Public Health Microbiology

Survival of *Mycobacterium avium* subsp. *paratuberculosis* in Dam Water and Sediment

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DOI: 10.1128/AEM.71.9.5304-5308.2005

ABSTRACT

In a previous longitudinal study, *Mycobacterium avium* subsp. *paratuberculosis* survived for 55 weeks in fecal material in the shade, but for much shorter periods in exposed locations. In this experiment, the survival of the organism was studied in 250 liters of dam water and sediment in large water troughs that were placed in either a semiexposed location or in a shaded location and compared to survival in fecal material and soil in the shaded location. Survival in water and/or sediment in the shade was for up to 48 weeks compared to 36 weeks in the semiexposed location. Survival in sediment was 12 to 26 weeks longer than survival in the water column. Survival in soil and fecal material in the terrestrial environment in the shaded location was only 12 weeks. Although disturbance to sediment could not be ruled out as a factor, there was evidence of dormancy in both the water column and the sediment, since the organism could not be recovered for several months before again becoming detectable. The results suggest that water may be a significant reservoir of *M. avium* subsp. *paratuberculosis* infection. Further research on the biology of the organism in aquatic environments is warranted. Animal health authorities will need to provide appropriate advice to farmers to minimize exposure of livestock to potentially infected water sources. Survival of the organism in water destined for human consumption will need to be addressed if the organism is found to be involved in the etiology of Crohn's disease.

KEYWORDS

Fresh Water, Geologic Sediments, Mycobacterium avium subsp. paratuberculosis

Paratuberculosis or Johne's disease is a chronic enteric infection of animals caused by *Mycobacterium avium* subsp. *paratuberculosis*, a member of the *M. avium* complex. This bacterium is defined as an obligate parasite of animals (27). The principal means of transmission is fecal-oral, a direct result of intestinal lesions and shedding of large numbers of organisms from those into the environment. Generally, young animals are infected by consumption of fecal material from pasture, off teats, or by consumption of water contaminated by feces. Transmammary and intrauterine transmission also occur, particularly in cattle.

Understanding the means of transmission of the organism has led to design of logical control strategies to reduce the impact of the infection or eliminate it completely. For control of paratuberculosis, the degree of environmental contamination can be reduced by culling affected animals and reducing contact between adult animals that may be shedders and juveniles that are most susceptible to infection. For eradication of the infection, it is thought that destocking and resting of pasture for a period sufficient for die-off of the organism, followed by restocking with healthy animals, will be effective (32, 33). To validate this, research on survival of the organism in feces, soil, and on pasture was reported recently, together with a review of findings in earlier studies (32). However, the risk of transmission of the infection through persistent organisms in water has not been addressed fully. Survival of *M. avium* subsp. *paratuberculosis* in water may pose a threat to control and eradication programs, and water may also be an efficient vehicle for transmission between farms. There is anecdotal evidence from southeastern Australia that ovine Johne's disease has been spread between farms within a water catchment by means other than movements of infected sheep.

Although the role of *M. avium* subsp. *paratuberculosis* as the etiological agent of Crohn's disease in humans is the subject of debate in the literature (11, 22), many authors have indicated the role of potable water as a source of *M. avium* complex infections for man (2, 9, 20, 28). Consequently, data on the survival of the organism in water is relevant also to current research on Crohn's disease (25).

There have been several microbiological studies of the survival of *M. avium* subsp. *paratuberculosis* in water, all using the cattle strain (C strain) of the organism in relatively artificial circumstances in the northern hemisphere. Survival was reported for about 6 to 18 months in tap or pond water in sealed bottles (16, 18) and for about 15 months in distilled water (5). Thus, the survival of *M. avium* subsp. *paratuberculosis* in water is potentially as significant for disease transmission as survival of the organism on soil and pasture.

The aim of the present study was to evaluate the duration of survival of *M. avium* subsp. *paratuberculosis* (S strain) in dam water and sediment and to

compare this with survival in feces and soil in an adjacent terrestrial environment in Australia. The data are particularly applicable to extensive grazing situations but have implications and relevance for other environments.

