Fate of *Escherichia coli* O157:H7 in irrigation water on soils and plants as validated by culture method and real-time PCR

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Abstract: One of the most common vehicles by which *Escherichia coli* O157:H7 may be introduced into crops is contaminated irrigation water. Water contamination is becoming more common in rural areas of the United States as a result of large animal operations, and up to 40% of tested drinking-water wells are contaminated with *E. coli*. In this study, 2 contrasting soil samples were inoculated with *E. coli* O157:H7 expressing green fluorescent protein through irrigation water. Real-time PCR and culture methods were used to quantify the fate of this pathogen in phyllosphere (leaf surface), rhizosphere (volume of soil tightly held by plant roots), and non-rhizosphere soils. A real-time PCR assay was designed with the *eae* gene of *E. coli* O157:H7. The probe was incorporated into real-time PCR containing DNA extracted from the phyllosphere, rhizosphere, and non-rhizosphere soils. The detection limit for *E. coli* O157:H7 quantification by real-time PCR was 1.2×10^3 in the rhizosphere, phyllosphere, and non-rhizosphere samples. *E. coli* O157:H7 concentrations were higher in the rhizosphere than in the non-rhizosphere soils and leaf surfaces, and persisted longer in clay soil. The persistence of *E. coli* O157:H7 in phyllosphere, rhizosphere, and non-rhizosphere soils over 45 days may play a significant part in the recontamination cycle of produce in the environment. Therefore, the rapidity of the real-time PCR assay may be a useful tool for quantification and monitoring of *E. coli* O157:H7 in irrigation water and on contaminated fresh produce.

Key words: real-time PCR, *Escherichia coli* O157:H7, irrigation, survival, quantification.

Résumé : Un des véhicules les plus communs introduisant du *Escherichia coli* O157:H7 dans les cultures est l'eau d'irrigation contaminée. La contamination par l'eau devient de plus en plus fréquente dans les régions rurales des États-Unis à cause de l'élevage intensif; jusqu'à 40 % des puits d'eau potable testés étaient contaminés avec *E. coli*. Dans cette étude, deux échantillons de sol distincts ont été inoculés avec *E. coli* O157:H7/pGFP (*E. coli*) à partir d'eau d'irrigation. Le PCR en temps réel et des méthodes de cultures ont été utilisés afin de quantifier l'aboutissement de ce pathogène dans la phyllosphère (surface de la feuille), la rhizosphère (volumes du sol solidement retenu par les racines des plantes) et dans des sols hors de la rhizosphère. Un essai de PCR en temps réel a été conçu avec le gène *eae* de *E. coli* O157:H7. La sonde a été incorporée au PCR en temps réel contenant de l'ADN extrait de la phyllosphère, la rhizosphère et des sols hors rhizosphère. La limite de détection quantitative de *E. coli* O157:H7 par PCR en temps réel étaient de $1,2 \times 10^3$ dans les échantillons de rhizosphère, phyllosphère, et hors rhizosphère. Les concentrations de *E. coli* O157:H7 étaient plus élevées dans la rhizosphère que dans les sols hors rhizosphère et les surfaces de feuilles, et ont persisté plus longuement dans un sol argileux. La persistance de *E. coli* O157:H7 dans la phyllosphère, rhizosphère, et les sols hors rhizosphère au-delà de 45 jours pourrait jouer un rôle significatif dans le cycle de recontamination de récoltes dans l'environnement. Ainsi, la rapidité du test de PCR en temps réel représente un outil précieux pour le décompte et le suivi de *E. coli* O157:H7 dans l'eau d'irrigation et sur des produits agricoles frais contaminés.

Mots clés : PCR en temps réel, *Escherichia coli* O157:H7, irrigation, survie, quantification.

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Introduction

One of the most common ways in which *Escherichia coli* O157:H7 may be introduced into crops is by flood irrigation with water contaminated with cattle feces or by contaminated surface runoff (Ackers et al. 1998; Hillborn et al. 1999; O'Conner 2002). Water contamination is becoming more common in rural areas of the United States, up to 40% of tested drinking-water wells being contaminated with *E. coli* (US EPA 1996). A 1-year study of cattle herds in the

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United Kingdom suggested that 36.8% of the herds carried the toxigenic strain of *E. coli* O157:H7 (Chapman et al. 1997). In Walkerton, Ontario, more than 1000 people fell ill, and 5 died of *E. coli* O157:H7 infections following a storm (O'Conner 2002). Intensification of regional livestock enterprises was named as the likely cause of contamination of drinking-water wells.

E. coli O157:H7 causes a wide spectrum of diseases in humans, ranging from mild to bloody diarrhea, hemorrhagic colitis, and complications including hemolytic uremic syndrome and seizures, which are particularly severe in children (Franke et al. 1995). The life-threatening complication of hemolytic uremic syndrome is acute kidney failure. It has been estimated that approximately 25 000 cases of foodborne illness are attributed to *E. coli* O157:H7 each year and an estimated 6 deaths (CDC 1999). *E. coli* O157:H7 outbreaks have been associated with ground beef, raw milk, lettuce, and minimally processed fresh fruit juices (Ackers et al. 1998; Hillborn et al. 1999). Recently, Solomon et al. (2002a, 2002b) demonstrated the transmission of *E. coli* O157:H7 from manure-contaminated soil and irrigation water to lettuce plants. These authors used laser scanning confocal microscopy, epifluorescence microscopy, and recovery of viable cells from the inner tissues of plants. They attributed the presence of *E. coli* O157:H7 in the edible portion of the plant to the direct migration through the conducting tissues of the root system.

