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Research Article

Leaching of *Mycobacterium avium* Subsp *paratuberculosis* in Soil under *In Vitro* Conditions

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Mycobacterium avium subsp *paratuberculosis* (Map), the causative agent of Johne's disease, has a robust ability to survive in the environment. However, the ability of Map to migrate through soil to drainage tiles or ground water, leave the farm, and leak into local watersheds is inadequately documented. In order to assess the ability of Map to leach through soil, two laboratory experiments were conducted. In the first study, 8 columns (30 cm long each) of a sandy loam soil were treated with pure cultures of Map. Two soil moisture levels and two Map concentrations were used. The columns were leached with 500 mL of water once a week for three weeks, the leachate was collected, and detection analysis was conducted. In the second experiment, manure from Map negative cows (control) and Map high shedder cows (treatment) were deposited on 8 similar columns and the columns were leached with 500 mL of water once a week for four weeks. Map detection and numeration in leachate samples were done with RT-PCR and culture techniques, respectively. Using RT-PCR, Map could be detected in the leachates in both experiments for several weeks but could only be recovered using culture techniques in experiment one. Combined, these experiments indicate the potential for Map to move through soil as a result of rainfall or irrigation following application.

1. Introduction

Mycobacterium avium subsp *paratuberculosis* (Map), the causative agent of Johne's disease (JD) in domestic and wild ruminants is known to be ubiquitous in the dairy farm environment [1]. Additionally, Map has long been implicated as a possible cause of Cohn's disease in humans [2]; however, this is still strongly debateable. Several reports indicate that Map will survive for long periods of time under various *in vitro* physical and environmental conditions, controlled by the amount of water, urine, manure, and temperatures [3–5]. Results suggest that long-term survival could occur near shaded animal management locations and locations that receive frequent introductions of manure [6]. Using dam water and sediment columns, one study [6] showed that Map could survive up to 48 and 36 weeks in shade or semiexposed location, respectively, and survival in sediment was 12 to 26 weeks longer than survival in water columns. In a Map-inoculated liquid manure slurry stored under anaerobic conditions, Map was shown to survive 252 days at 5°C and 98 days at 15°C [7]. One study [8] found that Map could survive up to 175 days in Map-inoculated liquid manure storage. A large study on 108 Minnesota dairy farms [1] found that a predictable location to recover Map isolates was in alleyways and manure storage areas. Similar results were obtained in another study, which used approximately 100 dairies across the USA [9]. This is important since land-spreading of bovine manure is a common practice on many dairy operations [10, 11]. Also, the survival of Map in the soil may be influenced by the type of soil present [11] as was established for other bacteria [12, 13]. The potential of Map to leach through soil to enter a farm from the environment or enter a local watershed is unknown. A recent study investigated the processes controlling Map transport through

aquifer material and found that, compared to other bacteria, Map transport in the soil is lower [14]. Another factor that remains unknown is the ability of Map to survive during the leaching process. Only recently, Map absorption in soil particles was studied and it was found that of the organism added to the columns 83% were estimated to be retained in chromatography columns packed with clay and silica soils [15]. Cho et al. [16] showed that fecal bacteria can migrate into the subsurface and cause significant contamination of vadose systems (subsurface soil) especially when manures are applied repeatedly. For other enteric bacteria it was shown [17] that bacterial leaching to tile-drains could exceed 71,000 organisms 100 mL^{-1} when driven with high rates of water infiltration. The objective of this study was to assess the potential of Map to leach through columns of soil under laboratory conditions. Our hypothesis was that Map has the ability to leach through a soil column when introduced as pure culture inoculum or via feces.

2. Material and Methods

2.1. Soil. The soil type used in the study was Tracy (Coarse-loamy, mixed, active, mesic Ultic Hapludalfs), collected from the Pinney-Purdue Agriculture Center located on the county line between Porter and LaPorte counties, Indiana, USA. We used this soil because it is the most common soil type in the US Midewest. An aggregate sample was generated by combining soil from ten separate locations across the field. The soil sample was obtained from area where there is no livestock production, sieved (4 mm) and stored in closed plastic bags at room temperature until use. Soil parameters for the Tracy soil includes pH 5.5, 1.5% organic matter, 6 ppm Na, 103 ppm NO_3^-–N, 72 ppm P (Bray I Olsen), 155 ppm K, 148 ppm Mg, 787 ppm Ca, 9 ppm SO_4^{2-}S, and 2.9 ppm Zn. Soil analysis was performed by Harris Laboratory (Lincoln, NE). Prior to packing the columns, the soil was passed through a 2 mm sieve. The soil moisture content was determined by drying subsamples of the soil at 105°C for 24 hours and determining its water loss gravimetrically.

