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# Quantification of Bacterial Indicators and Zoonotic Pathogens in Dairy Wastewater Ponds

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**Zoonotic pathogens in land-applied dairy wastewaters are a potential health risk. The occurrence and abundance of 10 pathogens and 3 fecal indicators were determined by quantitative real-time PCR (qPCR) in samples from 30 dairy wastewaters from southern Idaho. Samples tested positive for *Campylobacter jejuni*, $stx_1$- and *eaeA*-positive *Escherichia coli*, *Listeria monocytogenes*, *Mycobacterium avium* subsp. *paratuberculosis*, and *Salmonella enterica*, with mean recoveries of genomic DNA corresponding to $10^2$ to $10^4$ cells $ml^{-1}$ wastewater. The most predominant organisms were *C. jejuni* and *M. avium*, being detected in samples from up to 21 and 29 of 30 wastewater ponds, respectively. The qPCR detection limits for the putative pathogens in the wastewaters ranged from 16 cells $ml^{-1}$ for *M. avium* to 1,689 oocysts $ml^{-1}$ for *Cryptosporidium*. *Cryptosporidium* and *Giardia* spp., *Yersinia pseudotuberculosis*, and pathogenic *Leptospira* spp. were not detected by qPCR.**

In the United States, there are 9.3 million milk cows (33) producing an estimated 200 million metric tons of manure annually. Because cattle can be reservoirs of zoonotic pathogens, there is concern over the contamination of soil, water, air, and crops when the manure solids and liquids are land applied (6, 9, 44). At dairies, the solids are often removed from the manure slurries and the liquid fraction (i.e., urine, wash water) is then sent to a pond for storage or can be anaerobically digested first to produce biogas (43). During the crop-growing season, the pond wastewater is diluted with irrigation water and land applied through pressurized irrigation systems to improve the soil nutrient status. It is during spray irrigation that zoonotic pathogens could be aerosolized, increasing the risk of exposure to downwind receptors via inhalation or ingestion after deposition on fomites or food crops (8, 23). Once in the soil after manure addition, pathogens can be internalized by plants (49) and also reach recreational waters by overland flow transport during rainfall events (36, 53, 54), causing significant contamination.

Zoonotic pathogens of potential interest in cattle are *Campylobacter jejuni*, *Escherichia coli*, *Leptospira* spp., *Listeria monocytogenes*, *Mycobacterium avium* subsp. *paratuberculosis*, *Salmonella* spp., *Yersinia* spp., *Giardia lamblia*, and *Cryptosporidium parvum* (38, 39, 40). While numerous studies have measured the occurrence of these zoonotic pathogens in cattle manures and assessed their fate and transport in the environment (41, 48, 55), to our knowledge, no comprehensive studies have been conducted to quantify pathogens in dairy wastewaters. In addition, only a few studies to date have quantified a wide range of pathogens within cattle manures (20, 21, 26). Understanding the number of pathogens in any land-applied waste is particularly important when developing a quantitative microbial risk assessment (12, 17, 56). Estimation of the risk represented by pathogens in animal manures, however, has largely been based on the cultivation and enumeration of fecal indicator organisms (2, 10). It has been recently shown that molecular methods can be successfully applied with such difficult-to-analyze materials to complement culture-dependent approaches (26, 27).

In this study, we attempted to use quantitative real-time PCR (qPCR) to enumerate fecal indicator organisms and putative zoonotic pathogens in wastewaters from dairies in southern Idaho. qPCR is often used as a convenient alternative to culture-dependent techniques that has the added advantage of being able to detect viable but nonculturable (VBNC) cells of potential pathogens (7, 27, 59). While there are documented advantages and disadvantages with both molecular method- and culture-dependent approaches, it was our intent to provide the first quantitative survey of selected bacterial indicators and putative pathogens in dairy wastewaters. The bacterial indicators (enterococci, total coliforms, *E. coli*, and *Clostridium perfringens*) were also quantified using culture-dependent methodologies to gauge the effectiveness of qPCR as an enumeration technique for use with dairy wastewaters.