Little research has been done on the quantification of this pathogen in the rhizosphere (volume of soil adjacent to, and tightly held and influenced by plant roots) and phyllosphere (leaf surface) of plants. The recent availability of new technologies, such as real-time PCR, has greatly aided the study of pathogens such as *E.coli* O157:H7 in the environment (Oberst et al. 1998; Heid et al. 1996). Real-time PCR has been used in studies for the detection and relative quantification of *E. coli* O157:H7 in food and clinical samples (Bellin et al. 2001; Sharma et al. 1999). Recently, Ibekwe et al. (2002) and Ibekwe and Grieve (2003) described quantification of *E. coli* O157:H7 in soil, cattle feces, manure, and waste water using multiplex real-time PCR. These studies showed a very high correlation between real-time PCR results and plate counts of environmental samples naturally contaminated with the pathogen. In the present study, we used primers and probes specific for the intimin gene that have been developed to quantify *E. coli* O157:H7 in soil and fecal samples by real-time PCR (Ibekwe et al. 2002). The purpose of the study was to use real-time PCR to determine the persistence of *E. coli* O157:H7 from contaminated irrigation water in the rhizosphere and phyllosphere of lettuce grown under the flood irrigation system and to compare the results with those obtained from the culture method, based on the enumeration of *E. coli* O157:H7 expressing green fluorescent protein (pGFP).

Materials and methods

Bacterial strain, growth conditions, and inoculum preparation

E. coli O157:H7 strain 34 with pGFP was used for this study. Plasmid construction of the strain has previously been described (Fratamico et al. 1997). In short, competent cells

of *E. coli* O157:H7 strains were prepared, and plasmid pGFP was introduced into them by the calcium chloride method. Transformants were selected on the basis of growth on Luria–Bertani agar containing ampicillin 100 μg·mL^{-1}. *E. coli* O157:H7/pGFP was cultured at 37 °C overnight in modified tryptic soy broth (Difco Laboratories Inc., Cockeysville, Md.) supplemented with ampicillin 100 μg·mL^{-1} (Sigma, St Louis, Mo.). Cells were harvested by centrifugation at 3500*g* for 10 min and resuspended in PBS (Fisher Scientific, Pittsburgh, Pa.) to a concentration of ~10^8 CFU·mL^{-1}.

Soil, preparation of irrigation water and plants, and recovery of *E. coli* O157:H7/pGFP

Clay soil (Willows silty clay, saline-alkaline) and sandy soil (Dello loamy sand) samples were collected from Mystic Lake dry bed and the Santa Ana River bed, respectively. The clay soil has a bulk density of 1.51 Mg·m^{-3} with 3.7% sand, 49.1% silt, and 47.2% clay. The sandy soil has a bulk density of 1.67 Mg·m^{-3} with 99.1% sand, 0.20% silt, and 0.70% clay.

The soils were sieved through a 4-mm sieve before planting of the seedlings, and the high salt content (electrical conductivity = 15 ds·m^{-1}) in the clay soil was reclaimed as described by Ibekwe and Grieve (2004). The minimum soil water content prior to irrigation was about 0.14 cm^3·cm^{-3}, and the maximum water content during irrigation reached 0.28 cm^3·cm^{-3}. This range is observed in most field soils, as reported previously by Wang (2002).

Soils were tested by culture and PCR methods to make sure that they were negative for *E. coli* O157:H7. Seeds of green romaine lettuce *Lactuca sativa* (*L.*) 'Green Forest' were purchased from Johnny's Selected Seed Co. (Albion, Me.). The plants were grown at 20 °C with 70% relative humidity and a photoperiod consisting of 16 h of light and 8 h of darkness.

Lettuce seedlings were sprouted in 50% Hoagland's solution (Hoagland and Arnon 1950) and transplanted into the soils in 2 growth chambers. The experiment was a completely randomized design with 3 replications. There were 10 plants in each tray at transplanting, and 1 plant was harvested from each tray during analysis. *Escherichia coli* O157:H7/pGFP was grown overnight in tryptic soy broth with 100 μg·mL^{-1} ampicillin at 37 °C. The cells were suspended in 10 mmol·L PBS (Fisher Scientific, Pittsburgh, Pa.), pH 7, washed twice in PBS, and resuspended in sterile distilled water to a density of 10^7 cells·mL^{-1}. Cell concentrations were determined turbidimetrically and by plate counts.

