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Comparison of two poultry litter qPCR assays targeting the 16S rRNA gene of *Brevibacterium* sp.

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ABSTRACT

Chicken feces commonly contain human pathogens and are also important sources of fecal pollution in environmental waters. Consequently, methods that can detect chicken fecal pollution are needed in public health and environmental monitoring studies. In this study, we compared a previously developed SYBR green qPCR assay (LA35) to a novel TaqMan qPCR assay (CL) for the environmental detection of poultry-associated fecal pollution. We tested both assays against chicken litter ($n = 40$), chicken fecal samples ($n = 186$), non-chicken fecal sources ($n = 484$), and environmental water samples ($n = 323$). Most chicken litter samples (i.e., ≥98%) were positive for both assays with relatively high signal intensities, whereas only 23% and 12% of poultry fecal samples ($n = 186$) were positive with the LA35 and the CL assays, respectively. Data using fecal samples from non-target animal species showed that the assays are highly host-associated (≥95%). Bayesian statistical models showed that the two assays are associated with relatively low probability of false-positive and false-negative signals in water samples. The CL marker had a lower prevalence than the LA35 assay when tested against environmental water samples (i.e., 21% vs. 31% positive signals). However, by combining the results from the two assays the detection levels increased to 41%, suggesting that using multiple assays can improve the detection of chicken-fecal pollution in environmental waters.

1. Introduction

Poultry litter application is frequently applied to agricultural fields as a source of fertilizer in the United States. As a result, poultry litter is among the most important sources of watershed fecal pollution (ODEQ, 2005; USEPA, 1997; USEPA, 2001). Since poultry feces can include substantial concentrations of fecal bacteria and human pathogens, monitoring assays are needed to detect and quantify poultry fecal sources in environmental waters (McMurry et al., 1998). The primary fecal pollution source of poultry is the waste beddings or litter which is commonly applied in agricultural fields. Poultry litter is known to carry human pathogens and therefore represents a potential human health risks (Hofacre et al., 2000). The detection of fecal sources often relies on the use of PCR-based host-associated molecular assays (Lamendella et al., 2008,

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Table 1 – Summary of oligonucleotide primers and probes for poultry-associated qPCR assays.

Assay	Primer and probe sequences (5′ to 3′)	T_a (°C)[a]	Size (bp)	Reference
LA35-SYBR green	Forward: ACCGGATACGACCATCTGC Reverse: TCCCCAGTGTCAGTCACAGC	60	571	Weidhaas et al. (2010)
CL-TaqMan	CLF: CCCGGGAAACTGGGTCTAAT CLR: CCATCCCCAATCGAAAAACTT CLP[b]: 6FAM- CCGGATACGACCATCTGCCGCA -TAMRA	60	78	This study

[a] Optimum PCR annealing temperatures were determined using temperature gradients.
[b] FAM, 6-carboxyfluorescein, fluorescence reporter dye; TAMRA, 6-carboxytetramethylrhodamine, fluorescence quencher dye.

2009; Santo Domingo et al., 2007). Several assays directed toward poultry feces based on cryptic metagenomic sequences have been developed and have shown high host-specificity and host distribution (Lu et al., 2007). However, the detection of some metagenome-based markers in environmental waters has been sporadic and in some cases their detection is associated with the proximity of pasturing animals to streams (Lee et al., 2008) suggesting that the targeted bacterial populations do not survive long enough to be useful in environmental monitoring studies.

As the bacterial community structure of chicken feces and soiled chicken litter is different (Lu et al., 2003a,b) and manured litter application is a frequent agricultural practice, the likelihood of detecting chicken fecal bacteria in waterways is greater if methods are developed using litter rather than feces as the starting material. Using this rationale Weidhaas et al. (2010) recently developed a SYBR green-based, qPCR poultry-associated assay (i.e., LA35) targeting the 16S rRNA gene of *Brevibacterium* sp. Members of this genus have been found to persist and to be among the most abundant taxonomic groups in poultry litter (Dumas et al., 2011), while some of the abundant bacteria species in chicken feces are likely to rapidly die-off during the process of bedding deposition and land application. The LA35 assay was found to be host specific (i.e., 6.9% false positive against non-target hosts) and to exhibit high prevalence in chicken litter (100%) and chicken fecal samples (60%). More importantly, the LA35 marker was detected in all of tested water samples collected adjacent to chicken litter application areas. However, this study was conducted with only a limited number of water samples ($n = 10$) collected from run-off proximal to litter application areas. A more recent study by Weidhaas et al. (2011) in which the fate and transport of the LA35 marker was investigated showed low prevalence of the latter marker in environmental waters (i.e., <40% positive).

In this study, we further evaluated the LA35 assay against fecal samples from a wider variety of animals to test for potential cross-amplification (i.e., specificity), and more chicken litter and fecal samples to test for host-prevalence and marker relative abundance. A novel TaqMan-based qPCR assay (CL) was also developed and tested using the 16S rRNA gene sequences of *Brevibacterium* sp. obtained from chicken litter samples. The environmental monitoring value of these assays was tested by investigating the prevalence of these markers in poultry and non-poultry impacted water samples collected in Georgia, Delaware, and Ontario (Canada).

2. Materials and methods

2.1. Sample collection and DNA extraction

Chicken litter ($n = 40$) and poultry fecal ($n = 186$) samples were collected from Delaware, Georgia, Ohio, and Puerto Rico. Poultry litter was collected from barns in which chickens were being raised for meat production. The litter had been exposed to chickens for at least two weeks prior to sample collection. Litter from the surface scooped aseptically into sterile polypropylene screw-capped tubes, which were tightly closed and placed immediately on ice. Litter samples were shipped to the laboratory on ice by an overnight carrier. Approximately 0.2 g of dry-weight fecal and litter samples were used for DNA extraction. Additionally, fecal samples from a variety of host animals other than poultry (i.e., 213 samples from eight domesticated animals and sewage; 271 samples from 12 different avian species) were used in host specificity studies (Table 2). The assays were tested against environmental water samples with a history of chicken fecal contamination ($n = 293$) and by wildlife fecal sources other than poultry ($n = 30$). The first group of water samples consisted of potentially chicken impacted water samples ($n = 120$) collected biweekly in 2007 from four sites in one of the most intensive poultry watershed areas in Canada (Sumas watershed, British Columbia) (Smith et al., 2007). Other water samples ($n = 113$) were collected from three headwater streams (AH, DH and DF) located near Watkinsville, Georgia. AH and DH were associated with cattle pastures close to chicken houses and fertilized with broiler litter, while DF was located close to a hay field fertilized with composted chicken litter and no cattle was present. Besides chicken and cattle, other possible fecal sources affecting these streams and ponds were goat, horses and wildlife such as deer, geese, and raccoon. All streams presumably have no major human fecal impact. Water samples were collected weekly for the first month of monitoring and later sampled on a monthly basis between April 2006 and June 2007. Water samples collected were processed as previously reported (Lee et al., 2008). All samples were kept on ice after collection, transferred to the laboratory and processed within 6 h of collection. Another set of samples were collected from natural waterways adjacent to chicken farms where litter was land applied (DE) ($n = 6$). The fourth group of samples consisted of runoff collected from a pilot-scale rain simulation plots fertilized with non-composted chicken litter ($n = 54$). A detailed description of the simulated runoff