MATERIALS AND METHODS

This experiment was conducted at Camden (elevation 70 m above sea level, latitude 34° S) in the Sydney district of New South Wales, Australia, in parallel with another (experiment 3) to establish the duration of survival of *M. avium* subsp. *paratuberculosis* in the terrestrial environment (32). Water troughs were located on a protected partially shaded veranda on the northern side of a building. A shaded site was constructed on the veranda by using 70% knitted polypropylene cloth. For experiment 3, boxes composed of expanded polystyrene (58 by 38 by 23 cm) were filled to a depth of 20 cm with soil consisting of a dark yellow-brown light sandy loam with low organic matter content, pH 5.8 to 6.1, and iron levels of 12 to 30 mg/kg and sown with a commercial grass seed mixture 7 days before application of infected feces and then lightly watered to maintain the viability of the grasses, as described previously (32). More detailed chemical analysis of this soil was reported previously (32). The same soil mixture was added to the bases of two circular 1.2 m diameter plastic troughs to a depth of 120 mm. The troughs were then filled with about 250 liters of water from a farm dam. Lids of 70% shade cloth were placed on both troughs to prevent bird access. One trough was placed within the 70% shade enclosure, together with the pasture boxes in experiment 3, while the other was placed outside the shade enclosure on the same veranda. The sides and lid of the latter trough were exposed to direct sunlight for much of the day, which caused a greater degree of heating than that experienced by the trough in the adjacent shade enclosure. An automated weather data logger (Easydata Mk4; Environdata Australia Pty., Ltd., Warwick, Queensland, Australia) was used to record water temperatures in the troughs, soil temperature at 1-cm depth in the boxes, and other parameters relevant to the terrestrial experiment, as described previously (32).

Water in the reservoir lost due to evaporation was replenished using water from the dam via a tanker in June 2000 (time after contamination of water with feces, 32 weeks) and December 2000 (54 weeks). On both occasions the experimental troughs were also topped up from the tanker to compensate for evaporation; the sediment was grossly disturbed causing siltation of the water, which cleared over a period of 4 h, particularly in June (32 weeks). The troughs were also replenished from the reservoir by using buckets in August (40 weeks) and October (48 weeks) without gross disturbance to the sediment. Minor top-ups were performed at other times.

Dam water collected from the reservoir was submitted to the Environmental Centre of Excellence Laboratory, NSW Agriculture, Wollongbar, Australia, for analysis in August 2000. pH and conductivity were corrected to 25°C; hardness was determined titrimetically; nitrate nitrogen, ammonia nitrogen, and phosphorus free reactive were determined colorimetrically; and other parameters were determined by spectroscopy. Samples of dam water and sediment from the dam were collected at 10 sites around the periphery and were culture negative for *M. avium* subsp. *paratuberculosis*. Samples of feces were collected from sheep grazing surrounding pasture (n = 350) and tested by using pooled fecal culture (29), with negative results. When combined with negative flock history, this indicated a very low probability of infection of the flock with *M. avium* subsp. *paratuberculosis* (24).

A mixture of chaff, water, and feces from sheep with ovine Johne's disease was prepared. It contained 1.58×10^5 viable *M. avium* subsp. *paratuberculosis* (S strain) per gram. This was used to contaminate the pasture boxes in experiment 3 as described previously (32) and was also used to contaminate the water troughs. A total of 3.5 kg of fecal mixture was added to each water trough on 2 November 1999 to provide a final concentration of 2.21×10^6/liter viable *M. avium* subsp. *paratuberculosis*, whereas the concentration on the soil surface in the pasture boxes was $2.1 \times 10^4/cm^2$ at the start of the experiment.

A 1-liter sample from the water column (collected first) and duplicate samples (50 g) of sediment/soil to a depth of 50 mm were collected from each trough prior to contamination, the day after contamination, at 2-week intervals for 12 weeks, and then monthly or otherwise as indicated in Results. Samples of feces/soil were collected from the pasture boxes at similar times as previously described (32).

Methods for sample decontamination, culture, and identification of *M. avium* subsp. *paratuberculosis* from feces using the double incubation centrifugation method, BACTEC 460 radiometric culture, and PCR-based techniques, respectively, have been described previously (32). A 2-g subsample was removed from each water trough sediment or fecal/soil sample and cultured as for feces. The water samples (1 liter) were centrifuged at 11,000 × g for 20 min at 4°C, and the resulting pellet was cultured as for feces. The numbers of days for cumulative growth index to exceed 1,000 (dcgi1000) were extrapolated from weekly readings as previously described (23), and the approximate numbers of viable *M. avium* subsp. *paratuberculosis* inoculated to the BACTEC cultures were determined.