## MATERIALS AND METHODS

**Dairy operations and sample collection.** Wastewater samples were collected from 30 storage ponds at dairy operations of various levels of stocking density located in southern Idaho. Eight 500-ml near-surface samples were collected from the perimeter of each storage pond and then composited in a sterile 4-liter Nalgene container. The composite samples were transferred to our laboratory in coolers with ice packs and immediately stored in the dark under conditions of refrigeration at 5°C upon receipt. All samples were processed within 24 h of collection. Three sets of samples were collected from the ponds during the summer and fall of 2011.

**Culture-dependent quantification of fecal indicators.** Prior to cultivation as described below, 10-fold serial dilutions of thoroughly mixed wastewater samples were prepared in room temperature phosphate-buffered saline (PBS). The most probable numbers (MPNs) for *Escherichia coli* (and total coliforms) and enterococci were determined using Colilert-18 and Enterolert detection kits, respectively, in conjunction with a Quanti-

Received 8 August 2012 Accepted 6 September 2012
Published ahead of print 14 September 2012
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Supplemental material for this article may be found at http://aem.asm.org/.

doi:10.1128/AEM.02470-12
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**TABLE 1** Target genes, qPCR assays, and modified thermocycler conditions

| Target organism | Target gene | No. of targets per genome | Primers | Primer annealing temp (°C); extension time (s) | Reference(s)[a] |
|---|---|---|---|---|---|
| *Campylobacter jejuni* | VS1 | 1 | VS15-F, VS16-R | 55; 30 | 58 |
| *Clostridium perfringens* | Alpha toxin | 1 | cpa-F, cpa-R | 55; 30 | 15 |
| *Enterococcus faecalis* | 23S rRNA | 2 | ECF, ECR | 60; 30 | 18 |
| *Escherichia coli* | Glucuronidase | 1 | Eco-F, Eco-R | 60; 30 | 46 |
| Enterohemorrhagic *E. coli* | Shiga toxin | 1 | stx1-F, stx1-R | 55; 30 | 47 |
| | Shiga toxin | 1 | stx2-F, stx2-R | 55; 30 | 47 |
| | Intimin | 1 | eae-F, eae-R | 55; 30 | 47 |
| *Leptospira* spp. | Lipoprotein, *lipL32* | 1 | 45F, 286R | 60; 30 | 51 |
| *Listeria monocytogenes* | Listeriolysin O | 1 | F, R | 60; 30 | 35 |
| *Mycobacterium avium* subsp. *paratuberculosis* | IS*900* | 20 | F2, R2 | 65; 30 | 5, 25 |
| *Salmonella enterica* | Invasin | 1 | 139-F, 141-R | 65; 30 | 19 |
| *Yersinia pseudotuberculosis* | Invasin | 1 | inv-F, inv-R | 60; 30 | 52 |
| *Cryptosporidium* spp. | COWP[b] | 4 | P702-F, P702-R | 60; 30 | 16 |
| *Giardia* spp. | β-Giardin | 16 | P241-F, P241-R | 60; 30 | 16 |

[a] Probe sequences from the associated references were used for the PCRs, except SYBR green was used for *Y. pseudotuberculosis*.
[b] COWP, *Cryptosporidium* oocyst wall protein gene.

Tray 2000 (IDEXX Laboratories, Inc., Westbrook, ME). Vegetative cells and spores of *C. perfringens* were assayed using membrane filtration and mCP media (Neogen, Lansing, MI) with mCP selective Supplement I (Sigma-Aldrich, St. Louis, MO) as described by Armon and Payment (1). The mCP plates were anaerobically incubated at 44.5°C for 1 day, with exposure to ammonia hydroxide vapors afterward to quantify presumptive *C. perfringens* colonies. Use of negative controls was implemented, while positive controls consisted of *E. coli* (ATCC 13706), *Enterococcus faecalis* (ATCC 700802), and *C. perfringens* (ATCC 13124). All trays and plates were manually counted, and their numbers were reported as MPN and CFU per milliliter of wastewater, respectively.