2.2. Soil Columns Setup. The bottom of PVC tubes (40 cm length × 8 cm diameter) was covered with a wire mesh and cheese cloth (Figure 1). The lower 30 cm of the tubes was marked and enough soil added to provide a final bulk density of 1 gm cm^{-3} based on the mass of soil put into a specific volume of the column. In order to assess the impact of the initial soil moisture content on Map movement during leaching, the soil was adjusted to two moisture levels: −0.1 or −0.03 MPa (14 and 15.2%, resp.), before it was placed into the columns. These two soil moisture suctions were chosen because they bracket the range of optimal water potential in soil for microbial processes [18]. The soil was brought to the two moisture levels by adding water to the soil slowly in a dropwise fashion while mixing to achieve as uniform distribution as possible [19, 20].

2.3. Preparation of Map Inoculum. Map inoculums were prepared from fecal sample of naturally infected cattle previously confirmed to be positive based on serum ELISA and liquid fecal cultures (procedures described by Whitlcok and Rosenberg, 1990) [21]. The liquid culture was incubated for up to 6 weeks and acid fast staining was applied to samples at 4.5 and 6 weeks. Map specific PCR testing was performed to the sample with positive acid fast result. Samples positive by Map specific PCR were reported as Map positive. Liquid culture positive samples were streaked on to Herold's Egg York agar to obtain isolated colonies and subsequently passed to liquid culture medium again to propagate to desired concentration and used as the stock inoculums.

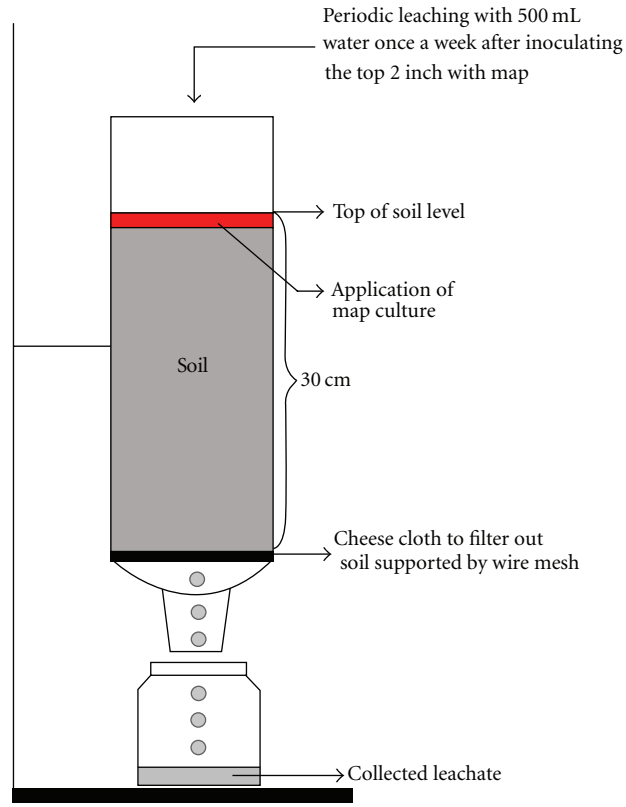

Figure 1: A column setup for leaching study of *Mycobacterium avium* subsp *paratuberculosis* in Tracy soil from northwest Indiana.

2.4. Map Recovery from Columns. To mimic natural rainfall, each soil column received once a week 500 mL distilled and deionized water in a dropwise fashion to achieve a uniform distribution, which is equivalent approximately 150 mm of rain. Leachates from each column were collected into a 100 mL sterile bottle placed below the PVC columns. Twenty-four hours after water was added, the leachates volume was measured and centrifuged (7500 g; 10 minutes). The supernatant was poured out and the remaining sediment was resuspended in 1 mL sterile water for Map detection and enumeration.

2.5. Preculture Map Detection with PCR. The recovered pellet in 1 mL of sterile water was centrifuged at 2500 g for 10 minutes. The supernatant was removed and the pellet was used

for DNA extraction and Real Time PCR using Tetracore MAP extraction system and DNA test kit (VetAlert, Tetracore, Inc., Rockville, MD). The manufacture's procedures were followed.