RESULTS

Weather and water temperature records. Weather data for Camden and soil temperatures for the pasture boxes in the 70% shade environment for the period of the experiment were reported previously (32). Briefly, mean weekly maximum and minimum soil temperatures ranged from about 5°C to about 40°C during a 12-month period. Weekly maximum, minimum, and average water temperatures in each trough are given in Fig. 1. The trough in the shaded enclosure had a narrower temperature range throughout the period of study (ca. 7 to 28°C) compared to the trough outside the shaded enclosure (ca. 6 to 36°C).

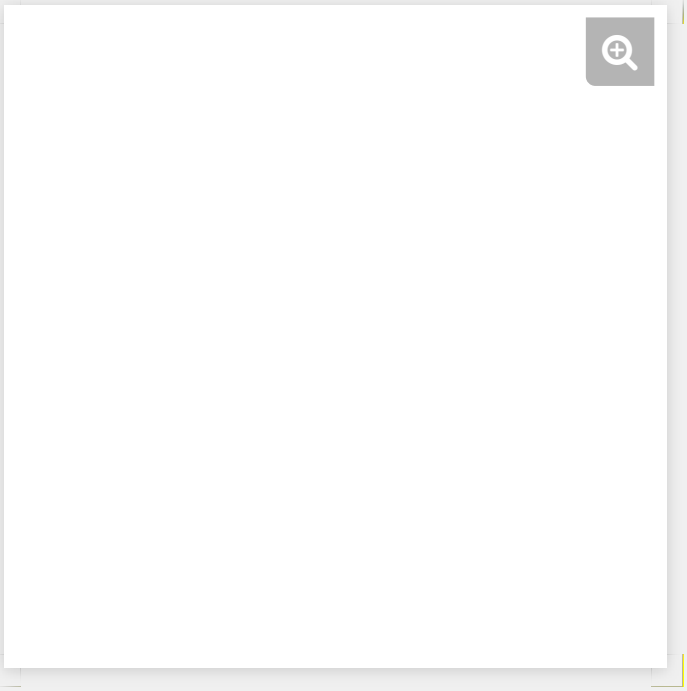

FIG. 1. Open in new tab | Download powerpoint

Weekly mean maximum, average, and minimum water temperatures in each trough. (A) Trough on the open veranda; (B) adjacent trough, in the 70% shade enclosure on the veranda.

Appearance and chemical analysis of dam water. Water in the troughs was initially slightly turbid but cleared upon standing. After addition of the fecal mixture, an algal bloom developed within weeks in both troughs. This subsided over 6 months and a population of invertebrates was then apparent and was visible in the water column for the remainder of the experiment. The dam water used in this experiment was pH 8.4 and had an electrical conductivity of 0.26 dS/m, a hardness of 81 mg/liter, and (per liter) 0.10 mg of nitrate nitrogen, <0.03 mg of ammonia nitrogen, <0.01 of phosphorus, <0.04 mg of boron, 22.1 mg of calcium, <0.02 mg of copper, <0.2 mg of iron, 2.76 mg of potassium, 6.27 mg of magnesium, <0.02 mg of manganese, 19 mg of sodium, <0.6 mg of phosphorus, 0.53 mg of sulfur, and 0.10 mg of zinc.

Survival of *M. avium* subsp. *paratuberculosis*. Water samples were culture positive to week 16 in the exposed trough and negative thereafter

(Fig. 2). Sediment samples were positive to week 16 in this trough and then negative except in weeks 32 and 36. In the shaded trough, the water sample and both sediment samples were positive to week 20, but after that the rate of culture-positive samples declined (Fig. 2). The last positive water sample was obtained in week 36, and the last positive sediment sample was obtained in week 48. The duration of survival of *M. avium* subsp. *paratuberculosis* in the water troughs was much longer than in feces and/or soil (feces/soil) in pasture boxes in the same shaded enclosure (maximum, 12 weeks) (Fig. 2). Note that survival to 55 weeks was recorded in feces/soil in a dry fully shaded environment as part of the experiment that was conducted in parallel (32).