**Isolation of microbial DNA from the wastewater.** Three aliquots were removed from each 4-liter container of wastewater after thorough mixing. Depending on the solids content, aliquots ranging in volume from 5 to 70 ml were dispensed into 85-ml Oak Ridge tubes (Nalge Nunc International, Rochester, NY) and brought up to volume with cold PBS. After centrifugation at 10,000 × *g* for 10 min, the supernatant was discarded and the remaining pellet was washed twice with PBS. The pellet was then resuspended with 600 μl of PBS and then transferred to a bead-beating tube from a FastDNA Spin kit for feces (MP Biomedicals LLC, Solon, OH) and processed using a FastPrep FP120 instrument at a speed setting of 6 m $s^{-1}$ for 45 s according to the manufacturer's recommendations. However, all wash and centrifugation steps were carried out twice to further reduce possible humic acid contamination. The DNA was eluted during the final step with 100 μl of *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer and then stored at −20°C until analysis by qPCR.

**Quantitative PCR.** qPCR was performed with a Bio-Rad (Hercules, CA) iQ5 multicolor real-time PCR detection system using dually labeled Black Hole Quencher (BHQ) probes or iQ SYBR green Supermix (Bio-Rad). The probes were manufactured by Biosearch Technologies (Novato, CA), with FAM (6-carboxyfluorescein) and BHQ-1 as the fluorophore and quencher, respectively. Organisms enumerated in the wastewater samples are listed in Table 1, along with their target genes, individual cycling conditions, and references for primers and probes used. Quantification standards were prepared from pure cultures of *C. jejuni* (ATCC BAA-1062), *C. perfringens* (ATCC 13124), *E. faecalis* (ATCC 700802), *E. coli* O157:H7 strain 3032 (courtesy of Tom Casey, USDA-ARS), *Leptospira interrogans* serovar Copenhageni (ATCC BAA-1198), *L. monocytogenes* (ATCC BAA-679), *M. avium* subsp. *paratuberculosis* (ATCC BAA-968), *Salmonella enterica* serovar Typhimurium (courtesy of Pina Frantamico, USDA-ARS), and *Yersinia pseudotuberculosis* (ATCC 29833). Genomic DNA was extracted from the cultures using a PowerMicrobial Maxi DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA), while DNA for *C. parvum* (PRA-67D) and *Giardia intestinalis* (30888D) was directly purchased from the American Type Culture Collection (Manassas, VA).

One microliter of nondiluted DNA template was used in a PCR with 0.3 μM (each) primer, 0.1 μM probe, 12.5 μl of iQ Supermix (Bio-Rad), and molecular analysis-grade water to achieve a final volume of 25 μl. SYBR green was used in lieu of probe only for the detection of *Y. pseudotuberculosis*. After an initial denaturation step (95°C for 180 s), 40 cycles of 94°C for 20 s followed by an extension time of 30 s at primer-annealing temperatures were performed as indicated in Table 1. The number of putative pathogens in the samples was calculated using the DNA concentration of the standards, genome molecular weight, and target gene frequency (26).

**Pathogen recovery and detection limits.** To determine the performance of the DNA extraction technique and qPCR to detect pathogens in the dairy wastewaters, one wastewater sample was individually spiked with *C. jejuni*, enterohemorrhagic *E. coli*, *L. monocytogenes*, *M. avium* subsp. *paratuberculosis*, *S. enterica* serovar Typhimurium, and *Y. pseudotuberculosis*. The wastewater chosen had a pH of 7.5 and total solids of 6.7 g $liter^{-1}$. In brief, an overnight culture was serially diluted in sterile PBS and then 1 ml of the appropriate dilution was dispensed into triplicate 9-ml aliquots of wastewater. The titer of the inoculum was determined by plate counting after growth of the organisms on appropriate media. For each pathogen, a total of 4 to 5 spiked dilutions were prepared, with final wastewater titer levels ranging from $\log_{10}$ 1.5 to 8.5. After spiking, the wastewater samples were processed in a vortex apparatus for 30 s and then a 5-ml aliquot was immediately processed as previously described for the DNA isolation.