2.6. Bacterial Culture. The TREK ESP liquid culture system (TREK Diagnostics Systems, Inc., Westlake, OH 44145) was used for the bacterial culture. The double incubation method as described previously [21] was used to prepare samples, with the modification that 1mL of sample replaced 2 g of feces. Samples were placed in 30 mL of sterile water, mixed and allowed to stand for 30 minutes. To reduce the number of other fast growing bacteria, five millilitres of the surface fluid were decontaminated with 25 mL of 0.9% hexadecylpyridinium chloride (Sigma-Aldrich, St Louis, MO) in half-strength brain heart infusion BHI broth and allowed to stand at 37°C for 24 hours After centrifugation at 900 g for 30 minutes, the pellet was resuspended in 1 mL of half-strength BHI broth with vancomycin (10 μg/mL; (Sigma-Aldrich, St Louis, MO), nalidixic acid (60 μg/mL; (Sigma-Aldrich, St Louis, MO), and amphotericin B (40 μg/mL; (Sigma-Aldrich, St Louis, MO) and incubated at 37°C for 24 hours [22]. Prepared soil was cultured using the ESP para-JEM culture bottles and incubated in the ESP machine (Trek Diagnostic Systems, Inc., Cleveland, OH). Samples were removed from the ESP machine at 4, 5, and 6 weeks and evaluated by acid-fast staining. This procedure was done to increase test specificity in order to ensure the presence of Map versus another acid-fast positive bacterium. Culture results were recorded as +, 2+, and 3+, which are equivalent to low, moderate, and high bacterial load (<10 colonies-per-tube, (CPT), 10–50 CPT, and >50CPT, resp.). Fecal culture sensitivity is estimated to be between 40 and 80% depending on the bacterial load. Using fecal culture, the test specificity is nearly 100%. Nevertheless, no such information is available on the test performance using soil leachates. Samples that were acid-fast positive were confirmed using IS*900* PCR to detect the IS*900* gene. DNA was extracted from ESP par a-JEM culture by the guanidine isothiocyanate-glass bead lysis method [23]. Alcohol-precipitated DNA extracts were resuspended in 40 μL of sterile water. The IS900 segment and primers used for amplification as well as the procedures performed for this PCR were reported previously [24–26]. Briefly, each 50 μL reaction contained 200 μM each of d ATP, dCTP, and dGTP; 1 μM dUTP; 3.0 mM $MgCl_2$; 10 mM Tris-HCl, pH 8.3; 50 mM KCL; 0.01% gelatine; 0.5 μM each of primers IS90/150C (5′-CCGCTAATTGAGAGATGC-GATTGG-3′) and IS900/921 (5′-AATCAACTCCAGCAG-CGCGGCCTCG-3′); 1U urail-*N*-glycosylase (UNG, Epicenter Technologies, Madison, WI); 2.5 *Taq* polymerase (PE Applied Biosystems, Foster City, CA); and 2 μL of DNA extract. In addition, each batch run included positive and template-negative controls, as well as UNG control (IS amplicons containing dU residues).

2.7. Experiment 1. In this experiment a total of 8 columns were used. Before the inoculation procedure soil samples from each column were cultured for Map and resulted negative. The pure culture strain of Map was introduced into the top 5 cm of eight of the columns. The cell suspension was uniformly applied to the top layer through injection by syringe. The cells were applied at two cell concentrations (10^8 and 10^7 cells mL^{-1}: 10 mL per tube). The soil columns were leached with 500 mL water as described above once a week for three weeks (Figure 1). The leachates were harvested as described above. The experiment was done in duplicates except for the control column.

2.8. Experiment 2. Feces from two cows known to be naturally infected with Map from previous testing were used for the Map-positive fecal inoculum. These samples were kept frozen (−70°C) after being collected rectally from the cows while a subset of the samples was cultured to assess bacterial load using a solid media as described elsewhere [27]. Results of this initial culture indicated that feces bacterial load was over >100 CFU/g. Once Map concentration was determined in the sample and soil columns were ready to be inoculated, the fecal samples were thawed at room temperature for 2 hours. Feces obtained from a fourth lactation cow known to be negative by repeated fecal culture and serum ELISA served as the negative fecal inoculums control specimen. These samples were frozen and thawed as described above.