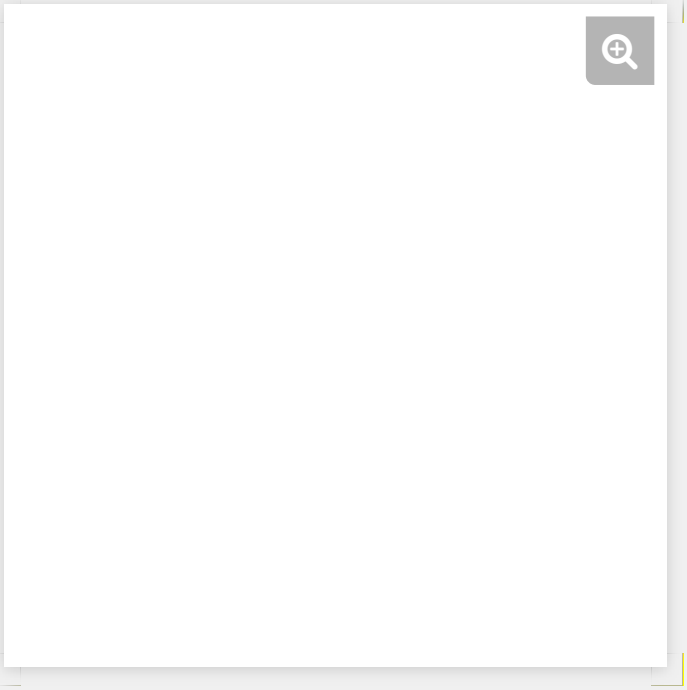

FIG. 2. Open in new tab | Download powerpoint

Number of samples culture positive for *M. avium* subsp. *paratuberculosis.* (A) Water trough exposed location; (B) water trough shaded location; (C) soil boxes shaded location. Bars: ▪, water; □, sediment; ▧, soil and feces.

The dcgi1000 in BACTEC radiometric medium is inversely correlated with the number of viable organisms present in the inoculum. On this basis, there appeared to be a decline in the numbers of viable organisms from those recorded in the first few weeks (dcgi1000 range, 18 to 32 days) to those recorded after 44 weeks (range, 57 to 59 days). There was a period during the study in which cultures from water and/or sediment were negative for two or three successive monthly samplings after 16 to 20 weeks but were followed by successive positive culture outcomes from week 32 or 36.

DISCUSSION

Nontuberculous mycobacteria in general and those in the *M. avium*

complex in particular are highly adapted to aquatic ecosystems, both fresh and saline, and to moist soil (4). It is surprising that there has been very little investigation of *M. avium* subsp. *paratuberculosis* in aquatic environments given its importance as a pathogen of livestock and as a suggested pathogen of humans. There is anecdotal evidence in Australia of lateral spread of ovine Johne's disease between farms within a catchment area, with water being one suggested means of spread.

This is the first study to evaluate the duration of survival of *M. avium* subsp. *paratuberculosis* in large volumes of water and sediment in a relatively natural setting. Livestock water troughs were used as reservoirs. Such troughs are commonly contaminated with soil and feces, algae, and invertebrates. The troughs were contaminated with a realistic number of *M. avium* subsp. *paratuberculosis*. The organism survived for 48 weeks in a shaded water trough, 36 weeks in an unshaded water trough, but only 12 weeks in fecal pellets and soil in a terrestrial location in the same environs (32). The mean weekly temperature range in the terrestrial environment was about 5°C to almost 40°C compared to 7 to 28°C in the shaded water trough. We previously hypothesized that temperature fluctuations were detrimental to this organism (32).

The organism survived for about 3 months longer in the sediment than in the water column in the troughs. Collectively, the data from the present study suggest that the aquatic environment is a greater risk than pasture and soil with respect to long-term persistence of the organism on a farm after destocking affected livestock.

Within the observed period of survival of the organism in water and sediment there were times when it could not be isolated from either type of sample (Fig. 2). Disturbance of the sediment with release of the organism into the water column during addition of water to the troughs may explain this as sediment had been consistently culture positive. However, dormancy of *M. avium* subsp. *paratuberculosis* in water and sediment is another explanation (32). Since one viable organism causes a growth index in BACTEC media of 999 within about 6 weeks (23), times in excess of this suggest that organisms are not in a cultivable state when inoculated into culture media. Extended cgi1000 (i.e., >49 days) are also suggestive of dormancy and were observed in the present study. Dormancy of the organism was reported in a dry shaded terrestrial environment (32).

