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Detection of *Mycobacterium avium* subsp. *paratuberculosis* in Drinking Water and Biofilms by Quantitative PCR▽†

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It has been suggested that *Mycobacterium avium* subspecies *paratuberculosis* has a role in Crohn's disease. The organism may be acquired but is difficult to culture from the environment. We describe a quantitative PCR (qPCR) method to detect *M. avium* subsp. *paratuberculosis* in drinking water and the results of its application to drinking water and faucet biofilm samples collected in the United States.

Mycobacterium avium subspecies *paratuberculosis* is a member of the *Mycobacterium avium* complex. *M. avium* subsp. *paratuberculosis* causes Johne's disease in bovine and ovine animals and has been hypothetically linked to Crohn's disease in humans. Several review articles have been written describing the association between *M. avium* subsp. *paratuberculosis* and Crohn's disease (1, 2, 10, 11, 16, 23). Most mycobacterial infections are acquired from the environment; however, *M. avium* subsp. *paratuberculosis* can elude laboratory culture from environmental samples (28). *M. avium* subsp. *paratuberculosis* has been cultured only once from drinking water in the United States; therefore, its occurrence in drinking water is unknown (17). There are several reasons one could expect to find *M. avium* subsp. *paratuberculosis* in drinking water. The bacterium has been isolated from surface water used as a source of drinking water (19, 20, 24, 26). It is resistant to chlorine disinfection (25). Also, other subspecies of *M. avium* have been detected in biofilms obtained from drinking water pipes in the United States (8, 22, 27).

Due to the potential for waterborne transmission of mycobacteria and the association of *M. avium* subsp. *paratuberculosis* with human illness, the focus of this study was to estimate the organism's occurrence in drinking water in the United States using quantitative PCR (qPCR) (15). A comprehensive method was developed for detection of *M. avium* subsp. *paratuberculosis* in drinking water and biofilms that includes the concentration of microorganisms from samples using membrane filtration, total DNA extraction and purification, and detection of two targets unique to this bacterium: IS*900* and target 251. IS*900* is a common target used to identify *M. avium* subsp. *paratuberculosis*, and the average number of copies per genome is 14 to 18 (13). Target 251 qPCR analysis, which corresponds to the *M. avium* subsp. *paratuberculosis* gene 2765c (David Alexander, personal communication), was developed by Rajeev et al. (21). Samples positive for both targets are considered positive for *M. avium* subsp. *paratuberculosis*. TaqMan primer and probe sequences and qPCR assay characteristics are described in Table 1. The complete method is described in Fig. S1 in the supplemental material.

A master standard curve was generated from six series of 10-fold dilutions of genomic DNA from *M. avium* subsp. *paratuberculosis* strain 49164 for quantification of IS*900* target copies (see Fig. S2A in the supplemental material). Each dilution series contained eight standards run in triplicate for a total of 18 threshold cycle (C_T) measurements per standard. A linear regression was performed on C_T versus log IS*900* copy number and R^2 was 0.997. The standard error of y was used to create two equations to estimate the upper and lower concentration, or range, of *M. avium* subsp. *paratuberculosis* IS*900* copy number.

The specificities of the IS*900* and target 251 primer/probe sets were evaluated by Rajeev et al. (21) on 211 *M. avium* subsp. *paratuberculosis* and 38 non-*M. avium* subsp. *paratuberculosis* isolates, and each assay was 100% specific for *M. avium* subsp. *paratuberculosis*. We further evaluated specificity using 22 *M. avium* subsp. *paratuberculosis* isolates from animals and 10 non-*M. avium* subsp. *paratuberculosis* ATCC reference strains (see Table S1 in the supplemental material) (18). Target 251 was 100% specific; however, one *M. avium* subsp. *paratuberculosis* isolate (3063) repeatedly produced a negative result by IS*900* qPCR. Results suggest that a small subset of *M. avium* subsp. *paratuberculosis* isolates may not contain the IS*900* element or may have a sequence that differs from that of the IS*900* primer/probe set.

