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How *Mycobacterium avium paratuberculosis* is affected by the composting process

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Abstract

The management of livestock manure has become increasingly important. Farms are separating, digesting and/or composting to create a value-added product that can either be used on site or sold off-farm. Regardless of the use, disinfection of this animal waste is important to the health of animals and humans. The high temperatures of composting have been shown to inactivate enteric pathogen indicator species such as *E. coli* but questions remain as to its ability to inactivate hardier pathogens such as *Mycobacterium avium paratuberculosis* (MAP). MAP, the causal agent of Johne's disease, is associated with economic losses worldwide. The disease is transmitted among livestock by the fecal-oral route, thus application of contaminated manure, slurry or compost to grazing or cropland could contribute to the spread of the disease. Manure from a Johne's free farm was mixed with manure from a heavily shedding cow and formed into a windrow. The windrow was turned weekly and temperatures reached > 55^{o}C for the course of the study. Thermophilic composting rendered MAP un-culturable as early as 5 days into the composting process and it remained un-culturable through day 70.

Key words: *Animal waste disinfection, composting, manure, Mycobacterium avium paratuberculosis*

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INTRODUCTION

Livestock manure may disseminate, transmit, or propagate pathogens such as Listeria and Salmonella as well as *Mycobacterium avium paratuberculosis* (MAP). Unless appropriately processed, manure can be a potential biohazard capable of transmitting infective agents. Composting manure has several benefits including decreased volume/weight, removal of odors, decreased risk of polluting surface and ground water, reduction of enteric organisms and production of a value-added product. Studies have shown that a variety of conditions in the composting process can influence the survival of pathogenic bacteria and viruses that subsequently infect livestock. These conditions include temperature, moisture content, pH, physical composition of the composting material and microbial population.

Most of the research done on pathogens or enteric indicator species in compost has examined *Salmonella*, *Escherichia coli*, and total fecal coliforms. Kudva et al. (1998) found that *E. coli* O157:H7 survived for more than 1 year in a non-aerated ovine manure pile that was exposed to environmental conditions. In similar aerated ovine and bovine manure piles, the organism survived for 4 months and 47 days, respectively. In other studies, when the manure is composted, *E. coli*, as well as *Salmonella*, was either inactivated or undetectable within 24 hours due to attainment of temperatures greater than 50^{o}C (Lung et al. 2001; Turner 2002; Hess et al. 2004). Larney et al. (2003) showed that more than 99.9% of total coliforms and *E. coli* was eliminated in the first 7 days in windrow composted beef feedlot manure when average temperatures ranged from 33.5 to 41.5^{o}C. Hardier bacteria, however, such as fecal streptococci and enterococci took longer to show a reduction (Shuval et al. 1991).

Additional pathogens have been studied to a smaller extent in manure and manure-based composts and rising temperatures have been found to decrease or eliminate pathogens such as *Yersinia, Campylobacter,*

Cryptosporidium, and *Giardia* (Kearney et al. 1993; Guan and Holley 2003). Temperatures reached in short time composting of poultry manure were sufficient to eliminate the vegetative forms of *Salmonella*, *E. coli*, *Proteus*, *Pasteurella*, and *Streptococci* investigated by Platz (1977) after exposure to an average 22-hour composting process. *Listeria* was eliminated when exposed to the athermic factors of composting, such as change in pH. *Clostridium perfringens* was not affected by either temperature or athermic factors.

