerning soil pH, has been made in England, France, the Netherlands, and the US adds credibility to the idea that somehow soil composition and paratuberculosis are connected. In addition, Johnson-Ilfearulundu and Kaneene (1997) recently showed by careful epidemiological analysis that in the state of Michigan in the US the practice of application of lime to pastures (a practice that should increase soil pH) in 1993 was associated with ten-fold lower odds of a dairy herd being serologically test-positive for *M. paratuberculosis* infection in 1996. As an aside, it is interesting to note that in a very different study an association was demonstrated between soil acidity and frequency of skin test reactions (diagnostic tests for exposure or infection with mycobacteria) in humans. These epidemiological observations have led to speculation concerning mechanisms by which soil pH, or its interaction with soil calcium and iron content, affect survival of *M. paratuberculosis*. It should be noted, however, that the relationships observed are indirect, i.e., simply the association between incidence of clinical bovine paratuberculosis and broadly characterized soil types. Laboratory studies to verify if a particular soil type affects *M. paratuberculosis* survival or to explain the mechanism have not been done.

## Survival in faeces injected into soils

Manure management is a component of domestic agriculture enterprises. Modes of dispersal include spreading it on fields, injection into the soil, and slurry pit storage for later dispersal.

Published reports on *M. paratuberculosis* survival after injection of contaminated faeces into soil have not been found. Intuitively, the advantage of this practice is that it lessens opportunities for contact between *M. paratuberculosis* and animals or forage crops, and decreases the chance for contamination of surface water by rainfall run-off. However, faeces injection might also favour survival of *M. paratuberculosis* by placing it away from more harsh conditions of drying and sunlight. Minimal research has been conducted in this area.

## Survival in faeces stored in slurry pits

Jörgensen (1977) published the first comprehensive study of its kind on survival of *M. paratuberculosis* in slurry in Denmark. In his work he used 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%). After spiking each slurry preparation with $3 \times 10^7$ *M. paratuberculosis/*ml, he bubbled a mixture of hydrogen and nitrogen gas through the slurry to secure anaerobic conditions and then stored the slurry at 5°C or 15°C. He reported that the number of colonies of *M. paratuberculosis* isolated on modified Lowenstein-Jensen medium dropped drastically between sampling day 1 and day 7 but then remained relatively stable until recovery of the organism stopped, indicating the limit of survival. 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. Comparable findings have been reported on survival of *M. bovis* in cattle slurry.

The second major study on *M. paratuberculosis* in slurry was reported by Olsen *et al.* (1985). Their study concerned conditions found during anaerobic digestion of slurry as in bio-gas plants. Slurry was spiked to yield initial counts of $3.3 \times 10^3$ to $2.7 \times 10^4$ *M. paratuberculosis/*gm slurry and held at mesophilic conditions (moderate temperatures: 35°C or 95°F) or thermophilic conditions (high temperatures: 53 to 55°C or 127 to 131°F). At mesophilic conditions *M. paratuberculosis* was re-isolated at 7, 14, and 21 days but not at 28 days. At thermophilic conditions viable *M. paratuberculosis* could not be detected in as short as three hours.

## Survival in compost

Research on survival of *M. paratuberculosis* in composted animal wastes was not found. However, the time-temperature profiles of properly composted animal waste suggests that such conditions would be lethal to the organism.

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