For *Giardia* and *Cryptosporidium* spp., the same spiking and DNA isolation procedures were utilized as described above, except that the cysts and oocysts were spiked into two different wastewaters to determine a possible influence of levels of solids on qPCR performance. The low and high total solids contents of the wastewaters were 3.6 g $liter^{-1}$ and 12.7 g $liter^{-1}$, respectively. *Giardia lamblia* and *C. parvum* at a titer of $10^7$ cysts/oocysts $ml^{-1}$ were purchased from Waterborne, Inc. (New Orleans, LA).

The percent recovery was determined for each pathogen by dividing the qPCR result by the calculated wastewater titer level, which was based on the organism density of each spiking dilution. Detection limits were determined via a linear regression analysis of the wastewater titer levels versus the qPCR cycle threshold ($C_T$) values, where $r^2 > 0.96$. The minimum detection limits were determined at a $C_T$ of no greater than 35, a value at which detection errors may become significant due to pipetting errors or potential traces of cross-contamination.

**Statistical analysis.** Our *a priori* hypothesis was that there would be no statistical difference between the titers as determined by the culture-dependent techniques and qPCR for the indicator organisms. The two-sample paired *t* tests for the means were performed on log-transformed data using SAS statistical software version 9.2 (45). Statements of statistical significance were based on an α value of 0.05.

## RESULTS AND DISCUSSION

**Dairy wastewater samples and chemical properties.** Thirty freestall and open-lot dairies with <1,000 to 10,000 milk cows were targeted for this study. Most of the dairies were stocked with Holstein cows; however, in some cases, they were stocked with a combination of Holstein and Jersey cows. The freestall dairies used either a flush or a vacuum system to remove the manure from the alleys, while the open-lot dairies used a scrape system. The flushed or vacuumed manure is then commonly transferred to solid separation cells, followed, in some cases, by processing with a separator to reduce the solids content prior to discharge to the wastewater storage pond.

During the months of August and October, the pH of the wastewaters ranged from 6.6 to 8.8, with a median value of 7.7 (see Table S1 in the supplemental material). The total solids content ranged from a very low value of 0.28 g liter$^{-1}$ to as high as 57.3 g liter$^{-1}$, with a median value of 8.2 g liter$^{-1}$. The total Kjeldahl nitrogen value ranged from 0.001 to 1.5 g liter$^{-1}$, with a median value of 0.24 g liter$^{-1}$. The low values, which are much lower than anticipated for dairy wastewaters, were related to the fact that a few of the wastewater ponds were flooded with irrigation water. This practice was implemented at a few of the dairies as a means to blend the wastewaters prior to land application via pressurized spray irrigation. Because at some point more irrigation water than wastewater enters the pond, the pond takes on the chemical characteristics of the irrigation water.

**Culture-dependent quantification of fecal indicators.** The fecal indicator organisms were quantified in the dairy wastewaters using a MPN (enterococci, total coliforms, and *E. coli*) or plate-counting (*C. perfringens*) technique (see Table S2 in the supplemental material). Median titer values for enterococci and *C. perfringens* were approximately $10^2$ MPN or CFU ml$^{-1}$, respectively, throughout the sampling period. Total coliform median values were 1 to 2 orders of magnitude greater, with a maximum titer of $10^7$ MPN ml$^{-1}$ occurring in August. As expected, the titer of generic *E. coli* was lower than that of total coliforms in all pond samples (data not shown). On average, the organism titer levels were greater in October than in June and August, except for the titers of total coliforms, which were greatest in August. In the United Kingdom, Hutchison and coworkers (21) found that *E. coli* O157, *Campylobacter* spp., and *Listeria* spp. were more prevalent in fresh cattle feces during the spring months and December. Additional studies have confirmed that the prevalences and levels of various zoonotic agents within fresh cattle manures were affected in a season-dependent manner (3, 50). While too few seasonal data points were collected for a statistical analysis, our results suggest that seasonal factors such as temperature or solar irradiation could have influenced the levels of fecal indicators in the dairy wastewaters. It should be noted that changes in the levels of fecal indicators in the wastewater ponds may not be seasonal in nature and could be related to the manure aging process or nutrient status of the pond. In general, it has been reported that levels of bacterial indicators and zoonotic agents in stored livestock wastes decline over time (22, 28, 34, 57). However, regrowth of indicator bacteria to some extent, due to the nutrient-rich conditions, has also been reported (13, 28).