These studies all show that most bacteria found in cow manure can be eliminated through the high temperatures achieved in the composting process. Others that require temperatures higher than those achieved via composting, greater than 60^{o}C, may be inactivated due to the changes in pH, solids content and bacterial populations. One such important bacterium to the dairy industry is *Mycobacterium avium paratuberculosis*. MAP is the causal agent of Johne's disease, a chronic inflammatory bowel disease of ruminants that is associated with economic losses worldwide. The disease is transmitted among livestock by the fecal-oral route, thus application of contaminated manure, slurry or compost to grazing or cropland could contribute to the spread of the disease. MAP is a very hardy organism that has been known to survive in soil or water for as long a year when fully shaded, (Whittington et al. 2004; Whan et al. 2006; Gumber and Whittington 2009) and for up to 252 days in cattle slurry stored at 5^{o}C (Jorgensen 1977). High temperature pasteurization (70 - 75^{o}C) had been considered the only way to eliminate MAP, but recent studies in milk have shown that this is not always effective. Grant et al. (2002), tested raw and pasteurized milk samples via polymerase chain reaction (PCR) and culture and found clear evidence that MAP bacteria in naturally infected milk are capable of surviving commercial high-temperature, short-time pasteurization if they are present in raw milk in sufficient numbers. Others showed a reduction of MAP at pasteurization temperatures, but not at temperatures that would be achieved during composting (Rademaker et al. 2007).

Few studies have looked at the survival of MAP in media other than milk. Jörgensen (1977) published the first comprehensive study of its kind on survival of *M. paratuberculosis* in slurry in Denmark. He found that the number of colonies of MAP dropped drastically between sampling days 1 and 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 and at 15°C it was between 98 and 182 days. In 1984, Olsen reported that the population of MAP in digested slurry was reduced by 90% after 6 days at mesophilic temperatures (35^{o}C) and after 1 hour at thermophilic temperatures (53-55°C). In 1985, Olsen and Jorgensen investigated the reduction of MAP in conditions found during anaerobic digestion. Bovine slurry was spiked to yield initial counts of 3.3 x 10^3 to 2.7 x 10^4 MAP/g slurry and held at mesophilic or thermophilic conditions. At mesophilic conditions MAP was re-isolated at 7, 14, and 21 but not 28 days. At thermophilic conditions viable MAP could not be detected in as short as 3 hours.

More recently, Wright et al. (2003) showed that mesophilic anaerobic digestion resulted in a 2-log reduction in MAP and further composting of the solids showed an even greater reduction. A more comprehensive study was done in 2006 to compare the persistence of MAP during the treatment of dairy manure under conditions that simulate three commonly used manure management methods: thermophilic composting at 55°C, manure packing at 25°C and liquid lagoon storage (Sukhbir et al. 2006). In this study, MAP was detected not only by culture, but also by PCR, which detects DNA, and is thus a more accurate detection method. Using the culture method for MAP detection, this study showed that MAP can persist for long periods in stored liquid manure under anaerobic conditions, but no MAP organisms were culturable after just 3 days in the composting treatments at both temperatures. In contrast to the culture-based assay results, MAP DNA was detected through day 56 in all treatments, and also at day 175 in the lagoon storage treatment. The authors state that since MAP was not detectable by culture after day 3 in the compost and pack treatments this suggests that cells were either dead or present below the detection limit of the conventional culture methods in these treatments. Alternately, MAP organisms may have been alive but un-culturable due to the severe physicochemical conditions and/or microbial competition.

Manure management on livestock farms has become a focal point around the world. More farms are digesting or separating their manure. The solid byproducts can be sold off site as a soil amendment, composted and sold as a value-added product or used on the farm as bedding or spread on fields. It is therefore important to know if pathogen reduction will occur as a result of manure management techniques. This study was designed to investigate the degree to which the composting process and temperatures reduce or kill MAP using a farm composting system that can be accomplished without specialized equipment

MATERIALS AND METHODS

Pile Set-up and Sampling

Approximately 32 cubic meters of manure from a confirmed Johne's free farm was brought to Cornell University's compost site on August 1st 2001. The test negative herd was closed and determined to be free of Johne's Disease based on repeated whole herd fecal culture testing (8 times) with continuous monitoring for over 11 years. At the same time, approximately 90 kg, or 2 cow days worth, of MAP infected manure from a heavily shedding cow was

mixed with the Johne's free manure and formed into a 2 x 2 x 9 m windrow. The infected manure was tested for MAP and contained > 300,000 colony forming units per gram (cfu/g). A sanitized loader was secured for the initial mixing and weekly turning. Temperatures were taken approximately daily for 7 weeks at twelve locations in the pile, 4 each in the top fringe (30.5 cm depth), bottom fringe (30.5 cm depth) and core (91 cm depth).