The results of the present study are comparable to those of several smaller studies that were conducted under more contrived conditions. The D value (time for 1 $\log_{10}$ reduction) of the S strain in water was not measured directly in the present study but can be inferred to be about 1.8 months based on a 6-log decline over 11 months. Survival of the C strain of *M. avium* subsp. *paratuberculosis* in distilled water was observed for 455 days with a D value of approximately 2 to 3 months in a controlled laboratory

study (5). Survival of the C strain in tap water and pond water in sealed bottles at a starting concentration of 10^5/ml at ambient temperature (ca. 9 to 26°C) was >9 but <13 months (18) and from 3 $\log_{10}$-higher starting concentrations was about 17 months in the dark at 38°C (16).

The dam water used in the present study contained numerous invertebrate and protozoal species that were not identified. Although prolonged survival of *M. avium* subsp. *paratuberculosis* can occur in distilled and/or sterilized water (5), there may be significant interactions of the organism with microbivorous invertebrates (crustaceans, nematodes, trematodes, and insects) and protozoa (amoebae, ciliates, and flagellates). Although *M. avium* subsp. *paratuberculosis* is believed to be an obligate parasite of animals, it has been cultured from trichostrongylid nematode larvae and other invertebrates (7, 17, 30). Protozoa are recognized environmental hosts for a range of pathogenic bacteria that may replicate to high titer within vacuoles, persist during encystment, and in that state remain protected from chemical disinfectants such as chlorine (1, 12, 13, 19). There are several reports confirming that amebas ingest *M. avium* (3, 15, 26).

Aerosolization of mycobacteria in surface water is one means suggested for tuberculin sensitization of animals and humans living near water (8, 10) and for disease in humans (14). It is possible that *M. avium* subsp. *paratuberculosis* behaves in a fashion similar to that of other members of the *M. avium* complex and be concentrated in bubbles in a water column and ejected at the water-air interface to form aerosols, which might travel laterally (21). Inhalation has recently been suggested as a route of infection for *M. avium* subsp. *paratuberculosis* (6); in any case, aerosols may settle on pasture and be ingested.

The results of the present study suggest that the aquatic environment may be a significant reservoir of *M. avium* subsp. *paratuberculosis* infection. In a recent survey of soil and water on six farms with Johne's disease-affected sheep flocks, *M. avium* subsp. *paratuberculosis* was isolated from ca. 20% of samples (31). On one farm, 17 water and sediment samples were collected, but only 1 of these was positive for *M. avium* subsp. *paratuberculosis*; no terrestrial sites were sampled. On the other five farms, too few water samples were collected to enable a comparison with isolation rates from terrestrial sites. Water and sediment samples were also taken from a further 20 farms that had been destocked of Johne's disease-affected sheep for at least 11 months; there were several culture-positive samples, all of which were sediments from low-lying areas near dams or water courses (31). From these results, it can be assumed that low-lying and aquatic environments where feces accumulates may harbor *M. avium* subsp. *paratuberculosis*. Pending the outcomes of surveys that more thoroughly address the sites of *M. avium* subsp. *paratuberculosis*

contamination on extensively grazed sheep farms, animal health authorities will need to provide appropriate advice to farmers to minimize exposure of livestock to potentially infected water sources during Johne's disease control programs. For example, it may be prudent to restrict access to dams if the catchment has been grazed by infected stock in the previous 12 months. Disturbance to dam sediments may also lead to higher levels of contamination of the water column as the organism appears to survive longer in sediment than the water column. For this reason provision of water pumped from a dam to clean water troughs may be preferable to allowing animals direct access to the dam, since they generally enter the water while drinking and disturb sediments. The duration of survival of the organism in water destined for human consumption and means of filtration or disinfection may also need to be addressed if the organism is found to be involved in the etiology of Crohn's disease (**25**, **28**).

Further research on the microbiology of *M. avium* subsp. *paratuberculosis* in aquatic ecosystems is warranted. Epidemiological research priorities would be to culture water sources in areas where there is local geographic clustering of Johne's disease and to model the spread of Johne's disease by water or aerosol, using geographic information system data and distribution maps of Johne's disease-affected farms.

ACKNOWLEDGMENTS

This study was funded by Meat and Livestock Australia as project TR.055A.

We thank David Skerman for support in the initiation of this project and Terry and Cecily Hayes, “Hillwood” Goulburn, for providing infected donor sheep. Skilled technical assistance was provided by Vanessa Saunders, Scott McAllister, Aparna Vadali, Shayne Fell, Anna Waldron, and Brian Maddaford.

FOOTNOTES

- Received 5 January 2005.
- Accepted 21 April 2005.

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