The first irrigation with water contaminated with *E. coli* O157:H7/pGFP occurred at transplanting (day 1) for both soils, and the second contamination event occurred 15 d later. Irrigation solutions were prepared in 1000-L reservoirs and pumped to provide irrigation to clay and sandy soil in polypropylene trays. A 1-L solution containing ~10^7 *E. coli* O157:H7/pGFP and ampicillin 100 μg·mL^{-1} was applied directly to the irrigation lines with a Cole-Parmer HPLC pump (Cole-Parmer, Chicago, Ill.) and delivered to each tray with 4 drip lines. Plants in the clay soil were irrigated with distilled water daily and received the nutrient solution weekly. The plants in the sandy soil received the nutrient solution twice daily. Overflow irrigation solution was returned to the

stainless steel containers and, by gravity flow, to a different reservoir that was subsequently decontaminated. After each contamination event, the irrigation system was completely decontaminated before use for the daily irrigation application. Microbiological analysis was performed to make sure that the irrigation system was truly decontaminated.

Plant phyllosphere, rhizosphere, and non-rhizosphere soil samples were aseptically sampled for analysis at 3, 5, 9, 12, 15, 18, 25, 29, and 45 d after transplantation. Non-rhizosphere soil (100 g) was aseptically collected with a sterile spoon in sterile plastic bags from around selected plants at 3.5 cm below the surface and about 6 cm from plants. From each tray, randomly selected plants were pulled from the soil and collected aseptically in sterile plastic bags. These plants were then separated by cutting the above-ground part of the plant into different sterile petri dishes or collection bags and treating the rhizhosphere in the same way. Samples were processed immediately in the laboratory. Bacteria were recovered from the phyllosphere by homogenization with 100 mL of PBS for 2 min at 260 r·min^{-1} in a Seward Stomacher 400 Circulator (Seward Ltd., London, UK). The same procedure was used to recover bacteria from the rhizosphere. The homogenate was centrifuged at 3000g for 10 min, the pellet was resuspended in 2 mL of PBS, 100 μL was plated on modified tryptic soy agar (mTSA) with ampicillin and incubated at 37 °C overnight. Serial dilutions of non-rhizosphere soil (1:10) were prepared in 100 mL of PBS, and 100-μL portions of each dilution in duplicate were spread onto mTSA with ampicillin and incubated at 37 °C overnight. *E. coli* O157:H7/pGFP colonies were enumerated under a hand-held Spectroline ultra-violet lamp (Spectronics Corporation, Westbury, N.Y.). Portions of the concentrated samples from rhizosphere, phyllosphere, and non-rhizosphere samples were used for extraction of total bacterial DNA, and the DNA was used for quantification of *E. coli* O157:H7 by real-time PCR.

DNA extraction, and primer and probe design for real-time PCR

For the construction of standard curves for real-time PCR, genomic DNA was extracted from pure culture of *E. coli* O157:H7/pGFP, grown for 8 h at 37 °C, and extracted with the Qiagen tissue kit (QIAamp DNA Mini Kit, Qiagen Inc., Valencia, Ca.). The standard curves from O157:H7/pGFP were used to determine detection limits of the *E. coli* by real-time PCR. Total bacterial DNA was extracted from rhizosphere, phyllosphere, and non-rhizosphere samples with the Ultra Clean Soil DNA Kit (MoBio Laboratories, Solana Beach, Calif.) and stored at −20 °C.

The primers and probe used for the detection and quantification of the *eae* gene in *E. coli* O157:H7 were as described by Ibekwe et al. (2002). They were developed from the 3′ end of a previously published sequence of an *eae* gene, encoding EHEC O157:H7-specific intimin (Beebakhee et al. 1992), and were selected to amplify and detect a 106-bp fragment specific for the *eae* gene of EHEC O157:H7 (*eae*$_{O157:H7}$). The primers and probe sequences were as follows: *eae*-F GTAAGTTACACTATAAAAGCACCGTCGA; *eae*-R TCTGTGTGGATGGTAATAAATTTTTG; and *eae*-Probe AAA TGG ACA TAG CAT CAG CAT AAT AGG CTT GCT. Fluorescent reporter dye Texas Red (sulfor-

hodamine 101) was conjugated at the 5′ end of the probe and quencher dye Black Hole Quencher (BHQ) II at the 3′ end (Black Hole Quencher dyes, Biosearch Technologies, Novato, Calif.). The BHQ dye was used as the quencher dye because of its broad quenching spectrum and lower signal-to-noise ratio (Biosearch Technologies 2000). Comparison of the nucleotide sequences of *eae*$_{O157:H7}$-specific primers and probe set with the sequences deposited in the GenBank revealed that this primer and probe set could distinguish EHEC O157:H7 from non-O157 EHEC and EPEC serotypes (with the exception of EPEC O55:H7, which carries an *eae* allele homologous to that of EHEC O157:H7) and from bacterial species such as *Citrobacter freundii* and *Hafnia alvei*, which harbor an *eae*-like gene (McGraw et al. 1999).

