Quantification of *Escherichia coli* O157:H7 in soils using an inhibitor-resistant NanoGene assay

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ABSTRACT

Humic acids are ubiquitous and abundant in terrestrial environments; therefore, they are often co-extracted with nucleic acids and interfere with quantitative PCR (qPCR) assays. In this study a recently developed NanoGene assay that is resistant to interference by humic acids was evaluated for gene detection in soil samples. The NanoGene assay utilizes a combination of magnetic beads, dual quantum dots labels, and DNA hybridization in solution. Seven soil samples containing different amounts of organic matter were tested to compare NanoGene and qPCR assays for their respective ability to detect a bacterial pathogen. We spiked the soils with *Escherichia coli* O157:H7, extracted genomic DNA, and conducted NanoGene and qPCR assays targeting the *E. coli* O157:H7-specific *eaeA* gene. To prevent the inhibition of PCR that is common when using DNA extracted from soils, we used a range of template DNA concentrations and BSA addition in the qPCR assay. Compared to the qPCR assay the NanoGene assay was significantly more resistant to the inhibitory effect of humic acids, successfully quantifying the *eaeA* gene within a linear ($R^2 = 0.99$) range of 10^5 through 10^8 CFU/g soil for all seven soil samples tested. In contrast, the qPCR assay was significantly inhibited using the same template DNA isolated from soils containing a range of organic content (2.0%–12%). Interestingly, the qPCR assay was still inhibited despite additional purification steps, suggesting that humic acids were still associated with DNA at a level that was inhibitory to qPCR. This study demonstrated that the NanoGene assay is suitable for quantitative gene detection in diverse soil types and is not susceptible to inhibition by humic acids and other organic compounds that commonly lead to false negative results in qPCR assays.

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1. Introduction

Escherichia coli O157:H7 is a bacterium commonly found in the intestinal tract of warm-blooded animals and is a significant food-borne pathogen. It caused 8598 cases of infection in the United States from 1982 to 2002 (Rangel et al., 2005). More recent reports have attributed 205 cases of illness and 3 deaths due to *E. coli* O157:H7 outbreak in spinach in 2006 (Jay et al., 2007; Grant et al., 2008) and 77 patients were reported ill during the outbreak in cookie dough in 2009 (Neil et al., 2012). One possible route of contamination is via healthy cattle (Grauke et al., 2002), from which bacteria are transferred to the soil through feces or manure (Lim et al., 2010). It has been shown that *E. coli* can survive for more than 200 days in manure-treated autoclaved soil in an ambient environment (Jiang et al., 2002). *E. coli* O157:H7 can be a serious threat to public health (Beuchat et al., 1998; Jablasone et al., 2005)

when transferred from soils to fruits and raw vegetables. Therefore, it is critical to monitor and quantify *E. coli* O157:H7 in soils associated with cattle ranches, farms, and orchards.

One method that is widely used for bacterial quantitative detection is qPCR (Leblanc-Maridor et al., 2011; Palacio-Bielsa et al., 2011; Troxler et al., 2011; Wu et al., 2011). However, when qPCR is used to quantify bacteria in soil samples it is common for false-negative results to be a significant problem due to the presence of PCR inhibitors that are co-isolated with genomic DNA (gDNA) (Janzon et al., 2009). False-negative results for pathogen detection can have severe public health and economic implications. Soil samples can contain many compounds that may inhibit PCR assays, including humic acids, fulvic acids, bile salts, polysaccharides and cations (Lantz et al., 1997; Miller et al., 1999; Watson and Blackwell, 2000; Demeke and Jenkins, 2010; Kim et al., 2011a). Among these, humic acids are the most commonly reported PCR inhibitor in terrestrial samples (Wilson, 1997). Studies showed that even trace amounts of humic acids in DNA can completely inhibit PCR (Tsai and Olson, 1992; Tebbe and Vahjen, 1993).

Humic acids are formed by the degradation of animal and plant matter and other biological activities of microorganisms (Ghabbour and Davies, 2001). They are dominant components of natural organic matter (Menezes and Maia, 2010), and thus humic acids are both abundant (Hartenstein, 1981) and persistent (Picard et al., 1992) in soils. Specifically, humic acids interfere with the binding between target DNA and *Taq* polymerase (Tebbe and Vahjen, 1993; McGregor et al., 1996), disrupting the successful amplification of target genes. Park et al. reported that river DNA samples completely inhibited qPCR due to co-isolated humic acids (Park et al., 2007). In similar experiments, Janzon et al. showed that qPCR inhibition was observed for approximately 50% of the bacterial samples collected from drinking water and aquatic environments (Janzon et al., 2009). Miller et al. found that the PCR was inhibited when using template DNA isolated from agriculture soil, forest soil or wetland sediment (Miller et al., 1999). The necessity for high purity DNA as a template for qPCR frequently requires additional purification steps when working with environmental samples (Zhang and Lin, 2005; Lin et al., 2006; Balleste and Blanch, 2010). Various purification methods have been developed for gDNA extracted from soil samples (Jacobsen and Rasmussen, 1992; Tebbe and Vahjen, 1993; Volossiouk et al., 1995; Chandler et al., 1997; Krsek and Wellington, 1999; Liles et al., 2008; Fitzpatrick et al., 2010; Levy-Booth and Winder, 2010; Manter et al., 2010; Musovic et al., 2010; Newman et al., 2010; Xiao et al., 2010).