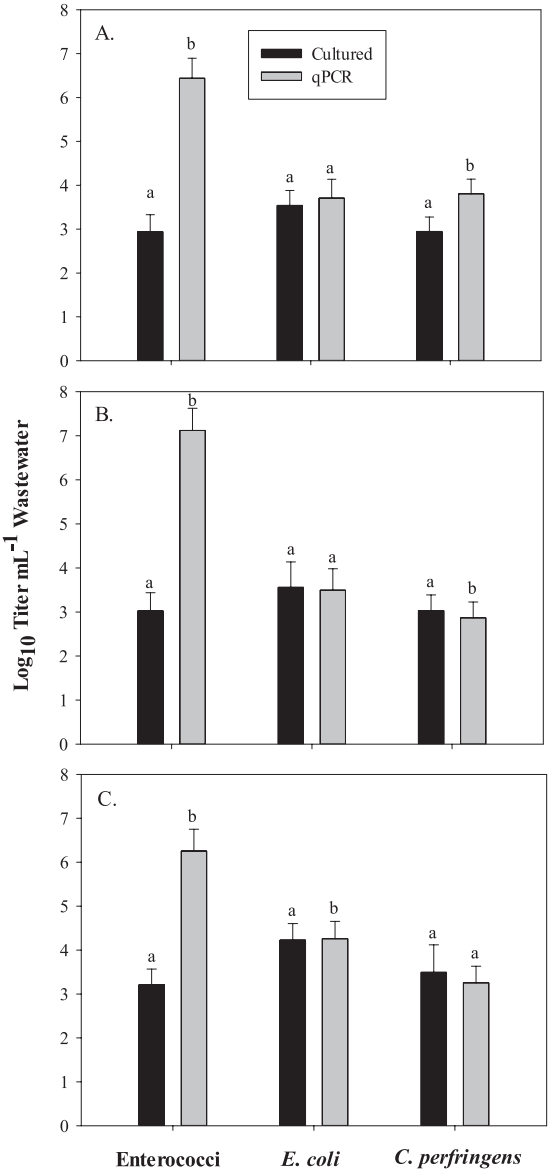


**FIG 1** Comparison of culture-dependent and qPCR estimates of numbers of enterococci, *E. coli*, and *C. perfringens* in the dairy wastewaters from (A) June, (B) August, and (C) October. Quantitative PCR was based on positive amplification of target genes using EC, Eco, and cpa primers for enterococci, *E. coli*, and *C. perfringens*, respectively. Columns represent means ± standard deviations ($n = 30$). Columns with different letters (a or b) indicate a significant difference between the two quantification techniques ($P < 0.05$).