After the windrow was completed on day 0, four samples of compost were taken from each of the top fringe, bottom fringe and core layers. These samples were taken using the following procedure: Fringe sampling was done first to avoid cross contamination of the fringe samples when taking core samples. Four bottom fringe samples were taken at separate locations approximately 30.5 cm above ground level and 20 cm inside the pile. The upper fringe samples were taken 20 cm inside the pile at a height of approximately 46 cm. Core samples were taken from the same locations as the bottom fringe at a height of 30.5 cm but were approximately 100 cm inside the pile. In addition to the individual samples taken on day 0, four composite samples were also taken using the compost sampling method for well-mixed windrows developed by Woods End Laboratories, Inc. (WEL), Mt. Vernon, ME. On day 5, prior to turning the windrow, 12 separate samples from the top, bottom and core were taken as described above. After turning on days 5, 12, 20, 27, 34 and 70, composite samples were taken.

Laboratory Analysis

All samples were sent to the Animal Health Diagnostic Laboratory at Cornell University, Ithaca, NY for determination of presence of MAP and beginning on day 5, the samples were split and the second set was sent to WEL for analysis of solids, water holding capacity, pH, volatile organic acids, organic matter, carbon to nitrogen ratio, total nitrogen, and fecal coliforms using EPA methods (EPA 1998). The procedure used to enumerate MAP was the Cornell Double Incubation Decontamination for *M. paratuberculosis* culture from feces:

Day 1: First Decontamination and Incubation: Two grams of feces are weighed out into 35 ml distilled water in a 50 ml plastic centrifuge tube. The tube is shaken and sits at room temperature for 30 minutes. A 5 ml sample is taken with a pipette from the top portion of the tube and put in 25 ml of 0.9% Hexadecylpyridinium chloride monohydrate (HPC) in ½ X Brain Heart Infusion (BHI) broth solutions, in a 50 ml plastic centrifuge tube. The HPC is a detergent used to kill bacteria and fungus and the BHI provides nutrients to the MAP bacteria. The tube is incubated for 18-24 hours (overnight) at 35 – 37^{o}C.

Day 2: Second Decontamination and Incubation: Centrifuge the tube for 20 minutes at 3000x g at 15^{o}C. The supernatant is discarded and the pellet is re-suspended by adding 1 ml of antibiotic brew. The antibiotic brew is made of BHI plus 100, 100, 50 µg NVA (Nalidixic acid, Vanco, Amphotericin B). The tube is incubated overnight.

Day 3: Set up for Herrold's Egg Yolk Media (HEY): Using a Liquipette, dispense 0.2 ml from the centrifuge tube onto 3 HEY tubes with Mycobactin J (MJ, a growth media needed for Johne's) and 1 HEY without MJ for comparison. Since MAP will not grow without MJ, if growth appears on all four tubes, it is not MAP. Set these tubes on a slant for 2 weeks to allow the liquid to dry then stand upright for 10 weeks. Start reading the tubes every week beginning at about 4 weeks by counting colonies. Take a final count at 12 weeks.

RESULTS AND DISCUSSION

The United States Environmental Protection Agency (EPA) regulates composts that contain sewage sludge or septage. These rules are part of a larger set of regulations dealing with sewage sludge referred to as the "Part 503" regulations (EPA 1993). EPA regulatory standards are designed to protect human health. As a result, criteria outlined in the Part 503 regulations are often used to measure the relative safety of non-sludge composts. According to Part 503, certain temperatures must be maintained during the compost process to classify the compost as Class A, suitable for unrestricted use, indicating that the pathogens have been destroyed. Using the windrow composting method, the temperature of the sewage sludge must be maintained at 55^{o}C or higher for 15 days or longer with a minimum of five turnings of the windrow.