The recently developed molecular diagnostic assay (hereafter, NanoGene assay) (Kim and Son, 2010) has previously demonstrated its ability to quantitatively detect the *eaeA* gene using serially diluted gDNA from a pure culture of *E. coli* O157:H7 in the presence of common PCR inhibitors such as humic acids, cations, surfactants, or alcohols (Kim et al., 2011a). The NanoGene assay uses quantum dot nanoparticles (QD_{655}) that are conjugated with a probe specific to the target DNA (QD_{655}-signaling probe DNA) as a signal and a magnetic bead (MB) coupled with a different carboxyl quantum dot nanoparticle (QD_{565}) and a target-specific probe DNA (MB-QD_{565}-probe DNA) as a carrier (Fig. 1). Unlike PCR assays that depend on enzymatic amplification, the NanoGene assay is based on hybridization of the target DNA to the two probes. After hybridization, the nanoparticles are separated from the solution using a magnet and the amount of target DNA is determined by the fluorescence ratio of the reporter to the internal standard (i.e., QD_{655}/QD_{565}).

In this study the NanoGene assay was used to quantitatively detect a bacterial pathogen in soil samples for the first time. We determined the presence of the *E. coli* O157:H7 *eaeA* gene in seven soil samples using the NanoGene assay in order to evaluate its resistance to inhibition when using gDNA isolated from soils. The *eaeA* gene encodes the outer membrane protein intimin that contributes to the virulence of *E. coli* O157:H7, and the strain-specificity of *eaeA* was previously reported (Kaper et al., 2004).

Fig. 1. The schematic diagram of particles and DNA configuration in the NanoGene assay.

The qPCR assay was also conducted with each sample for comparative purposes. The seven soil samples consisted of six soils with varying humic acid contents, and one sand sample that served as a humic-negative control.

2. Material and methods

2.1. Soil collection

This study tested seven soil samples: sterilized Ottawa sand as a negative control (S), soil from a walking path in Auburn, AL (W), soil from the lake bank in Auburn, AL (L), soil from the arboretum in Auburn, AL (A), soil from a garden in Auburn, AL (G), potting soil (P) and farm soil in Shorter, AL (F). The Ottawa sand (S soil) was purchased from Durham Geo (Stone mountain, GA) and sterilized (121 °C for 15 min) after being washed with DI water three times. The W, L, A, and G soils were collected from the vicinity of Auburn University in Alabama. The P soil (Potting mix, Hyponex, Imlay, MI) was purchased locally from a general supply store. The F soil was collected from the E.V. Smith agricultural research center (Shorter, AL). A standard soil sampling technique was used, and 2 kg of soil was collected from a depth of 30 cm. The soil samples were immediately transported to the laboratory, dried at 105 °C for 6 h for dehydration, and reduced to powder form with a mortar and sieved with 2 mm mesh for further soil testing. Soil characteristics including soil texture, pH, organic matter, and soil types for each of the seven soil samples were determined at the soil testing laboratory at Auburn University.

2.2. Humic acid analysis in soils

The humic acid content of each soil sample was measured in order to compare different soil types for their relative inhibitory effects on the gene quantification assays (Ting et al., 2010). Briefly, 10 g of each soil was dissolved in 30 mL of 1 N NaOH (pH >> 10). Precipitates were removed by filtration with a 0.45 μm syringe filter. The supernatant was subsequently acidified with 10 mL of 1 N HCl to pH < 2 to precipitate humic acids while retaining free metals in solution at ambient temperature. The precipitated humic acids were collected by centrifugation at $3000 \times g$ for 30 min (AccuSpin™ 400, Fisher Scientific, Waltham, MA). The alkaline and acid treatments above were repeated to further purify humic acids. The purified humic acids were dissolved in 20 mL of 1 N NaOH.

To establish a standard curve for humic acid quantification, the optimal humic acid absorbance wavelength was determined. The absorbance was scanned from 200 nm to 800 nm using a Spectramax M2 microplate reader (MDS, Sunnyvale, CA), and the maximum absorbance was determined at 320 nm. The humic acids used for constructing the standard curve were purchased from the International Humic Substances Society (St. Paul, MN). A 1 N NaOH solution without humic acids was used as a negative control. The standard curve for humic acids was constructed using various humic acid concentrations and their absorbance at 320 nm ($R^2 = 0.99$). Subsequently, the amount of humic acids extracted from each soil sample was determined based on extrapolation using the standard curve. In addition, the amount of humic acids co-isolated with the purified gDNA was also quantified to indicate the degree of co-extraction of humic acids during gDNA extraction.

2.3. Inoculation of E. coli O157:H7 bacteria into soil samples

The freeze-dried culture of *E. coli* O157:H7 (ATCC 43888) was revived according to the ATCC's protocol by incubating the lyophilized cells in 1 mL trypticase soy broth (TSB) (Difco Laboratories, Detroit, MI) at 37 °C for 2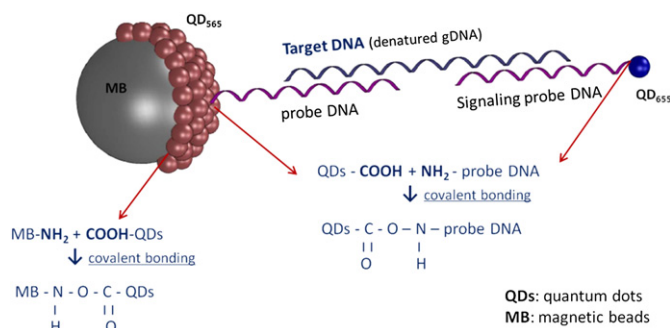0 h. The optical density at 600 nm (OD_{600})