**Quantification of fecal indicators by qPCR.** In addition to quantification by culture-dependent techniques, qPCR of bacterial DNA was used to calculate the titers of enterococci, *E. coli*, and *C. perfringens* in the dairy wastewaters. The qPCR results are presented in Fig. 1 along with the MPN and CFU values for comparison. During each of the 3 months in which samples were collected, the qPCR values for enterococci corresponded to $10^6$ to $10^7$ cells ml$^{-1}$ wastewater. The qPCR method was specifically designed to detect *E. faecalis*, which is one of the most common enterococcus species found in the gastrointes-

**TABLE 2** Recovery and detection limits for bacterial pathogens in a dairy wastewater determined by qPCR after spiking

| Target organism | Wastewater titer range after spiking ($log_{10}$ CFU $ml^{-1}$ wastewater) | Range of recovery values (%) | Mean recovery (%)[a] | qPCR detection limit (no. of cells $ml^{-1}$) |
|---|---|---|---|---|
| *C. jejuni* | 3.4–8.4 | 41–836 | 326 | 768 |
| *E. coli* | | | | |
| *stx1* | 3.2–8.2 | 38–120 | 72 | 1,229 |
| *stx2* | 3.2–8.2 | 54–238 | 121 | 232 |
| *eaeA* | 2.2–8.2 | 41–117 | 75 | 140 |
| *L. monocytogenes* | 3.5–8.5 | 45–127 | 81 | 410 |
| *M. avium* subsp. *paratuberculosis* | 1.5–6.5 | 25–52 | 37 | 16 |
| *S. enterica* | 3.3–8.3 | 13–133 | 56 | 325 |
| *Y. pseudotuberculosis* | 3.2–8.2 | 126–179 | 160 | 234 |

[a] Data represent mean values as determined by spiking triplicate wastewater samples with pathogens at several titer levels.

tinal tract of cattle (14, 32). qPCR values were about 3 logs greater than those determined by the MPN technique (a statistically significant difference; $P < 0.0001$), which indicated that the majority of enterococci might have gone into a VBNC state such as was seen in earlier studies involving bovine feces and manure (28, 30). However, since the DNA could also have been extracted from damaged or dead cells, qPCR analysis may have resulted in overestimation of actual titer values in the samples (7, 31, 37).

In contrast to the results seen with enterococci, the average qPCR and culture-dependent values for both *E. coli* and *C. perfringens* were markedly but not statistically significantly similar in all cases (Fig. 1). In June and August, the qPCR and CFU values for *C. perfringens* were statistically significantly different ($P < 0.0001$), while the October titer values for *E. coli* were also different ($P = 0.02$). During the 3 months in which wastewater samples were collected, the titers of *E. coli* and *C. perfringens* (as determined by qPCR) ranged from about $10^3$ to $10^4$ cells $ml^{-1}$.

**Quantification of zoonotic pathogens by qPCR.** Prior to the enumeration of zoonotic bacterial pathogens in the dairy wastewaters, wastewater samples were spiked to determine the efficiency of the DNA extraction kit and precision of the qPCR technique. On average, recovery levels ranged from 56% for *S. enterica* to as high as 326% for *C. jejuni* (Table 2). Although these recovery levels are presumed to be representative of all wastewaters examined in this study, spiking was not performed on all of the wastewaters due to the logistics of conducting this task. The qPCR detection limits for the bacterial pathogens were determined to range from 16 to 1,229 cells $ml^{-1}$ (Table 2). Due to the presence of multiple copies of the target gene within *M. avium* (i.e., 14 to 20 copies per genome), analysis performed using those sequences is more sensitive than analysis using the other targeted sequences (5), thus explaining the low detection limit of 16 cells $ml^{-1}$.

The putative bacterial pathogens detected in the dairy wastewater by qPCR were *C. jejuni*, *E. coli* ($stx_1$ and *eaeA* positive), *L. monocytogenes*, *M. avium*, and *S. enterica* (Table 3). All of these organisms were detected at least twice in samples from June, but *L. monocytogenes* and *S. enterica* were not detected in the August and October samples. The organisms with the greatest number of positive detections were *C. jejuni* and *M. avium*, with 21 and 29 of 30 ponds showing the presence of those species in samples collected in June, respectively. While the greatest number of detections for all organisms occurred in that first month of analysis, the number of positive pond results generally decreased during each subsequent month. The decrease in the number of detections may have been affected by the pathogen-shedding rate of the cattle, environmental variables (e.g., solar irradiation, temperature), pond characteristics (e.g., solids content), or competition from other indigenous microorganisms leading to a reduction of pathogen

