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# Application of cattle slurry containing *Mycobacterium avium* subsp. *paratuberculosis* (MAP) to grassland soil and its effect on the relationship between MAP and free-living amoeba

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ABSTRACT

Slurry from dairy farms is commonly used to fertilize crops and pastures. This mixture of manure, urine and water can harbor multiple microbial pathogens among which *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is a major concern. Persistence of MAP in soil and infection of soil *Acanthamoeba* was evaluated by culture, real-time IS*900* PCR, and by staining of amoeba with acid-fast and vital stains comparing soils irrigated with MAP-spiked or control dairy farm slurry. MAP DNA was detected in soil for the 8 month study duration. MAP was detected by PCR from more soil samples for plots receiving MAP-spiked slurry ($n$ = 61/66) than from soils receiving control slurry (**n** = 10/66 samples). Vital stains verified that intracellular MAP in amoeba was viable. More MAP was found in amoeba at the end of the study than immediately after slurry application. There was no relationship between MAP presence in soil and in amoeba over time. Infection of amoeba by MAP provides a protected niche for the persistence and even possibly the replication of MAP in soils. As others have suggested, MAP-infected amoeba may act like a "Trojan horse" providing a means for persistence in soils and potentially a source of infection for grazing animals.



## 1. Introduction

Intensification of dairy production results in larger herds and thus the quantity of organic waste, *e.g.* feces, waste water and urine (collectively called slurry), applied onto small areas of land (Salazar et al., 2007). Commonly, slurry is used to fertilize pastures and forage crops because the phosphorus, nitrogen and potassium in slurry are important for maintenance of high quality pastures and crops.

A disadvantage of using slurry as a fertilizer is that it often contains microbial pathogens that could threaten

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both animal and public health (Mawdsley et al., 1995; Pell, 1997; Hooda et al., 2000). Thus, it is vital to understand pathogen survival during slurry storage or after application to soils and also in surface or groundwater that may become contaminated after rainfall events in areas with slurry application. Slurry treatment prior to application onto land can lower pathogen concentrations (Grewal et al., 2006).

Among the many pathogens of concern in slurry is *Mycobacterium avium* subsp. *paratuberculosis* (MAP). MAP is the causative agent of paratuberculosis, also known as Johne's disease, a chronic intestinal disease of worldwide distribution (Harris and Barletta, 2001) with high herd-level prevalence (44–87%) among dairy herds in southern Chile (Kruze et al., 2013). MAP mainly infects ruminants and is excreted from infected animals in feces. Prior studies demonstrated that after application of MAP-contaminated slurry, MAP tends to remain on the upper layers of soil rather than moving down through soil profile and persists for about a year (Salgado et al., 2011, 2013). Environmental persistence of this intracellular parasite (Lambrecht and Collins, 1993) may be affected by moisture, clay content, and the soil depth from which the samples were collected for MAP detection (Pribylova et al., 2011). Other possible persistence mechanisms are by formation of dormant endospore-like structures (Lamont et al., 2012) or by infection of free-living amoeba (Mura et al., 2005).

Persistence or even amplification of MAP numbers in the environment after application of MAP-contaminated slurry to soils was the focus of the studies herein described. The findings are important to a fuller understanding of the epidemiology of paratuberculosis (Whittington et al., 2004, 2005; Pickup et al., 2005; Salgado et al., 2011, 2013).

## 2. Materials and methods

### 2.1. Experimental design

The experimental work was conducted at the Institute for Agricultural Research (INIA) Remehue Research Centre located in southern Chile (40°35′S, 73°12′W) May to December 2012. The study was a field experiment (ambient environmental conditions) using 12 soil plots of 1 m × 2 m × 15 cm deep blocks of volcanic ash soil typical for the region (series Osorno, typical Hapludands). These blocks of soil were maintained on raised wooden platforms (Fig. 1). Slurry that was not spiked with MAP (control) or spiked with cultured MAP to achieve $\geq 10^6$ MAP/mL was applied to the surface of soil blocks (Fig. 2).

### 2.2. Soil plots

The soil blocks were obtained from a pasture with a slope less than 5%, belonging to INIA Remehue. No domestic ruminants had grazed the pasture for at least four years prior to soil collection. Soil blocks were obtained by digging to a depth of 15 cm around the perimeter of the plot with a shovel. The soil block was gently removed from the surface, placed on a sheet of plywood and handled flat as it was moved to the experimental platform at the study site (Salgado et al., 2013). Following extraction and transportation, soil blocks were mounted on 1 m × 2 m wooden platforms, which were angled to mimic different slopes. The soil blocks were exposed to ambient temperatures and rainfall. These same soil blocks were used for a previous study of MAP in water runoff (Fig. 1) (Salgado et al., 2013). Thus, this slurry application was the second application made one year after the first. Soil blocks receiving control (not MAP-spiked) slurry in the first trial again received non-spiked slurry in the present trial.

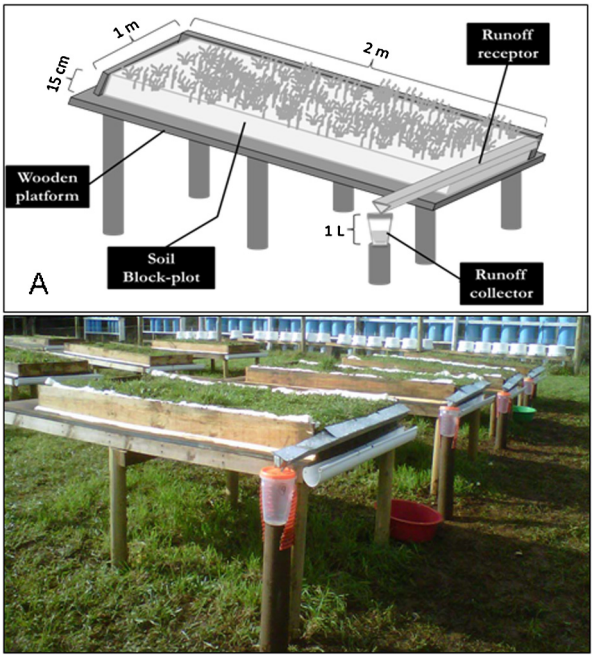


**Fig. 1.** Soil plot experimental platform. Diagram of the platform and soil plot with key components labeled; and photograph of the platforms with soil plots.

### 2.3. Slurry

Slurry was collected from a storage lagoon belonging to a commercial dairy farm at INIA Remehue. No previous test was done to categorize the herd's infection status. However, this herd had no history of clinical paratuberculosis cases.

### 2.4. Preparation of MAP inoculum

For slurry spiking, a pure culture of the MAP reference strain ATCC 19698 was cultured in Middlebrook 7H9 liquid medium supplemented with 10% OADC (BD DIFCO® Becton Dickinson and Company, USA) and 2 mg/mL of mycobactin J (Allied monitor USA). Cultures were incubated for two months at 37 °C in 40 mL flasks. Organisms were harvested when in mid-exponential phase growth ($OD_{600}$ = 1.00 equating to roughly $10^8$ CFU/mL of MAP) based on weekly spectrophotometer (Helios Gamma® Thermo Scientific) readings (Sung et al., 2007), and 1.5 L of this pure liquid culture was used for slurry spiking.