Temperatures within the research compost pile reached 55^{o}C in the top and bottom fringe within 24 hours after the pile was built, while it took the core a little over a week to reach 55^{o}C. All remained at or above that temperature for the duration of the trial (figure 1). These temperatures, combined with turning six discrete times, should indicate that pathogen reduction had occurred. The test data confirmed that. Fecal coliforms decreased from 6.6 $\log_{10}$ MPN/g on day 5 to 2.5 $\log_{10}$ MPN/g on day 70 (table 1). Table 1 also shows the change in other compost parameters over the study period. Over the 70 days of the trial, % solids increased from 38.1 to 49.7, organic matter decreased from 78.9 to 65.4% and the carbon to nitrogen ratio went from 23.7 to 17.8. All of this indicates that composting is progressing but not completed. Finished compost should generally have a solids and organic matter content between 50 to 60%, and a carbon to nitrogen ratio of 10 – 15:1. Coupled with the fact that the compost was still in the thermophilic temperature range indicates that this was not yet a mature compost and if used now could have implications for re-growth of fecal coliform and other environmental pathogens.

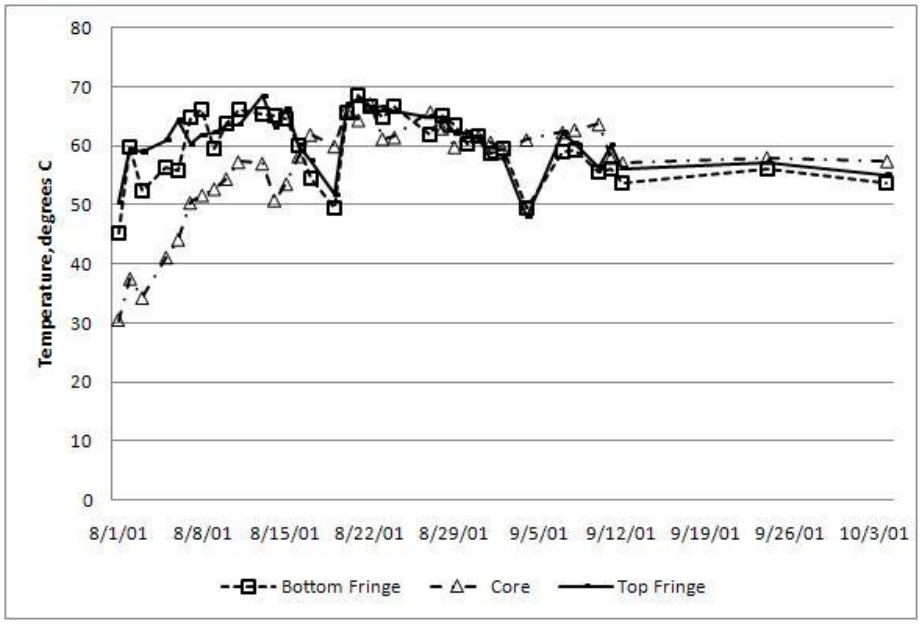

Figure 1. Temperatures in the compost pile at 3 depths over time.

Table 1. Average compost parameters over time

Date	Day	Solids (%)	Water Holding Capacity (% as is)	pH	Volatile Organic Acids (ppm)	Organic Matter (% dry basis)	Carbon to Nitrogen Raio	Total Nitrogen (% dry basis)	Fecal Coliforms (log_{10} MPN[1]/g)
8/6/01	5	38.1	70.8	8.3	1383.3	78.9	23.7	1.8	6.6
8/13/01	12	37.3	69.5	9.2	2233.8	74.6	20.7	1.9	2.5
8/21/01	20	39.8	69.8	9.4	1336.8	74.1	22.9	1.8	3.7
8/28/01	27	43.1	69.5	8.8	1681.8	73.9	22.4	1.8	3.7
9/4/01	34	44.4	69.0	9.0	1371.3	71.6	21.1	1.8	3.2
10/10/01	70	49.7	67.3	8.8	575.8	65.4	17.8	2.0	2.5

[1]MPN = most probable number

Table 2 shows cfu/g of MAP recovered from the samples taken from the compost pile. Although the inoculum used had counts in excess of 300,000 cfu/g, once it was mixed with the other compost feedstocks, it was diluted to an average of 183.8 cfu/g and several of the day 0 samples found no MAP detectable. By day 5, MAP was undetectable and was not detected in any of the samples taken through day 70. MAP levels were analyzed using the JMP Statistical Package version 8.0 (SAS, Cary, NC). Non-linear regression of the MAP levels in the compost pile show an exponential decay rate of 2720 cfu/day with a half-life of 0.3 days, or 7 hours. This is similar to that which was found by Olsen and Jorgensen (1985) where MAP contaminated slurry exposed to thermophilic temperatures was not found after 24 hours in one experiment, and after 3 hours in another.