Real-time, quantitative PCR was performed with the iCycler iQ Real-Time PCR as described by Ibekwe et al. 2002. Briefly, PCR was performed in a 50-μL volume containing 200 μmol·L^{-1} of deoxynucleoside triphosphate, 2 μL of DNA from each concentration, 2.5 U of AmpliTaq Gold polymerase, 5 μL of 10× TaqMan buffer (PE Applied Biosystems, Foster City, Calif.), 0.3 μmol·L^{-1} of each primer, 0.1 μmol·L^{-1} of probe, and 3 mmol·L^{-1} of MgCl$_2$. Genomic DNA purified from *E. coli* O157:H7/pGFP was used as a template for the positive control, and no template was used for the negative control. Reaction mixtures were dispensed into a 96-well, thin-well PCR plate (Bio-Rad Laboratories, Hercules, Calif.), covered with optically clear sealing film and centrifuged briefly. PCR was performed with the iCycler iQ thermal cycler (Bio-Rad Laboratories) using the following cycle conditions: denaturation at 95° C for 10 min, 50 cycles of 94 °C for 20 s, of 55 °C for 30 s, and of 72 °C for 40 s followed by a 5 min extension at 72 °C and a hold at 4 °C. This system used a thermal cycler, an optical module, and detection software to quantify PCR products in real time, as revealed by the increase of fluorescence signal by 5′ nuclease activity during the amplification process. The threshold cycle (C$_T$) for each standard was plotted against the log of starting quantity to construct the standard curve used to quantify genes in the unknown samples.

Assay sensitivity, amplification efficiency, and data analysis

Standard curves generated from plotting the threshold cycle (C$_T$) vs. log$_{10}$ of starting DNA quantities (pg) were used for determining the detection limit of the assay. The standard curve was constructed from known quantities of genomic DNA extracted from *E. coli* O157: H7. The concentrations of *E. coli* O157:H7 present in unknown samples was determined from the standard curve by calculating the concentration of the extracted DNA as measured by an Ultrospec 4000 spectrophotometer with SwiftII application software (PharmaciaBiotech, Cambridge, England). The CFU·mL^{-1} was determined from the concentrations by plating culture dilutions containing 2.3 × 10^{-2} to 2.3 × 10^8 CFU·mL^{-1} of *E. coli* O157: H7 on cefixime/tellurite-sorbitol MacConkey (CT-SMAC) agar with BCIG (5-bromo-4-chloro 3-indoxyl-β-D-glucuronide) containing cefixime 0.05 mg·L^{-1}) and tellurite 2.5 mg·L^{-1} (LAB M, International Diagnostics Group, Bury, Lancs., England). The titers (CFU·mL^{-1}) of *E. coli* O157:H7 present in unknown samples were determined from the standard curve. The slopes of the standard

curves were calculated by performing linear regression analysis within the iCycler iQ software to compare the PCR amplification efficiency and detection sensitivity among different experiments. Amplification efficiency (E) was estimated by using the slope of the standard curve and the following formula: $E = (10^{-1/slope}) - 1$. Reaction with 100% efficiency generated a slope of −3.32.

Standardization of DNA quantities between known and unknown samples was accomplished by dividing total $CFU \cdot mL^{-1}$ of *E. coli* O157:H7/pGFP by the mean starting DNA concentration of that $CFU \cdot mL^{-1}$ from the instrument analysis, as described previously (Ibekwe et al. 2002). This resulted in a $CFU \cdot mL^{-1}$ index, which was used as a multiplier to calculate the $CFU \cdot mL^{-1}$ of all unknown samples. The $CFU \cdot mL^{-1}$ index was obtained from the highest DNA quantity to estimate $CFU \cdot mL^{-1}$ from lower DNA quantities. This approach was used because the instrument can give reports either in concentrations or copy numbers, and for environmental samples it is easier to understand results in actual numbers than concentration or copy number. *Escherichia coli* O157:H7/pGFP concentrations were then converted to log $CFU \cdot g^{-1}$ for regression analysis. Comparisons between pairs of treatment means at any date were accomplished with the Tukey's test. All calculations were performed using the general linear model procedure of the Statistical Analysis System (SAS Institute Inc. 1991).

Results and discussion

Quantification of heterotrophic bacteria by selective media

There were no differences in the levels of background bacteria as determined by heterotrophic plate count in the phyllosphere, rhizosphere, and non-rhizosphere soil during the first 12 d of the study (data not shown). This showed that the antibiotic used to grow the bacteria in the broth did not have any significant impact on the bacteria population, since the concentration was too low in the soil. The phyllosphere consistently showed concentrations of heterotrophic bacteria of about 2.3×10^5 as compared with about 2.5×10^7 in the rhizosphere. The $CFU \cdot mL^{-1}$ values from the phyllosphere, rhizosphere, and non-rhizosphere soil samples were similar in both the sand and clay soils. This trend was also observed after the second contamination event, when the numbers of heterotrophic plate counts followed the same pattern as observed after the first 12 d.

