Journal of Microbiological Methods 98 (2014) 122–127

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Visual endpoint detection of *Escherichia coli* O157:H7 using isothermal Genome Exponential Amplification Reaction (GEAR) assay and malachite green

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ARTICLE INFO

Article history:
Received 28 October 2013
Received in revised form 2 January 2014
Accepted 2 January 2014
Available online 11 January 2014

Keywords:
E. coli O157:H7
Molecular assay
Isothermal amplification
Malachite green
Water microbiology

ABSTRACT

Rapid and specific detection methods for bacterial agents in drinking water are important for disease prevention and responding to suspected contamination events. In this study, an isothermal Genome Exponential Amplification Reaction (GEAR) assay for *Escherichia coli* O157:H7 was designed specifically to recognize a 199-bp fragment of the lipopolysaccharide gene (*rfb*E) for rapid testing of water samples. The GEAR assay was found to be specific for *E. coli* O157:H7 using 10 isolates of *E. coli* O157:H7 and a panel of 86 bacterial controls. The GEAR assay was performed at a constant temperature of 65 °C using SYTO 9 intercalating dye. Detection limits were determined to be 20 CFU for the GEAR assay. When SYTO 9 fluorescence was measured using a real-time PCR instrument, the assay had the same detection limit as when malachite green was added to the reaction mix and a characteristic blue color was visually observed in positive reactions. The study also found that 50 and 20 CFU of *E. coli* O157:H7 seeded into 100-liter of tap water could be detected by the GEAR assays after the sample was concentrated by hollow-fiber ultrafiltration (HFUF) and approximately 10% of HFUF concentrate was cultured using trypticase soy broth–novobiocin. When applied to 19 surface water samples collected from Tennessee and Kentucky, the GEAR assay and a published real-time PCR assay both detected *E. coli* O157:H7 in two of the samples. The results of this study indicate that the GEAR assay can be sensitive for rapid detection of *E. coli* O157:H7 in water samples using fluorometric instruments and visual endpoint determination.

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1. Introduction

Escherichia coli O157:H7 is a bacterial pathogen that can be transmitted through food and water (Gould et al., 2013; Hlavsa et al., 2011). *E. coli* O157:H7 infection is typically associated with bloody diarrhea and can cause hemolytic uremic syndrome (HUS), especially in the young and immunocompromised individuals (Sanchez et al., 2010). *E. coli* O157:H7 are gram negative bacteria that produce shiga toxin (*stx*) and are often referred as shiga-toxin *E. coli* (STEC) (Dean-Nystrom et al., 1998) or enterohemorrhagic *E. coli* (EHEC) (Cebula et al., 1995). A small percentage of other shiga toxin producing serotypes of *E. coli*, including O26 (stx_1 and stx_2), O45 (stx_1), O103 (stx_2 and *eae*A), O111 (stx_1, stx_2, and *eae*A), O121 (stx_1 and *eae*A) and O145 (stx_2) also cause similar illness (Beutin et al., 2009; Couturier et al., 2011; Fratamico et al., 2011; Madic et al., 2010; Madic et al., 2011; O'Hanlon et al., 2004). Other shiga-toxin producing serotypes of *E. coli* O91, O113 and O128 also cause HUS and bloody diarrhea (Friedrich et al., 2003; Kappeli et al., 2011; Orden et al., 1998). With such a diversity of serotypes potentially present in food and environmental samples, it is important to distinguish *E. coli* O157:H7 from other bacteria using appropriate virulence markers. The molecular identification of *E. coli* O157:H7 often targets the marker genes of stx_1 (shiga toxin 1), stx_2 (shiga toxin 2), *eae*A (intimin gene — A/E lesions widely present in enteropathogenic *E. coli*), *hly* (60 mDa plasmid pO157 encoding enterohemolysin gene), *rfb*E (O antigen cluster — locus containing lipopolysaccharide gene present in *E. coli* O157 serogroup) and *fli*C (H7 flagellin gene present in all serotypes of H7 serogroup) (Prendergast et al., 2011; Wang et al., 2002). Assays for specific detection of *E. coli* O157:H7 often require screening for virulence markers of stx_1, stx_2, *hlyA* and *eae*A, and serotype specific markers of *rfb*E and *fli*C genes (Bai et al., 2010).