**TABLE 3** Enumeration of pathogens in dairy wastewater samples as determined by qPCR[a]

| Organism | June | | | | August | | | | October | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. of ponds | No. of cells ($ml^{-1}$) | | | No. of ponds | No. of cells ($ml^{-1}$) | | | No. of ponds | No. of cells ($ml^{-1}$) | | |
| | | Min | Max | Mean | | Min | Max | Mean | | Min | Max | Mean |
| *C. jejuni* | 21 | $<7.7 \times 10^2$ | $2.7 \times 10^4$ | $4.8 \times 10^3$ | 17 | $<7.7 \times 10^2$ | $1.8 \times 10^4$ | $2.5 \times 10^3$ | 13 | $<7.7 \times 10^2$ | $2.5 \times 10^4$ | $3.4 \times 10^3$ |
| *E. coli* | | | | | | | | | | | | |
| $stx_1$ | 13 | $<1.2 \times 10^3$ | $6.3 \times 10^3$ | $2.0 \times 10^3$ | 2 | $3.6 \times 10^3$ | $9.9 \times 10^3$ | $6.8 \times 10^3$ | 5 | $<1.2 \times 10^3$ | $2.7 \times 10^3$ | $1.0 \times 10^3$ |
| *eaeA* | 10 | $1.5 \times 10^2$ | $6.3 \times 10^3$ | $2.0 \times 10^3$ | 2 | $2.4 \times 10^3$ | $4.2 \times 10^3$ | $3.3 \times 10^3$ | 1 | $1.4 \times 10^2$ | $1.4 \times 10^2$ | $1.4 \times 10^2$ |
| *L. monocytogenes* | 2 | $1.4 \times 10^3$ | $6.6 \times 10^3$ | $4.0 \times 10^3$ | 0 | $<4.1 \times 10^2$ | | | 0 | $<4.1 \times 10^2$ | | |
| *M. avium* | 29 | $<1.6 \times 10^1$ | $7.1 \times 10^4$ | $2.9 \times 10^3$ | 24 | $<1.6 \times 10^1$ | $1.3 \times 10^3$ | $1.5 \times 10^2$ | 22 | $<1.6 \times 10^1$ | $1.4 \times 10^3$ | $2.2 \times 10^2$ |
| *S. enterica* | 5 | $3.0 \times 10^3$ | $8.3 \times 10^4$ | $2.1 \times 10^4$ | 0 | $<3.2 \times 10^2$ | | | 0 | $<3.2 \times 10^2$ | | |

[a] No sample had detectable *L. interrogans*, *Y. pseudotuberculosis*, *Cryptosporidium* spp., *Giardia* spp., or $stx_2$ gene of *E. coli* O157:H7 during any month. No. of ponds, number of wastewater ponds containing the indicated pathogen ($n = 30$ ponds). Min, minimum; Max, maximum.

**TABLE 4** Recovery of and detection limits for *Giardia* and *Cryptosporidium* spp. in dairy wastewaters as determined by qPCR with low and high total solids

| Target organism | Total solids (g liter$^{-1}$) | Wastewater titer range after spiking ($\log_{10}$ cysts/oocysts ml$^{-1}$ wastewater) | Range of recovery values (%) | Mean recovery (%)[a] | qPCR detection limit ($\log_{10}$ cysts/oocysts ml$^{-1}$) |
|---|---|---|---|---|---|
| *Giardia* spp. | 3.6 | 3–6 | 8–20 | 15 | 130 |
| | 12.7 | | 15–25 | 19 | 182 |
| *Cryptosporidium* spp. | 3.6 | 3–6 | 22–110 | 61 | 1,485 |
| | 12.7 | | 71–426 | 210 | 1,689 |

[a] Data represent mean values as determined by spiking triplicate wastewater samples with pathogens at several titer levels.

survival in the ponds (29, 41, 42). The fact that *Leptospira* spp. were not detected in the ponds could be related to the performance of the DNA extraction, as spiking studies were not conducted with these organisms. However, sufficient recovery levels of these organisms in cattle manure have been reported from studies using similar DNA isolation techniques (26), making it more likely that they were present at levels below the detection limits. *Yersinia pseudotuberculosis* was also not detected, but, based on the spiking results, it can be expected that the titer in the ponds was below the detection limit of 234 cells ml$^{-1}$.