Sensitivity, standard curve, and amplification efficiency of real-time PCR assay

Previous work (Ibekwe et al. 2002) showed the *eae* gene to be a much conserved marker for distinguishing *E. coli* O157:H7 from other serotypes of *E. coli* after analysis of 33 Shiga toxigenic *E. coli* (STEC) and non-STEC *E. coli* strains. The sensitivity of the Texas Red-labeled probe to specifically detect and quantify the *eae* gene was determined by plotting the log DNA starting quantities of *E. coli* O157:H7/pGFP. The mean C_T values were between 21 and 23 when 5 to 10 pg DNA·mL^{-1} was used as a template. The dynamic range for cell detection of the quantitative real-time PCR was determined to be between 10^3 and 10^8 $CFU \cdot mL^{-1}$ of *E. coli* pGFP (Fig. 1). Concentrations of *E. coli* O157:H7

Fig. 1. Standard curves for the real-time, quantitative PCR analysis of *Escherichia coli* O157:H7 strain 34 green fluorescent protein (pGFP). C_T values of known (○) and unknown (◇) samples were plotted against corresponding $CFU \cdot mL^{-1}$ of genomic DNA. Concentrations of *E. coli* were determined in plant and soil samples from 3 assays: (A) $r = 0.992$, slope = −3.314, Y = −3.29X1 + 20.96; (B) $r = 0.998$, slope = −3.779, Y = −3.779X + 22.367; and (C) $r = 0.957$, slope = −3.234, Y = −3.234X + 21.148.
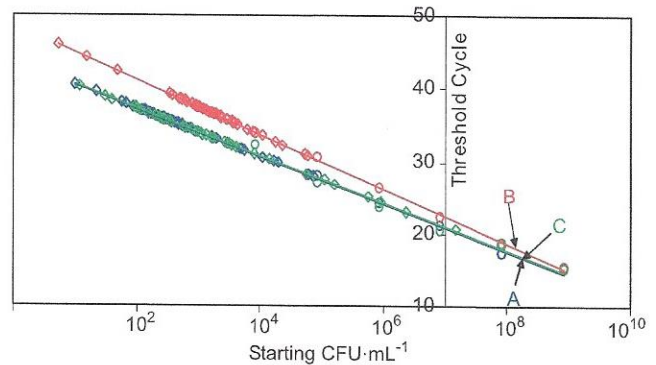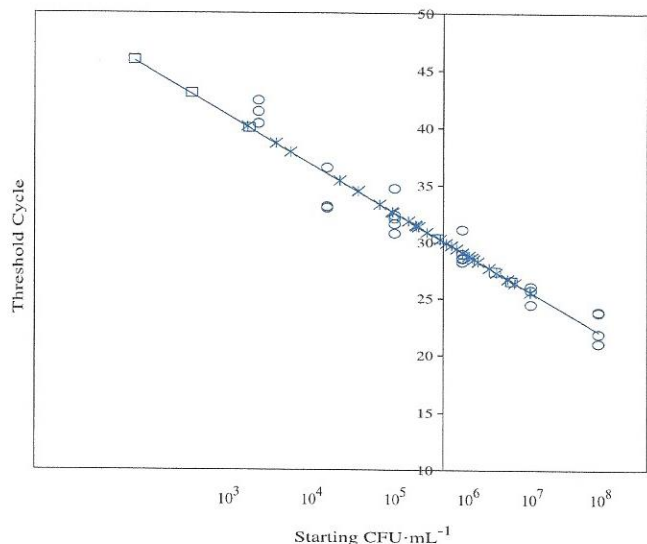

Fig. 2. Serial dilutions of *Escherichia coli* O157:H7 DNA were used as standard (○) and also added to DNA isolated from soil that did not contain *E. coli* O157:H7 (□), and then quantified using real-time PCR. Values for known amounts of *E. coli* O157:H7 DNA recovered from rhizosphere (×) are also shown.


in plant and soil samples were calculated from this standard curve. On the basis of this approach, a correlation was observed between the C_T and the $CFU \cdot mL^{-1}$ of the starting quantity of *E. coli* O157:H7 DNA, with a detection limit of 1.2×10^3 $CFU \cdot mL^{-1}$. The efficiency of each assay, calculated from the slope of each standard curve, was above 99.9% with correlation coefficients of about 0.99 for each curve.

To determine whether the quantity of *E. coli* O157:H7 in a complex mixture of bacterial DNA could be determined accurately using the real-time PCR assay, known amounts of *E. coli* O157:H7 DNA were added to DNA isolated from soil

