Food Control 62 (2016) 150–156

Contents lists available at ScienceDirect

Food Control

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

Optimization and validation of a PMA qPCR method for *Escherichia coli* quantification in primary production

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ARTICLE INFO

Article history:
Received 22 July 2015
Received in revised form 13 October 2015
Accepted 14 October 2015
Available online 17 October 2015

Keywords:
Viable cells
Microbial sampling program
Indicator microorganisms
Cultivation-based techniques
Propidium monoazide

ABSTRACT

The microbial requirements defined in many quality assurance guidelines and standards of primary production demand the establishment of microbial sampling programs. Recently, a q-PCR *Escherichia coli* assay has been reported as a good method to quantify the presence of fecal indicator bacterial in groundwater samples. This study focuses on the optimization, validation and application of a qPCR method combined with propidium monoazide (PMA) treatment to exclude DNA from dead cells. A first screening consisting of six primer sets targeting single and multi-copy of *E. coli* were tested to evaluate the sensitivity of the assay. After that, four primer sets were selected, combined with PMA treatment and their capacity to distinguish viable cells when combined with a background of dead cells was assessed. A primer set targeting the 23S rRNA gene was 10-fold more sensitive than the rest of primers, enabling the detection of low concentrations of viable *E. coli* cells. This assay also exhibited good repeatability and reproducibility, which indicates the robustness of the method. Optimized and validated PMA-qPCR was used to enumerate *E. coli* in environmental samples including irrigation water and fresh produce. The results were compared with the levels quantified using qPCR and cultivation-based techniques. Counts of *E. coli* using plate count assay were significantly lower than the levels obtained by molecular techniques (PMA-qPCR and qPCR) in both irrigation water and fresh produce. *E. coli* PMA-qPCR enumeration method showed similar results as qPCR quantification, although the PMA-qPCR treatment seemed to a good alternative to distinguish between viable and dead cells. It can be concluded that the optimized PMA-qPCR assay can be used by the industry in microbial sampling programs, helping them with the implementation of Good Agricultural Practices (GAP).

1. Introduction

A food safety management program focused on primary production is the result of the implementation of available and relevant quality assurance guidelines and standards such as Codex Alimentarius, guidelines on good agricultural practices (GAP), hygiene legislation, and private standards (Kirezieva, Jacxsens, Uyttendaele, Van Boekel, & Luning, 2013). In the current European legal framework, microbiological criteria is not defined at the primary production stage, but growers must be able to demonstrate that their operations do not represent a food safety risk (EC, 2004). However, microbiological requirements have been defined in many quality assurance guidelines and standards, which make, the establishment of microbial sampling programs, necessary for growers (Allende & Monaghan, 2015).

Traditionally, sampling programs are based on the detection of specific pathogens and the enumeration of indicators of fecal contamination. However, the prohibitive cost and time required for pathogen detection, makes enumeration of microbial indicators a good strategy to characterize microbial contamination in the environment of field cultivation and fresh produce (Park et al., 2013).

Indicator microorganisms, such as *Escherichia coli*, are usually used by the industry and competent authorities to assess the implementation of Good Agricultural Practices (GAP). At primary production, *E. coli* have been identified as suitable hygiene criterion in leafy greens as its presence provides evidence of an increased likelihood of potential contamination by ecologically closely related

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http://dx.doi.org/10.1016/j.foodcont.2015.10.014
0956-7135/

pathogens (Castro-Ibáñez, Gil, Tudela, Ivanek, & Allende, 2015; EFSA, 2014; Holvoet, Sampers, Seynnaeve, Jacxsens, & Uyttendaele, 2015). Traditional culture techniques using pour and spread plate count methods are used to enumerate *E. coli* in water and produce samples. However, these cultivation-dependent assays may not represent the most efficient method to predict presence of bacterial pathogens. Ferguson et al., (2012) reported that a qPCR-based *E. coli* assay was the best indicator for the presence of bacterial pathogens in groundwater samples.

Many studies have focused on the development and optimization of qPCR methods to detect and enumerate *E. coli* in environmental samples as a more sensitive, rapid and specific test that plate counts (Ahmed, Richardson, Sidhu, & Toze, 2012; Ferguson et al., 2012; Pitkänen et al., 2013). However, qPCR-based assays cannot differentiate between viable and dead bacterial cells as DNA of dead cell can persist in the environment, leading to a substantial overestimation of *E. coli* concentrations (Rudi Moen, Drømtorp, & Holck, 2005; Varma et al., 2009). To avoid false-positive results, a recently proposed strategy is the treatment of samples with propidium monoazide (PMA) before DNA extraction, allowing the differentiation between viable and dead cells. This technique is based on the ability of PMA to penetrate into the dead cells with compromised membrane integrity and in turn inhibit DNA amplification by PCR following light-induce cross-linking (Fittipaldi, Codony, Adrados, Camper, & Morató, 2011; Nocker & Camper, 2009; Nocker, Sossa-Fernandez, Burr, & Camper, 2007; Varma et al., 2009). The use of PMA has been successfully integrated with qPCR assays for the differentiation of viable and dead *E. coli* cells in different environmental samples, mostly in water samples (van Frankenhuyzen, Trevors, Flemming, Lee, & Habash, 2013; Gensberger et al., 2014; Kim, Gutiérrez-Cacciabue, Schriewer, Rajal, & Wuertz, 2014; Li et al., 2014). However, discrepancies regarding the most recommended primers and PMA concentrations make the selection of a PMA-qPCR method difficult based on the available literature.