Sukhbir et al. (2006) also found that MAP was undetectable by culture after 3 days of thermophilic composting. However, MAP DNA was detected by PCR through day 56 in that experiment, indicating that there may be some un-culturable MAP remaining. This may be due to the fact that MAP, although a non-sporulating bacteria, has the ability to go into a dormant state (Gumber and Whittington 2009). Dormancy, as defined by Kaprelyants et al. (1993) means a reversible state of low metabolic activity, in which cells can persist for extended periods without division. This often corresponds to a state in which cells are not alive in the sense of being able to form a colony when plated on a suitable solid medium, but one in which they are not dead, in that when conditions are more favorable, they can revert to a state of aliveness.

Table 2 MAP levels over time

Date	Day	ID	Replicate	MAP (cfu[1]/g)	Average MAP
7/31/01	0	Inoculum	1	300,000	
8/1/01	0	Top Fringe	1	2,810	
			2	0	
			3	0	
			4	0	
		Bottom Fringe	1	0	
			2	0	
			3	0	
			4	0	
		Core	1	10	
			2	40	
			3	10	
			4	10	
		Composite	1	0	
			2	0	
			3	0	
			4	0	183.8
8/6/01	5	All[2]		0	0.0
8/13/01	12	All[3]		0	0.0
8/21/01	20	All[3]		0	0.0
8/28/01	27	All[3]		0	0.0
9/4/01	34	All[3]		0	0.0
10/10/01	70	All[3]		0	0.0

[1]cfu = colony forming units
[2]Sixteen samples were taken: 4 in the top fringe, 4 in the bottom fringe, 4 in the core and 4 composite samples
[3]Four composite samples were taken

This dormancy state was further verified by Whittington et al. (2004) who studied natural pasture plots and boxes of soil containing sown grasses contaminated with MAP infected sheep feces. Fecal, soil, and pasture samples were collected at intervals for up to 117 weeks and cultured to detect viable MAP. In all of the experiments, there were culture positive results after one or more time points at which all samples were culture negative. PCR showed MAP DNA in almost all of the samples that had been culture negative. Similar results were found in carcass compost piles where MAP infected dairy manure was seeded in the abdominal cavities of road-killed deer and removed at weeks 0, 3, 6, 9, 12 and 36 for analysis (Schwarz et al. 2010). MAP levels decreased immediately from 4.5 $\log_{10}$ cfu/g at week 0 to 0.1 to 0.2 $\log_{10}$ cfu/g through week 12. Each week, 8 out of 9 samples analyzed were culture negative. However, at week 36, 6 of the samples had values of around 2 $\log_{10}$ cfu/g (actual counts of 1–30 colonies) and one of the samples had colonies that were too numerous to count (>3000). These two studies are consistent with MAP being able to enter a dormant or viable non-culturable state and later reverting to a vegetative form.

All of the studies showing MAP to be inactivated during thermophilic composting or digestion were short term studies that analyzed for the presence of MAP only during the thermophilic stage. Since the current study also only took samples during the thermophilic stage, it is possible that conditions could become favorable for dormant MAP to revert to the vegetative form and become culturable again.

The presence of MAP in manure can contribute to the spread of Johne's disease if manure is handled improperly. Windrow composting is one method by which manure can be treated. This study showed that MAP was un-culturable in the first 5 days of composting and was not found by culture again over a 70 day period. The duration of high temperatures achieved during the thermophilic process were unfavorable for bacterium survival. The study did not examine for the presence of MAP in the finished compost, at which time conditions may become favorable for MAP to revert to its vegetative form.

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