Fig. 3. Quantification of *Escherichia coli* O157:H7 in the phyllosphere after (A) 12 d and (B) from day 15 to 45. *E. coli* O157:H7 enumerated from clay and sandy soils by plate count of green fluorescent protein (pGFP), from clay soil and sandy soil by real-time PCR (RT-PCR).
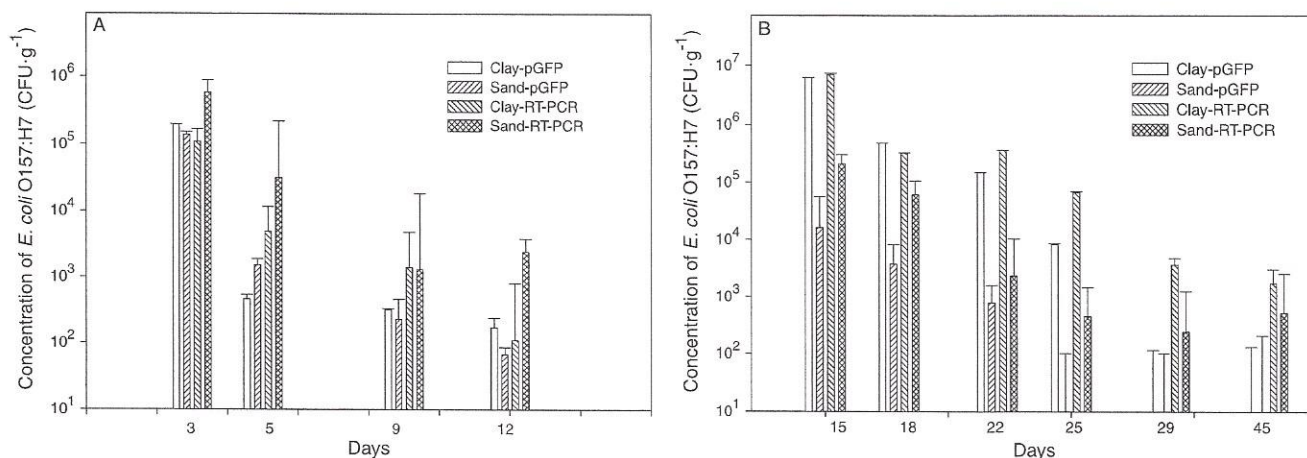
samples that were negative for *E. coli* O157:H7, as determined by either agarose gel electrophoresis and ethidium staining or analysis with the real-time PCR procedure (data not shown). The fluorescent signal was compared with a standard curve generated from *E. coli* O157:H7 DNA alone (Fig. 2). Each point represents an average of measurements from 3 to 4 independent samples. Similar values were obtained from *E. coli* O157:H7 genomic DNA alone and the same DNA mixed with DNA from soil, indicating that *E. coli* O157:H7 levels can be determined accurately even in the presence of DNA from other species. Rhizosphere samples that were part of the growth chamber study were also included to determine the influence of rhizosphere environment.

Fate of *E. coli* O157:H7 in phyllosphere, rhizosphere, and non-rhizosphere as determined by plate count of pGFP colonies

E. coli O157:H7 populations in the phyllosphere samples after the first contamination event decreased by an average >3 logs between day 3 and 12 in the clay soil and <4 logs for the sandy soil (Fig. 3A). On the average, about 170 CFU of *E. coli* O157:H7·g^{-1} was recovered from lettuce phyllosphere grown on clay soil and about 67 CFU from lettuce phyllosphere grown on sandy soil after the first 12 d. Following the second contamination event, the concentration of the pathogen in the phyllosphere of both soils after 45 days dropped to ≤10 CFU·g^{-1} (Fig. 3B). Throughout the 45-d study, the *E. coli* O157:H7 population decline was linear in the phyllosphere. The leaf surface of any plant is a very harsh environment for bacterial growth, and the survival may be limited by competition from other epiphytic microorganisms and nutrient availability. The number of *E. coli* O157:H7/pGFP in this study may be due to cross-contamination between the phyllosphere and soil irrigated with contaminated water during the early stages of growth, as pathogens may reside and survive between the leaves and the stems of the plants. It may also be through direct transmission from the roots through the veins to the leaves, as previously reported by Solomon et al. (2002*a*). We did not examine this process microscopically, as previously de-

scribed by Solomon et al. (2002*a*), since our objective was to determine the fate of the pathogen on the 3 matrices.

The concentration of *E. coli* O157:H7/pGFP in rhizosphere soils was 10^5 CFU·g^{-1} in both soils at day 12 (Fig. 4A), the clay soil concentration being significantly higher than that of the sandy soil. However, the trend in survival after the second contamination event (from day 15 to 45) was significantly different from that in the first contamination event (Fig. 4B). There were no differences in concentration between the 2 soil types by the end of the study. The survival of *E. coli* O157:H7/pGFP in the rhizosphere was 2 logs higher than in the leaves of the plants. Furthermore, the decline rate constant of the *E. coli* O157:H7/pGFP population was significantly different in the clay and sandy soils (r^2 = 0.99 and P = 0.001). Thus *E. coli* O157:H7/pGFP survived best and remained culturable for a longer period of time in the rhizosphere. This agreed with a recent report by Gagliardi and Karns (2002) that *E. coli* O157:H7 persistence was enhanced in the rhizosphere of rye and legumes grown in clay soil. It also suggested that the rhizosphere and soil types had significant influences on the persistence of *E. coli* O157:H7 in plants. Soil types may have a lesser influence on the persistence of the pathogen after many weeks compared with the first few days after the contamination event.

In this study, a higher concentration of pathogens was observed in the non-rhizosphere clay soil than in the sandy soil (Fig. 5A) during the first contamination event. This trend continued for 30 days after the second contamination event (Fig. 5B). While the decrease in population of culturable *E. coli* O157:H7/pGFP cells on non-rhizosphere soil was apparent, the decline rate constants of the *E. coli* O157:H7 population for the 2 soils were insignificant (r^2 = 0.35 and P = 0.42). Our study was conducted in a controlled environment with a constant temperature (20 °C), photoperiod (12 h), and relative humidity (70%) to minimize the influence of nonrelated soil variables on the survival and growth of *E. coli* O157:H7. The antagonistic effect of indigenous soil microorganisms may play a significant role in the survival of *E. coli* O157:H7 in the non-rhizosphere soils. Our study revealed that *E. coli* O157:H7/pGFP can survive for extended periods of time in soil contaminated with irrigation