2.9. Experiment Procedure. In this experiment we used eight PVC columns filled with 30 cm of Tracy soil packed to a bulk density of 1 gm cm^{-3} at a starting moisture content corresponding to −0.03 MPa. For the treatment group, 50 g of the manure was mixed well and evenly spread on the top 2 cm of soil of each of the 4 treatment columns (no. 5–8) in similar fashion to the control group. Similarly, 50 g of Map negative feces were evenly spread on top of the 4 control columns and mixed into the top 2 cm of soil to on farm mimic manure spreading. Twenty-four hours after the manure was deposit, columns were leached with 500 mL of distilled water as described above. Thereafter this procedure to harvest samples was repeated once a week for 7 weeks. Samples collected on weeks 0, 2, 4, 5, 6, and 7 were processed for Map detection. The rest of the procedure was the same as described for experiment one.

2.10. Statistical Analysis. Map data that are expressed as −, +, 2+, and 3+ in Table 1 were ranked as 1, 2, 3, and 4, respectively, for the purpose of statistical analysis. The ranks were then subject to a nonparameteric one way ANOVA analysis (Chi-Square and Wilcoxon tests) to investigate the statistical significance of the effect of antecedent soil moisture level, initial Map inoculum size, and time on the level of Map in the leachats. Leachate volume data were also subject to one-way ANOVA to test for statistical significance on the effect of the antecedent soil moisture level. All statistical analyses were done in SAS (2002-2003, SAS Institute, Inc., North Carolina) at significance level of $\alpha = 0.05$.

3. Results

3.1. Experiment 1. On average 322 mL and 345 mL leachates were collected from the soil columns with initial soil

Table 1: Result summary from Experiment 1 after application of a pure *Mycobacterium paratuberculosis* culture to the top of 30 cm soil column of sandy loam soil from Indiana, USA.

Moisture content	Initial noculumsize (cfu ml^{-1})	Week after bacterial inoculation					
		1		2		3	
		RT − PCR	Culture + IS900	RT − PCR	Culture + IS900	RT − PCR	Cultur + IS900
−0.1	10^8	+	−	−	−	+	−
−0.1	10^8	−	−	−	−	−	−
−0.1	10^7	−	−	−	−	−	−
−0.1	10^7	−	−	−	−	−	−
−0.03	10^8	−	−	−	−	+	−
−0.03	10^8	+	3+	−	−	+	−
−0.03	10^7	+	3+	−	−	−	−
−0.03	10^7	+	2+	+	−	+	−

Culture results were recorded as +, 2+, and 3+, which are equivalent to low, moderate, and high bacterial load on agar (<10 colonies-per-tube, (CPT), 10–50 CPT, and >50 CPT, resp.).

moisture content of −0.1 MPa and −0.03 MPa, respectively. The difference in leachat volume between the two treatments was not statistically significant ($P = .79$). The results of RT-PCR, bacterial culture, and PCR-IS900 show that Map was detected in the leachate samples collected from the treatment columns with both concentrations of Map (10^7 and 10^8 CFU (Table 1) over three weeks time. Week by week statistical analysis of the data indicated that the initial inoculum size did not have any significant effect on PCR and culture-based Map levels in the leachate (e.g., $P = 1.0$, week-1 PCR; $P = .3173$, week 2 PCR; and $P = .186$, week 3 PCR). The same was true for the antecedent soil moisture level except for week 1 in which the culture-based Map level in the leachate was significantly higher ($P = .045$) from the columns in which the antecedent soil moisture level was 0.03 MPa. Culture-based Map level decreased with time in the leachate, and this decrease was statistically significant ($P = .038$). The PCR-based Map levels, however, did not show any significant decrease over time ($P = .362$).

3.2. Experiment 2. On average 410 mL and 425 mL of leachate were collected from each of the control and the treatment soil columns, respectively, 24 hours after the columns were leached with 500 mL distilled water. This difference was not statistically significant but traceable to the fact that despite all efforts to maintain the same soil density in all columns, column no. 6 of the treatment group produced little leachate over the course of the study (on average <100 mL).

Results from RT-PCR indicate that immediately following fecal material application to the surface of the treatment columns, all of the leachates were negative for Map. At two weeks post application three of the four treatment columns were positive, but near the limits of detection which is 35 cycle threshold (Ct). The three columns produced Ct values of 34.9, 34.81, and 34.43. At four weeks post manure application, one column of the treatment group was positive (Ct = 34.31). At weeks five, six, and seven post treatment, all columns were RT-PCR negative. At all time points, when tested with the liquid media culture, no fecal Map bacteria were detected in the leachate recovered from any of the eight columns.