Environmental samples from the primary production are complex matrices that may interfere with the efficacy of the PMA treatment. Factors such as the ratio between viable and dead bacterial cells, pH and salt concentrations as well as the natural presence of PMA inhibitors have been highlighted as potential inhibitors for the PMA treatment, DNA extraction and qPCR yield (van Frankenhuyzen, Trevors, Lee, Flemming, & Habash, 2011; Fittipaldi, Nocker, & Codony, 2012). Therefore, optimization and validation of previously developed PMA-qPCR methods as well as their suitability as monitoring systems in the primary production are essential before their application as routine tools in microbial sampling programs.

Thus, the aim of the present study was the optimization of a previously described PMA-qPCR assay for the quantification of viable generic *E. coli* cells in irrigation water and produce samples covering; (i) selection of specific primer sets for *E. coli* qPCR with TaqMan technology; (ii) characterization of the PMA-qPCR capacity to differentiate between viable and dead cells and (iii) validation of the assay in irrigation water and produce samples taken from a commercial-scale production field of leafy greens.

2. Material and methods

2.1. Bacterial strains and inoculum preparation

A seven-strain cocktail of *E. coli* (CECT 434, 471, 515, 516, 533 and LFMFP 679) was used in this study. Strains were obtained from the Spanish Type Culture Collection (CECT) (Valencia, Spain) and the Laboratory of Food Microbiology and Food Preservation (LFMP) (Ghent University, Belgium). Cultures were inoculated in Brain Heart Infusion (BHI) (Scharlau Chemie, Barcelona, Spain) and incubated without agitation at 37 °C for 24 h. Then, 2 ml of each strain were combined and washed twice by centrifugation (3500 g, 20 min) at 4 °C in 14 mL of 0.1% buffered peptone water (BPW; AES Chemunex, Marcy l'Etoile, France). The washed cell pellets were suspended in 2 mL of 0.1% BPW.

2.2. Sample collection

Water samples were collected at five sampling points from a stream bordering farmlands and urban areas in Murcia (Spain) at six different sampling times. A total volume of 3L was collected using sterile polypropylene plastic bottles. Iceberg lettuces were purchased from a local supermarket at the day of harvest. Samples were transported to the laboratory (within 30 min) and stored at 4 °C for maximum 16 h until further processing. Lettuce heads, which were confirmed to be free of *E. coli* by Chromocult agar (Oxoid, Hampshire, UK), were sprayed with irrigation water obtained from the urban stream (A) and a mixture of irrigation water and heat-treated irrigation water at a 1:1 ratio (B). Non-sprayed lettuce was used as negative control. After being sprayed with water, lettuce samples were incubated at room temperature for 16 h.

2.3. Cultivation-based E. coli quantification

Cultivation-based enumeration of *E. coli* in irrigation water and fresh lettuce samples was performed according to ISO 9308-1:2014. In the case of water samples, different volumes (10 mL and 100 mL) were filtered through 0.45 μm pore size nitrocellulose membranes (Sartorius, Madrid, Spain). For *E. coli* quantification, membranes were aseptically removed from the filter base and placed on Chromocult agar. For iceberg lettuces, external leaves were cut and samples (25 g each) were homogenized for 3 min in 225 mL of 0.1% BPW using a Stomacher (IUL instruments, Barcelona, Spain). Tenfold dilution series were made in 0.1% BPW and plated onto Chromocult agar. The plates were incubated at 37 °C for 24 h.

2.4. Molecular-based E. coli quantification

Water samples were taken from the surface water as previously described. Two 500 mL aliquots per sampling point were centrifuged at 3000 g for 20 min and supernatant was removed. In the case of iceberg lettuces, external leaves were cut and three samples (25 g each) were homogenized in 125 ml of sterile 0.1% BPW using a Stomacher at medium speed for 3 min. The homogenate of each sample was divided to obtain two pellets by centrifugation at 3000 g for 10 min. From each water or lettuce sample, one of the pellets was stored at −20 °C for genomic DNA extraction while the other pellet was resuspended in sterile water for PMA treatment.

2.5. PMA treatment for qPCR

Propidium monoazide (PMA) (Biotium Inc, Hayward, CA, USA) was dissolved in 20% dimethyl sulfoxide (DMSO); (Sigma–Aldrich, Ameresco, USA) as a 20 mM stock solution. Aliquots of 2 mM were transferred to light-impermeable 2 mL microtubes at kept at −20 °C in the dark. For PMA treatment cell suspensions were diluted to a cell density of 10^4 cells/mL in 0.1% BPW. Aliquots of 1 mL of the diluted cell suspensions, irrigation water and lettuce samples were transferred to clear transparent 2-mL microtubes. These aliquots were then centrifuged at 9000 *g* for 10 min and supernatants removed. Cell pellets were resuspended in 1 ml of sterile water with variable PMA concentrations (10 and 40 μM). PMA treated microtubes were shaken at 400 rpm for 5 min in darkness. The tubes laid on ice were then exposed to a 500-W halogen light source (GE lighting, Cleveland, USA) for 5 min. The distance between microtubes and light source was 20 cm. After light exposure, the bacteria were harvested by centrifugation at 9000 g for 10 min.