Real-time PCR based rapid detection of *E. coli* O157:H7 has been reported for several marker genes individually or in multiplex format for confirmation (Carey et al., 2009; Jothikumar and Griffiths, 2002; Oberst et al., 1998; Singh et al., 2009; Suo et al., 2010). Real-time PCR machines, and associated reagents, are relatively expensive, which limits the implementation of molecular testing in resource-limited laboratories. Other molecular techniques have been reported for the rapid identification of *E. coli* O157:H7 that are based on isothermal amplifications such as nucleic acid based amplification (NASBA) (Won

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0167-7012/$ – see front matter. Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.mimet.2014.01.002

and Min, 2010), ramification amplification (Li et al., 2005) and loop mediated isothermal amplification (LAMP) (Ohtsuka et al., 2010; Zhao et al., 2010). The GEAR technique offers advantages over established isothermal amplification methods, such as the widely used loop mediated isothermal amplification (LAMP) assay. The GEAR technique differs from the LAMP technique in that the pair of core GEAR primers (FT and BT) targets three regions (Prithiviraj et al., 2012), while the pair of core LAMP primers (FIP and BIP) targets four regions (Notomi et al., 2000). Additionally, the reagent, malachite green, can be used in conjunction with GEAR to enable visible observation of positive GEAR assays. Isothermal amplification of target nucleic acids can be performed in a water bath or a heat-block that maintains a constant temperature at 65 °C and the amplified products result in color change that can be visually observed without the need for any reader.

The objective of the present study was to develop a rapid isothermal molecular assay method using the GEAR technique for rapid detection of *E. coli* O157:H7. The GEAR assay was applied to the detection of *E. coli* O157:H7 in 100 L drinking water samples concentrated by tangential flow ultrafiltration (UF). Previous studies have investigated molecular detection of *E. coli* O157:H7 in water (Mull and Hill, 2009), but no previous study has investigated the use of isothermal amplification methods for detection of this pathogen in water. The study incorporated experiments designed to determine the method detection limit for recovery of *E. coli* O157:H7 in 100-L drinking water samples using hollow-fiber ultrafiltration (HFUF) and broth culture, followed by application of the GEAR assay for *E. coli* O157:H7.

2. Materials and methods

2.1. Bacterial strains

The specificity of the *E. coli* O157:H7 assay was determined using a panel of 86 bacterial isolates, including 56 isolates representing a diverse array of pathogenic serotypes. Pathogenic *E. coli* isolates used for the study were previously characterized by the CDC's National *Escherichia coli* Reference Laboratory, as follows: EHEC *E. coli* O157:H7 (10 isolates), EHEC non-*E. coli* O157:H7 [O26:H11 (3 isolates); O103:H2 (1 isolate), O121:H19 (2 isolates), O45:H2 (3 isolates), O60:H8 (1 isolate), O145:NM (1 isolate)], DAEC *E. coli* (1 isolate), EAEC *E. coli* [O126:H27 (1 isolate), O111:H2 (2 isolates) and O44:H18 (1 isolate)], EIEC *E. coli* (4 isolates), O22:NM (1 isolate), O28ac:NM (1 isolate), EPEC *E. coli* [O55:NM (1 isolate), O128:H1 (1 isolate), O111:NM (1 isolate), O127:NM (1 isolate), O55:H6 (1 isolate), O119:H6 (1 isolate), O86:H34 (1 isolate)], ETEC *E. coli* (4 isolates), other pathogenic *E. coli* [ExPEC/NMEC/UPEC; O1:H7 (1 isolate), O4-H5 (1 isolate), O6:H1 (3 isolates), O6:H31 (1 isolate), O7:NM (2 isolates), O157:H18 (1 isolate), O18:H7 (1 isolate)], and STEC *E. coli* [O111:H8 (2 isolates), O11:NM (1 isolate)]. Non-pathogenic *E. coli* used in this study were obtained from ATCC (8 isolates). Non-*E. coli* used in this study were: *Escherichia albertii* (1 isolate), *Escherichia vulneris* (2 isolates), *Escherichia hermannii* (2 isolates), *Escherichia fergusonii* (3 isolates), *Escherichia blattae* (1 isolate), *Salmonella enterica* subsp. enteric serovar Typhimurium (2 isolates), *Yersinia pestis* (1 isolate), *Shigella boydii* (2 isolates), *Shigella dysenteriae* (3 isolates), *Shigella sonnei* (2 isolates) and *Shigella flexneri* (3 isolates). After overnight culturing of bacterial isolates, 200 μL of each sample was subjected to nucleic acid extraction as described previously (Hill et al., 2007), and DNA was eluted from the silica column with 200 μL of TE buffer (pH 8.0).