4. Discussion

Contamination of food and water by microorganisms from animal manure has become a topic of concern in the last decade especially in regard to non-point-source manure contamination as a result of pastured animals or manure intentionally spread onto fields as fertilizer or waste [28]. The results of our first experiment using Map inoculum showed that it is possible to recover Map from soil columns that are leached with water. We, however, do not have an explanation why two samples from both moisture contents were negative on week two and positive on week 3. One possible explanation is that Map was retained in the soil and only after sufficient water was poured it could be washed to the bottom of the column. Map trends to clump and intermittent detection are not uncommon. Our second experiment assessed the leaching ability of Map which originated from a manure matrix that contains fecal bacteria and possibly other inhibitors such as organic and inorganic materials to migrate in the column. The results of this experiment indicated that with manure as a source of Map, only a few cells could leach through the 30 cm soil column. Nevertheless, we could not assess if these were viable cells. The authors are well aware that epidemiologically this is a crucial factor in terms of Map transmission whether to livestock or to human. Despite the fact that RT-PCR results were very close to the recommended cut-off value, we are confident that they were accurate because these results were constant over several sampling period and over 3 of the 4 treatment columns. Hexadecylpyridinium chloride (HPC), alone or in combination with the antibiotics vancomycin and natamycin, is used in the decontamination process during the sample preparation for culture [21]. Several studies reported that HPC decontamination resulted in a significant reduction in the number of culture-positive milk samples [12, 29]. It is possible that this process killed the few viable cells that were

shown on RT-PCR and hence culture results were negative. It was shown that the interaction of enteric bacteria with the soil environment can differ from that in the absence of manure components and this can affect the retention and transport through the soils [30]. Manure application, alters the organic and inorganic components of the soil, which have a great influence on the survival and mobility of bacteria in the soil [30]. Since the number of bacteria added via feces was lower than with the pure culture application, a comparison between the two methods is difficult. It is plausible, however, that the difference in Map leaching ability between the two experiments is partly due to the manure environment with its biotic and abiotic components. PCR test sensitivity and specificity have been previously compared to fecal culture, whether solid or liquid media [31–34]. In one study, however, test sensitivity was shown to be directly associated with the manure bacterial load [33]. Specifically to the PCR method used in the current study Alinovi et al. [34] assessed test sensitivity and specificity of 60% and 97%, respectively. In this initial study we have demonstrated that Map could be mobile in the environment and this finding suggests that a large-scale investigation is warranted. The possibility that Map can leach through soils to possibly enter groundwater or exit the system in tile water where it could enter the water supply used for human consumption is especially alarming in light of the considerable evidence that Map may be involved in the etiology and pathophysiology of Crohn's disease in humans [35]. It is important to consider that we only used one type of soil (Tracy), but this soil is the most common soil found near Indiana's dairy operations. Map's movement through soil may also be influenced by the physical, chemical, and biological properties of the soil as was established for other bacteria [12–15]. Further understanding of Map leaching and survival in different soil types will enable us to better explain the spatial cluster of Map seropositivity found to be associated with loamy soils with silt or sand content in Indiana [11]. Thus, the results from our study indicate that further research is needed to determine how Map may move through the environment, especially as leachate in soils.

References

[1] E. A. Raizman, S. J. Wells, S. M. Godden et al., "The distribution of *Mycobacterium avium* subsp. *paratuberculosis* in the environment surrounding Minnesota dairy farms," *Journal of Dairy Science*, vol. 87, no. 9, pp. 2959–2966, 2004.

[2] R. J. Chiodini and C. A. Rossiter, "Paratuberculosis: a potential zoonosis?" *The Veterinary Clinics of North America. Food animal practice*, vol. 12, no. 2, pp. 457–467, 1996.

[3] P. P. Vishnevskii, E. G. Manatsev, V. V. Chernyshev, and N. S. Ustoichivost, "Paratuberkuliznykh batsill Ione," *Soviet Veterinary*, vol. 11–12, pp. 89–93, 1940.

[4] R. Lovel, M. Levi, and J. Francis, "Studies on the survival of Johne's bacilli," *Journal of Comparative Pathology*, vol. 54, pp. 120–129, 1944.