To determine the effect of solids on the recovery and detection of pathogens, wastewaters with low and high total solids contents were spiked with *G. lamblia* cysts and *C. parvum* oocysts (Table 4). Regardless of the solids content, the mean levels of recovery of cysts from the low and high wastewater totals, at 15% and 19%, respectively, were very similar. In contrast, the recovery of oocysts from the high total content of wastewater solids was 210%, which is 3.4 times higher than in the low total content. Although this test was performed with only two pathogens, these results suggest that recovery of organisms might be slightly enhanced in some wastewaters with a higher solids content. Despite our efforts to quantify *Giardia* and *Cryptosporidium* spp. in the dairy wastewaters, no pond samples were found to contain them above their average detection limits of 156 cysts and 1,587 oocysts ml$^{-1}$. Our results, however, do not exclude the possible presence of *Giardia* and *Cryptosporidium* spp. in the wastewaters, since they are generally found at relatively low concentrations in cattle feces (20, 21).

The bacterial pathogen titers were generally consistent, with mean values of between $10^2$ and $10^4$ cells ml$^{-1}$ (Table 3). The organisms detected at the highest titer levels were *M. avium*, *S. enterica*, and *C. jejuni* at $10^4$ cells ml$^{-1}$ in June, while *C. jejuni* was also detected at titer values of up to $10^4$ cells ml$^{-1}$ in August and October. The mean titer value for *L. monocytogenes* and $stx_1$- and *eaeA*-positive *E. coli* was $10^3$ cells ml$^{-1}$, except in October, where the titer for *eaeA*-positive *E. coli* was lower by 1 order of magnitude. The fact that the $stx_1$ and *eaeA* gene markers were detected suggests that some of the wastewaters were presumptive for enterohemorrhagic or enteropathogenic *E. coli*. While most studies do report the presence of virulence genes encoding Shiga toxin 2 in dairy manures (4, 11), the $stx_2$ gene was not detected in the wastewater ponds. Although our qPCR detection limit for $stx_2$ was 232 cells ml$^{-1}$, the lack of detections suggests that our methodology may not have been sensitive enough, as the majority of Shiga toxin-producing *E. coli* isolates from dairy cattle are known to possess both $stx_1$ and $stx_2$ genes (24).

**Concluding remarks.** Based on our results, the oligonucleotide primers and probes used in this study appeared to be suitable for quantitation of zoonotic bacterial pathogens in dairy wastewaters. While some of the key pathogens in the wastewaters were below their qPCR detection limits, this study successfully detected and quantified bacterial pathogens of public health concern at levels similar to those reported in the literature in related environments. In addition, the qPCR and culture-dependent results were in general agreement, further suggesting the suitability of using qPCR as an alternative to laborious culture-dependent techniques when analyzing dairy and (potentially) other livestock wastewaters. Once more, qPCR has turned out to be a reliable method to quantify pathogens in materials that are often difficult to analyze. Despite the fact that some of the values tended to be more conservative due to a possible codetection of irreversibly damaged or dead organisms, this approach allows the detection of putative pathogens in a dormant state which would not be detectable using culture techniques alone.

## ACKNOWLEDGMENTS

We gratefully acknowledge the efforts of Sheryl Verwey in conducting the laboratory analyses. We also thank the anonymous individuals who spent considerable amounts of time reviewing our manuscript and providing many helpful suggestions.

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA.

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