Fig. 4. Quantification of *Escherichia coli* O157:H7 in the rhizosphere after (A) 12 d and (B) from day 15 to 45. *E. coli* O157:H7 enumerated from clay and sandy soils by plate count of green fluorescent protein (pGFP), and from clay and sandy soils by real-time PCR (RT-PCR).
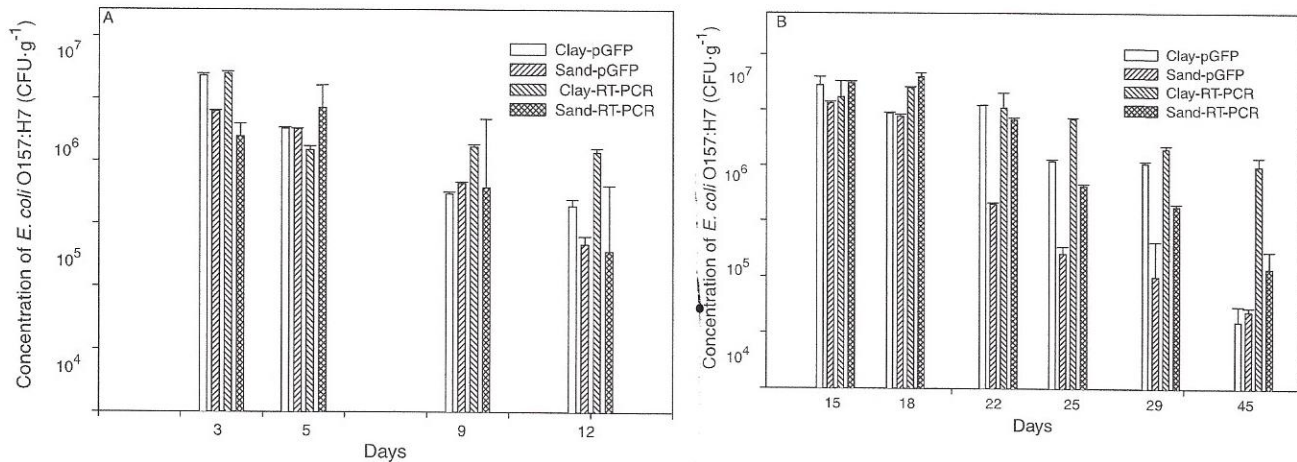
Fig. 5. Quantification of *Escherichia coli* O157:H7 in the non-rhizosphere soil after (A) 12 d and (B) from day 15 to 45. *E. coli* O157:H7 enumerated from clay and sandy soils by plate count of green fluorescent protein (pGFP), and from clay and sandy soils by real-time PCR (RT-PCR).
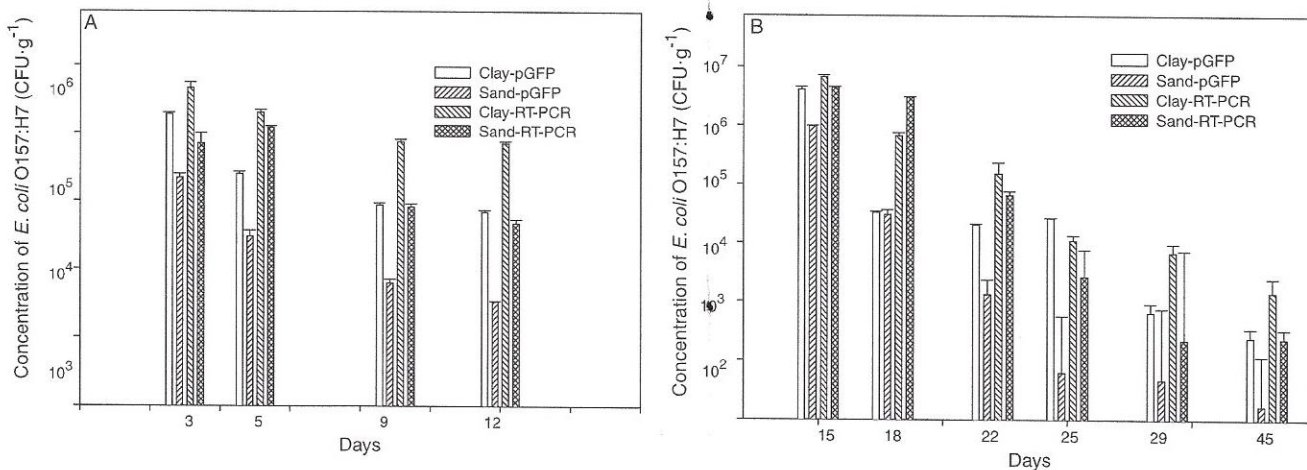
water, but the numbers of pGFP-expressing cells were consistently lower than cell numbers obtained by real-time PCR. Maule (1997) reported the survival of *E. coli* O157:H7 for 130 days at 18 °C when inoculated into a laboratory-prepared soil and grass microcosm.