[5] A. B. Larsen, S. Richard, B. S. Merkal, and T. H. Vardman, "Survival time of *Mycobacterium paratuberculosis*," *American Journal of Veterinary Research*, vol. 17, no. 64, pp. 549–551, 1956.

[6] R. J. Whittington, I. B. Marsh, and L. A. Reddacliff, "Survival of Mycobacterium avium subsp. paratuberculosis in dam water and sediment," *Applied and Environmental Microbiology*, vol. 71, no. 9, pp. 5304–5308, 2005.

[7] J. B. Jørgensen, "Survival of *Mycobacterium paratuberculosis* in slurry," *Nordisk Veterinaermedicin*, vol. 29, no. 6, pp. 267–270, 1977.

[8] S. K. Grewal, S. Rajeev, S. Sreevatsan, and F. C. Michel Jr., "Persistence of *Mycobacterium avium* subsp. *paratuberculosis* and other zoonotic pathogens during simulated composting, manure packing, and liquid storage of dairy manure," *Applied and Environmental Microbiology*, vol. 72, no. 1, pp. 565–574, 2006.

[9] J. E. Lombard, T. M. Byrem, B. A. Wagner, and B. J. McCluskey, "Comparison of milk and serum enzyme-linked immunosorbent assays for diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* infection in dairy cattle," *Journal of Veterinary Diagnostic Investigation*, vol. 18, no. 5, pp. 448–458, 2006.

[10] D. Morse Meyer, I. Garnett, and J. C. Guthrie, "A survey of dairy manure management practices in california," *Journal of Dairy Science*, vol. 80, no. 8, pp. 1841–1845, 1997.

[11] M. P. Ward and A. M. Perez, "Association between soil type and paratuberculosis in cattle herds," *American Journal of Veterinary Research*, vol. 65, no. 1, pp. 10–14, 2004.

[12] C. P. Gerba, C. Wallis, and J. L. Melnick, "Fate of wastewater bacteria and viruses in soil," *Journal of Irrigation and Drainage Engineering*, vol. 101, no. 3, pp. 157–174, 1975.

[13] W. T. Frankenberger Jr., "Fate of wastewater constitutents in soil groundwater: pathogen," in *Irrigation with Reclaimed Municipal Wastewater*, G. S. Pettygrove and T. Asano, Eds., pp. 14-1–14-25, Lewis Publishers, Chelsea, Mich, USA, 1985, California State Water Resources Control Board Report Number 84-1.

[14] C. H. Bolster, K. L. Cook, B. Z. Haznedaroglu, and S. L. Walker, "The transport of *Mycobacterium avium* subsp. *paratuberculosis* through saturated aquifer materials," *Letters in Applied Microbiology*, vol. 48, no. 3, pp. 307–312, 2009.

[15] N. K. Dhand, J. A. L. M. L. Toribio, and R. J. Whittington, "Adsorption of *Mycobacterium avium* subsp. *paratuberculosis* to soil particles," *Applied and Environmental Microbiology*, vol. 75, no. 17, pp. 5581–5585, 2009.

[16] J. C. Cho, H. B. Cho, and S. J. Kim, "Heavy contamination of a subsurface aquifer and a stream by livestock wastewater in a stock farming area, Wonju, Korea," *Environmental Pollution*, vol. 109, no. 1, pp. 137–146, 2000.

[17] C. A. Scott, L. D. Geohring, and M. F. Walter, "Water quality impacts of tile drains in shallow, sloping, structured soils as affected by manure application," *Applied Engineering in Agriculture*, vol. 14, no. 6, pp. 599–603, 1998.

[18] R. F. Harris, "Effect of water potential in microbial growth and activity," in *Water Potential in Soil Microbiology Special Publication no. 9*, J. F. Parr, M. R. Gardner, and L. F. Elliott, Eds., pp. 23–95, Soil Science Society of America, Madison, Wis, USA, 1981.

[19] R. L. Mahler and A. G. Wollum, "The influence of water potential on survival of rhizobia in a Goldsboro loam sand," *Soil Science Society of America Journal*, vol. 44, pp. 988–992, 1980.

[20] R. L. Mahler and A. G. Wollum, "The influence of irrigation and Rhizobium japonicum strains on yield of soybean grown in a lakeland sand," *Agronomy Journal*, vol. 73, pp. 647–651, 1981.