2.1.1. Sensitivity of the GEAR assay

A standard curve for the GEAR assay was performed using a 10-fold dilution series of DNA extracted from a stock of 8×10^8 CFU/mL of *E. coli* O157:H7 in nuclease free-water. The standard curve was generated using triplicate reactions of 10^0 to 10^5 CFU per reaction.

2.1.2. Genome Exponential Amplification Reaction (GEAR) assay

The GEAR assay developed in the present study was used for detecting the *rfb*E gene (coding for the O antigen for O157). The GEAR assay was designed using Tab primers (FT and BT) that are complementary at their 5′ end (Fig. 1A). The primer sequences for the GEAR assay and its locations are shown in (Fig. 1B and C). In a previously reported GEAR assay (Prithiviraj et al., 2012), the primers DF and DR were not included since the GEAR assay targeted a high copy number gene. In the present study, additional primers (DF and DR) were included to improve the sensitivity and speed of the reaction (Fig. 1B and C). Amplification of DNA targets was performed using an ABI 7500 instrument, but the instrument was programmed to maintain a constant temperature at 65 °C for 90 min. Fluorescence was acquired at the end of every minute and collected up to 90 min. An amount of 2 μL of DNA template was tested in 20 μL reactions. Each 20 μL reaction mix contained 10 μL of 2× Loopamp DNA Amplification mastermix (Eiken Chemical Co., Ltd., Japan), 0.8 μL of *Bst* DNA polymerase (New England Biolabs, Ipswich, MA, USA), 0.8 μL of 25 μM SYTO 9 (Invitrogen, Carlsbad, CA, USA), 2 μL of DNA template, and six primers [outer displacement primers DF and DR (each at 0.2 μM), GEAR primers FT and BT (each at 1.2 μM), inner primers IR and IF (each at 1.2 μM)]. Positive and negative controls were included in each run.

2.2. Malachite green

Experiments were also performed to determine the detection limit of the *E. coli* O157:H7 GEAR assay when malachite green was used to enable observation of positive reactions using a visual color change instead of using SYTO 9 fluorescence detection by a real-time PCR instrument. Malachite green was obtained as a 5% stock solution and further diluted with nuclease free water to obtain a stock solution of 0.2%, then stored at room temperature. The reaction mixture (20 μL reaction) was overlaid with 20 μL mineral oil. The malachite green (4 μL) was added on top of the mineral oil. Mineral oil was used to initially separate malachite green from the GEAR reaction mixture because direct addition of malachite green was associated with inhibition of the GEAR assay (data not shown). At the end of the 90 min reaction time, amplification of target DNA was determined by observation of a blue color in the reaction mix. Negative GEAR assays were associated with a colorless reaction mix.

2.3. Detection of E. coli O157:H7 in seeded tap water samples

The GEAR assay was evaluated for the detection of *E. coli* O157:H7 seeded into 100 L volumes of tap water. The tap water samples were obtained from the study laboratory and were dechlorinated prior to seeding. The 100 L tap water samples were concentrated using UF according to the method of Hill et al. (2007). *E. coli* O157:H7 (ATCC 43895) was seeded into tap water samples at two different levels (50 and 20 CFU). Non-seeded 100 L control samples were also processed by UF and assayed for background *E. coli* O157:H7. Three replicate experiments were performed at each seeding level (including non-seeded controls). The UF procedure resulted in concentrated samples with average volumes of approximately 450 mL. To assay UF concentrates for *E. coli* O157:H7, 10% of the volume of the UF concentrate was filtered through a 0.45-μm pore size mixed cellulose ester membrane filter. Membrane filters were incubated separately at 37 °C in 100 mL of modified tryptic soy broth with novobiocin (Merck, Darmstadt, Germany) and agitation for 18–24 h (Mull and Hill, 2009). Following culture, the genomic DNA was obtained by direct heating. Two hundred microliters of each log-phase bacterial broth culture was subjected to heating at 95 °C for 5 min in a heating block and cell debris was removed by centrifugation at 10,000 ×*g* for 1 min. Two microliters of the supernatant containing DNA was added to reaction tubes.

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