This study agrees with the report by Takeuchi and Frank (2001), who showed the percentage of pGFP-expressing cells to be consistently lower than total viable cells obtained by microscopy. The study concluded that some cells lost their ability to express detectable pGFP, although they retained ampicillin resistance, and the percentage of pGFP expression could be improved only through repeated subculturing. Fratamico et al. (1997) showed that cells of the pGFP strain used in our study were stable through 15 cycles of daily subculturing at 37 °C, both with and without antibiotics, but declined to undetectable levels after 24 d at 4 °C in apple cider. Our study agrees with these studies that pGFP is highly stable and would not denature immediately but may not be a good marker for monitoring long-term survival of

bacteria if the plasmid insert is extrachromosomal, as it was in this study. The higher concentrations of bacteria determined by real-time PCR after 10 to 14 d from the start of inoculation compared with plate counts based on pGFP confirmed this observation. The real-time PCR assay also has the advantage of higher throughput, reproducibility, and less time required to screen many samples, as discussed in the next section.

Fate of *E. coli* O157:H7 in soil, rhizosphere, and leaf surfaces as determined by real-time PCR

Successful quantification of *E. coli* O157:H7 in soils, manure, and fecal samples in previous studies (Ibekwe et al. 2002; Ibekwe and Grieve 2003) led to the evaluation of this pathogen in the rhizosphere and on leaf surfaces. These matrices are known to harbor different microbial communities (Yang et al. 2001; Yang and Crowley 2000). The standard curves from the first and second inoculation for the quantification of *E. coli* O157:H7/pGFP from phyllosphere, rhizo-

sphere, and non-rhizosphere soil samples by real-time PCR are shown in Fig. 1. Quantitative real-time PCR analysis of these samples revealed linearity between the C_T values and the starting quantities of DNA representing 10^3 to 10^8 CFU·g^{-1}. Amplification efficiencies and the goodness of fit analysis for the standard curves were higher than 99%.

Figures 3, 4, and 5 show the quantification of *E. coli* O157:H7/pGFP detected over several weeks in the phyllosphere, rhizosphere, and non-rhizosphere soil samples, respectively, by real-time PCR. The concentrations of *E. coli* O157:H7 in the rhizosphere soils (Fig. 4A–B) were very close to the numbers of pGFP colonies obtained by the traditional culture methods on mTSA during the first 3–5 d of the first contamination event (Fig. 4A), and days 15 to 18 of the second contamination event (Fig. 4B). After this point, except in a few instances, the concentration obtained from real-time PCR was higher by 1 to 3 logs. This was observed in clay soil on day 9 and in sandy soil on day 9 and 15.

Our study shows that pGFP is highly stable for a few days, but it may not be a good marker for monitoring the long-term survival of bacteria. The higher concentrations of bacteria determined by real-time PCR versus plate count, 9 days after inoculation in some instances, confirmed this observation. While PCR may sometimes detect dead cells and degraded DNA, the data from this study showed that quantification of *E. coli* O157:H7 in the environment using DNA as template for amplification may be very effective during the first 7 days of contamination. The quantification strategy used here was successful because of prior knowledge of *E. coli* O157:H7 DNA copy numbers and genome size, as the instrument can determine results as copy numbers or concentrations. This strategy has also recently been applied to the detection and quantification of methyl *tert*-butyl ether-degrading strain PM1 by real-time PCR (Hristova et al. 2001), and *E. coli* and *Pseudomonas fluorescens* (Ludwig and Schleifer 2000). Real-time PCR also has the advantage of higher throughput, reproducibility, and less time required to screen large volumes of samples.

The ability to quantify *E. coli* O157:H7 in fresh produce and other food matrices without using culture methods will be very helpful for developing risk assessment models. Currently, most data demonstrating the risk of *E. coli* O157:H7 in food depend on culture techniques (Duffy and Schaffner 2001). For example, Duffy and Schaffner used a quantitative risk assessment of *E. coli* O157:H7 in apple cider with data on plate count from scientific journals to develop probability distribution functions. Other investigators have looked at the detection of *E. coli* O157:H7 in food samples (Fratamico et al. 2000; Sharma and Carlson 2000). The present study used a new method of converting fluorescent signal into target cell densities or copy numbers and relating the results directly to cell densities in the phyllosphere, rhizosphere, and non-rhizosphere soil samples by using a standard curve. The results suggest that *E. coli* O157:H7 can exploit the nutrient resources on leaves under conditions in which the physical environment does not limit its activities and therefore can survive in large numbers as part of the community. This observation was confirmed in a related study by Ibekwe and Grieve (2004), which showed that microbial community development in lettuce took about 7–12 d and that this may be

the most likely period for maximum pathogen contamination in plants.

Our procedure is in contrast to other studies of *E. coli* O157:H7, in which presumptive detection by TaqMan PCR was used to estimate the population size of *E. coli* O157:H7 (Bellin et al. 2001; Oberst et al. 1998), a strategy recently applied to the detection and quantification of *E. coli* O157, O111, and O26 in beef and bovine feces (Sharma 2002). The automated PCR amplification and detection of target gene amplicons described in the present study is conducive to screening large numbers of samples in a single assay. The real-time PCR can be a useful method for processing plants to monitor the contamination of fresh produce for risk analysis before sending produce to the consumers.

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