[21] R. H. Whitlock and A. E. Rosenberger, "Fecal culture protocol for *Mycobacterium paratuberculosis*: a recommended procedure," *Proceedings, Annual Meeting of the United States Animal Health Association*, vol. 94, pp. 280–285, 1990.

[22] J. S. Sung, H. H. Jun, E. J. B. Manning, and M. T. Collins, "Rapid and reliable method for quantification of Mycobacterium paratuberculosis by use of the BACTEC MGIT 960 system," *Journal of Clinical Microbiology*, vol. 45, no. 6, pp. 1941–1948, 2007.

[23] P. Kirchner, M. Meier, and E. C. Bottiger, "Genotypic identification and detection of mycobacteria-facing novel and uncultured pathogens," in *Diagnostic Molecular Microbiology: Principles and Applications*, D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White, Eds., pp. 173–190, American Society for Microbiology, Washington, DC, USA, 1993.

[24] P. H. Vary, P. R. Andersen, E. Green, J. Hermon-Taylor, and J. J. McFadden, "Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease," *Journal of Clinical Microbiology*, vol. 28, no. 5, pp. 933–937, 1990.

[25] T. E. Secott, A. M. Ohme, K. S. Barton, C. C. Wu, and F. A. Rommel, "*Mycobacterium paratuberculosis* detection in bovine feces is improved by coupling agar culture enrichment to an IS900-specific polymerase chain reaction assay," *Journal of Veterinary Diagnostic Investigation*, vol. 11, no. 5, pp. 441–447, 1999.

[26] C. C. Wu, T. E. Secott, T. E. J. Stewart, and T. L. Lin, "Identification and subspeciation of *Mycobacterium avium* isolates by PCR amplification of a fibronectin-attachment protein gene element," in *Proceedings of the 8th International Colloquium on Paratuberculosis*, pp. 438–442, 2005.

[27] S. J. Wells, R. H. Whitlock, C. J. Lindeman, and T. Fyock, "Evaluation of bacteriologic culture of pooled fecal samples for detection of *Mycobacterium paratuberculosis*," *American Journal of Veterinary Research*, vol. 63, no. 8, pp. 1207–1211, 2002.

[28] J. V. Gagliardi and J. S. Karns, "Leaching of Escherichia coli O157:H7 in diverse soils under various agricultural management practices," *Applied and Environmental Microbiology*, vol. 66, no. 3, pp. 877–883, 2000.

[29] A. Gao, J. Odumeru, M. Raymond, and L. Mutharia, "Development of improved method for isolation of Mycobacterium avium subsp. paratuberculosis from bulk tank milk: effect of age of milk, centrifugation, and decontamination," *Canadian Journal of Veterinary Research*, vol. 69, no. 2, pp. 81–87, 2005.

[30] A. Unc and M. J. Goss, "Transport of bacteria from manure and protection of water resources," *Applied Soil Ecology*, vol. 25, no. 1, pp. 1–8, 2004.

[31] Y. Fang, W. H. Wu, J. L. Pepper et al., "Comparison of real-time, quantitative PCR with molecular beacons to nested PCR and culture methods for detection of Mycobacterium avium subsp. paratuberculosis in bovine fecal samples," *Journal of Clinical Microbiology*, vol. 40, no. 1, pp. 287–291, 2002.

[32] J. R. Stabel and J. P. Bannantine, "Development of a nested PCR method targeting a unique multicopy element, ISMap02, for detection of Mycobacterium avium subsp. paratuberculosis in fecal samples," *Journal of Clinical Microbiology*, vol. 43, no. 9, pp. 4744–4750, 2005.

[33] S. J. Wells, M. T. Collins, K. S. Faaberg et al., "Evaluation of a rapid fecal PCR test for detection of Mycobacterium avium subsp. paratuberculosis in dairy cattle," *Clinical and Vaccine Immunology*, vol. 13, no. 10, pp. 1125–1130, 2006.

[34] C. A. Alinovi, M. P. Ward, T. L. Lin, G. E. Moore, and C. C. Wu, "Real-time PCR, compared to liquid and solid culture media and ELISA, for the detection of Mycobacterium avium ssp. paratuberculosis," *Veterinary Microbiology*, vol. 136, no. 1–2, pp. 177–179, 2009.

[35] R. J. Greenstein, "Is Crohn's disease caused by a mycobacterium? Comparisons with leprosy, tuberculosis, and Johne's disease," *The Lancet Infectious Diseases*, vol. 3, no. 8, pp. 507–514, 2003.