The sensitivity of the method for detection of *M. avium* subsp. *paratuberculosis* in different drinking water matrices was evaluated by spiking serial dilutions of strain 1112 cells, ranging from 10^4 cells to no addition of cells, into 1-liter tap water samples obtained from five locations in the United States. The number of *M. avium* subsp. *paratuberculosis* cell equivalents was estimated by dividing the IS*900* copy number obtained from the master standard curve by 18 (mean, 18 IS*900* copies/*M. avium* subsp. *paratuberculosis* genome). The method provided consistent detection (5/5 samples) in a spiked sample of 100 cells/liter. In a spiked sample of 10 cells/liter, the IS*900* target was detected 40% (2/5 samples) of the time, and at 1

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TABLE 1. qPCR assay primers, probes, DNA targets, and assay characteristics[a]

DNA target	Primer or probe (sequence, 5′→3′)	Product (bp)	LOD[b]	LOQ[c]	Reference
IS*900*	IS*900*F (CCGCTAATTGAGAGATGCGATTGG) IS*900*R (ATTCAACTCCAGCAGCGCGGCCTC) IS*900*P (6-FAM-TCCACGCCCGCCCAGACAGG-TAMRA)	230	1.8	1.8	13
Target 251	251F (GCAAGACGTTCATGGGAACT) 251R (GCGTAACTCAGCGAACAACA) 251P (6-FAM-CTGACTTCACGATGCGGTTCTTC-TAMRA)	200	ND	ND	21

[a] FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; ND, not determined.
[b] The limit of detection (LOD) of the IS*900* qPCR assay was defined as the lowest copy number resulting in a C_T of <40, determined from six independent dilution series.
[c] The limit of quantification (LOQ) was defined as the lowest copy number per assay yielding a coefficient of variation (CV) of less than 25% (33).

cell/liter we did not detect the target in any spiked sample. Percent recovery was variable and decreased as the number of spiked cells decreased (Fig. 1). At a spike level of 1×10^4 cells/liter, the average percent recovery was 64%; this decreased to 9.2% at 1×10^2 cells/liter. Cell surface hydrophobicity, a property of mycobacteria, may have influenced clumping of the spiked sample or partitioning of *M. avium* subsp. *paratuberculosis* onto the sample bottle or filtration unit, affecting recovery of the bacterium (3).

Midwest and temporal study. Two liters of cold drinking water and a biofilm sample were collected from 33 homes or commercial buildings in two metropolitan areas in the Midwest from May to November 2007. The first liter, or "first-pull" sample, was collected immediately upon turning on the tap. The second liter, or "standard methods" sample, was collected according to sections 9060A and B of *Standard Methods for the Examination of Water and Wastewater* (7). Biofilm samples were collected by swabbing the surface of the faucet grating at the end of the tap with a sterile cotton swab. The swab was broken off in a tube containing 10 ml sterile molecular biology-grade water and transported back to the lab. Samples were analyzed by the method described in Fig. S1 in the supplementary material. No template controls and standards were included with every set of qPCR analyses. Method controls were prepared by filtering sterile molecular biology-grade water and processed with every set and on the same day as that for water and biofilm samples.

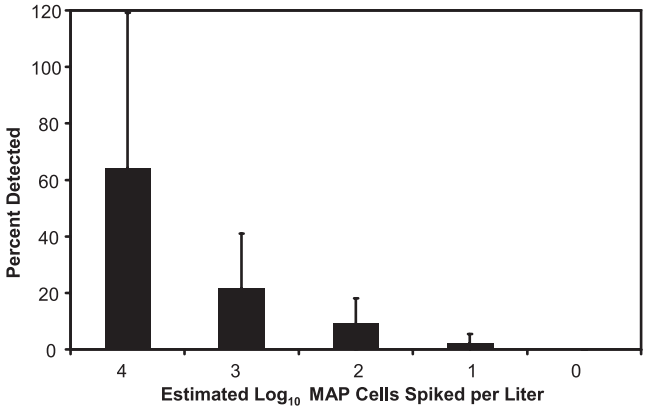

FIG. 1. Average percent recovery of *M. avium* subsp. *paratuberculosis* spiked into drinking water collected from five sites in the United States. Error bars denote standard deviation. MAP, *M. avium* subsp. *paratuberculosis*.

Eighty-one percent of first-pull water samples, 88% of standard methods samples, and 76% of the biofilm samples were positive for both IS*900* and target 251 in the qPCR assays (n = 33). IS*900* copy concentrations varied widely among sites (see Table S2 in the supplemental material). IS*900* copy concentrations in first-pull and standard methods samples from the same site were not significantly different ($\alpha = 0.05$; $P > 0.095$). Additionally, 82% of IS*900*-positive samples contained <100 target copies per liter of water or biofilm sampled, and 68% contained <500 target copies/liter. These results are similar to what Hilborn et al. (12) found in a geographically localized survey of home taps in the Pacific Northwest, where greater than 50% of water samples were positive for *M. avium*. Five *M. avium* subsp. *paratuberculosis*-positive biofilm samples had negative results for water samples. Conversely, three *M. avium* subsp. *paratuberculosis*-positive water samples had negative results for biofilm samples. Little is currently known about the ability of *M. avium* subsp. *paratuberculosis* to survive or grow in drinking water biofilms (9).

In order to determine if *M. avium* subsp. *paratuberculosis* persists in drinking water, 1 liter of water was collected from two homes in the Midwest once a month for 4 months (January to April 2008). The homes were positive for *M. avium* subsp. *paratuberculosis* in 2007. *M. avium* subsp. *paratuberculosis* was consistently detected at both taps, though the quantity was highly variable during the period sampled (Fig. 2). Repeated detection at drinking water taps supports the findings of Hilborn et al. (12), who isolated the same *M. avium* genotypes from three taps during a 2-year period.

National study. Drinking water samples (n = 238) were collected from January to December 2009 from taps in homes or commercial buildings at 41 sites in 25 states, one district, and one U.S. territory. A spectrum of source waters (surface and ground water), system sizes (large and small utilities, private wells), and disinfection types (chlorine, chloramine, and chlorine dioxide) was included. Twenty-nine samples were analyzed using TaqMan universal master mix, and 209 samples were analyzed using TaqMan environmental master mix 2.0. All reaction mixtures included the TaqMan exogenous internal positive-control reagent to detect PCR inhibition. A standard curve using the new reagents was generated (see Fig. S2B in the supplemental material) and was not significantly different

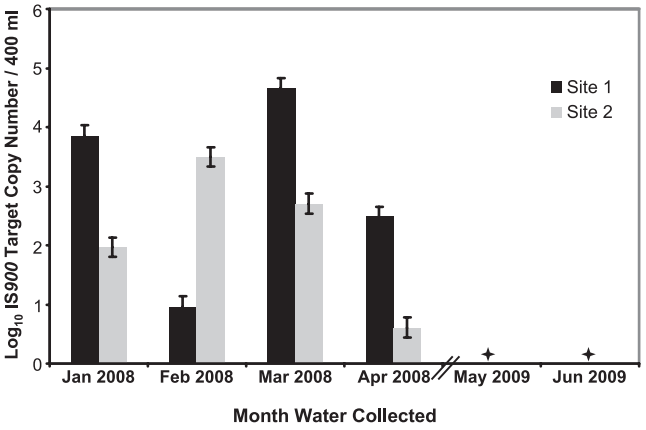

FIG. 2. Results of the temporal study showing the change in $\log_{10}$ estimated *M. avium* subsp. *paratuberculosis* IS*900* target copy number over time between sites. Error bars denote the range between high and low estimates. ✦, negative detection for IS*900* on those dates; Jan, January; Feb, February; Mar, March; Apr, April; Jun, June.

from the curve obtained with the original reagents ($\alpha = 0.05$; $P > 0.31$).

In contrast to the Midwest study, no samples in the national study were positive for *M. avium* subsp. *paratuberculosis*, though five were positive for IS*900* but not the target 251 qPCR assay. Additionally, five Midwestern sites positive for *M. avium* subsp. *paratuberculosis* in 2007 were negative for that bacterium in 2009. Such disparate findings between the two studies were unexpected because a large number of Midwest samples were positive for *M. avium* subsp. *paratuberculosis* in the Midwest. In a similar survey of the United States, Covert et al. (5) measured the occurrence of nontuberculous mycobacteria (NTM) in drinking water from geographically dispersed sites. In that study, only one sample (<1%) was positive for *M. avium*. The studies of Hilborn et al. (12) and Covert et al. (5) demonstrate the variability of detecting *M. avium* in a localized versus national survey of drinking water. The cause of the variability observed in these studies is unknown, though it is important to note that the Midwest experienced a severe drought in 2007 but not in 2009, as reported by the National Climatic Data Center. Furthermore, the area sampled in the Midwest was in the top 10th percentile for temperatures in August 2007 (http://www.ncdc.noaa.gov/oa/climate/research/2007/ann/drought-summary.html#regdrot). Only one of five IS*900*-positive samples from the national study was from a state experiencing a drought in 2009. Additional abiotic and biotic factors which could influence the occurrence of *M. avium* subsp. *paratuberculosis* in drinking water include water temperature, the effect of drinking water disinfection, and the source of drinking water and its proximity to *M. avium* subsp. *paratuberculosis*-infected herds. Further research is needed to understand the geographical and temporal differences in the occurrence of this bacterium, as has been described for other NTM (4, 8, 14). This is the first report on the occurrence of *M. avium* subsp. *paratuberculosis* in drinking water in the United States using a molecular method that may prove useful for understanding the ecology and epidemiology of the organism.

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Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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