LWT - Food Science and Technology 86 (2017) 586–593

Contents lists available at ScienceDirect

LWT - Food Science and Technology

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

Selective and concurrent detection of viable *Salmonella* spp., *E. coli*, *Staphylococcus aureus*, *E. coli* O157:H7, and *Shigella* spp., in low moisture food products by PMA-mPCR assay with internal amplification control

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ARTICLE INFO

Article history:
Received 26 October 2016
Received in revised form
5 August 2017
Accepted 8 August 2017
Available online 11 August 2017

Keywords:
Food safety
Food-processing industry
Propidium monoazide
Multiplex polymerase chain reaction

ABSTRACT

Salmonella enterica, enterohemorrhagic *Escherichia coli*, and *Staphylococcus aureus* are major pathogens contaminating low moisture foods. *E. coli* and *Shigella* spp. may acquire significance in future since cross-contamination plays a critical role in outbreaks involving low moisture foods. This study investigated a PMA-IAC-mPCR for rapid, reliable and simultaneous detection of viable *Salmonella* spp., *E. coli*, *Staphylococcus aureus*, *E. coli* O157:H7, and *Shigella* spp., in low moisture foods. Propidium monoazide (PMA) was applied to detect only viable cells by eliminating PCR signal from dead cells. In addition, an internal amplification control (IAC) was included in the multiplex PCR as an indicator of false negative results arising due to inhibitors in low moisture foods. The sensitivity of the assay for viable cells with PMA treatment was 10^2–10^3 CFU/mL for all the target pathogens reflecting the non-influence of PMA treatment on sensitivity. After 10 h enrichment, the PMA-IAC-mPCR could detect 10^1 CFU/g of *Salmonella* spp., *E. coli*, *Staphylococcus aureus*, *E. coli* O157:H7, and *Shigella* spp., in artificially inoculated low moisture foods (peanut butter, chocolate, milk powder and egg powder). This PMA-IAC-mPCR assay would find its promising application in simultaneous detection of these viable target pathogens in low moisture foods.

1. Introduction

Low moisture foods have been an important nutritional ingredient of the human diet. Low moisture foods are those that have water activity (a_w) of 0.85 or below (FAO/WHO, 2016). Due to their low water activity, these foods were perceived to be harmless from a microbiological food safety point of view in the past (FAO., 2014). However, in recent years there has been a rise in the burden of foodborne illness and the number of food recalls related to microbial contamination of low moisture foods (Dey, Mayo, Saville, Wolyniak, & Klontz, 2013; Vij, Ailes, Wolyniak, Angulo, & Klontz, 2006). Major bacterial pathogens implicated in outbreaks involving low moisture foods include *Salmonella enterica,* enterohemorrhagic *Escherichia coli*, and *Staphylococcus aureus*. *Salmonella* spp. and *S. aureus* was responsible for 44.9% and 7.5% cases of these outbreaks, respectively (FAO., 2014). The relevance of *E. coli* O157:H7, pathotypes of *E. coli* in contamination of low moisture food are evident from the recent outbreaks in Germany (European Food Safety Authority, 2011), USA and Canada (CDC Centers for Disease Control and Prevention, 2016). The sources of contamination in these outbreaks were fenugreek seeds, raw cookie dough, in-shell hazelnuts, and raw shelled walnuts most of which come under the category of nut and nut products of low moisture foods.

Due to the broad range of low moisture foods, FAO/WHO has categorized them into seven major groups to facilitate in ranking the foods of greatest concern from a microbiological food safety approach. Within this category, dried protein products, nut and nut products top the ranks in terms of disease burden caused by these low moisture food products (FAO., 2014). It is noteworthy that the former category includes milk powder, egg powder and the latter includes tree nuts, peanuts and peanut products such as peanut

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http://dx.doi.org/10.1016/j.lwt.2017.08.023
0023-6438/

butter. In particular, peanut and peanut containing products are associated with at least seven salmonellosis outbreaks of low-moisture food reported in past two decades (Schaffner et al., 2013). Moreover, another category of concern comes from confections and snacks (e.g. chocolate) due to their susceptibility to microbial contamination. The risk of microbial contamination of these products arise from their present status in international trade, consumption levels and risks of contamination post killing step in processing (FAO., 2014). The vulnerability of these low moisture food products highlights their due importance from a microbiological food safety perspective. Evidence from major outbreaks reveals that cross-contamination plays a critical role in the contamination of low moisture foods. Moreover, low water activity and high-fat content of many of these foods contribute to heat resistance of pathogens to thermal processing commonly used in food industry (Mondal, Buchanan, & Lo, 2014). Therefore, the survival of these pathogens through heat treatment can lead to further contamination of processing equipments, new lots in production. In addition to this, cross-contamination could be caused by contaminated equipment, environment, and food handlers in the supply chain (The Association of Food Beverage and Consumer Products Companies, 2009). In this regard, *E. coli* and *Shigella* spp. demand future concern since the water used in the processing step and personnel involved in processing play a critical role in cross-contamination of dry foods. These pathogens can cause potential damage due to their heat resistance in low moisture foods. Furthermore, their ability to cross-contaminate end products such as peanut butter, chocolate, milk powder and egg powder necessitates their rapid and accurate detection to ensure food safety.

Conventional culture based methods that are traditionally used for the detection and enumeration of foodborne pathogens are laborious, time-consuming (Chen, Tang, Liu, Cai, & Bai, 2012) and are known to have poor sensitivity and specificity (Elizaquível & Aznar, 2008). Hence, there is urgency in the need for rapid and accurate methods for the detection of foodborne pathogens. Among molecular methods, PCR is a rapid and simple method used as an alternative to detect various pathogens. In particular, multiplex PCR has gained substantial importance due to its capability to simultaneously detect several pathogens in a single reaction. This capability to detect several pathogens in a single reaction lowers laboratory cost and saves time and effort (Perry et al., 2007). However, conventional mPCR cannot discriminate between viable and non-viable cells due to persistence of DNA even after death (Nogva, Drømtorp, Nissen, & Rudi, 2003). This limitation of conventional mPCR can be an obstacle in discriminating cross-contamination occurring after processing in low moisture foods. This can be overcome only when a method to differentiate live/dead cells using DNA as a viability marker is applied. The concept of viability PCR using nucleic acid intercalating dyes such as propidium monoazide (PMA) recently has attracted significant attention. PMA can selectively penetrate membrane compromised dead cells and cross-link DNA using its azide group upon photo-activation to make it unavailable for polymerase activity during PCR (Nocker, Cheung, & Camper, 2006).

Therefore, in the present work, we have combined PMA pretreatment and IAC-mPCR to enable rapid, accurate and simultaneous detection of viable *Salmonella* spp., *E. coli*, *Staphylococcus aureus, E. coli* O157:H7, and *Shigella* spp., in peanut butter, chocolate, milk powder and egg powder. Five specific genes, *invA* gene, *uidA* gene, *nuc* gene, 92* *uidA* gene, and *ipaH* gene were used as targets for *Salmonella* spp., *E. coli*, *Staphylococcus aureus, E. coli* O157:H7, and *Shigella* spp. Internal amplification control (IAC) using pUC18 plasmid primer was added to exclude false negative results occurring due to inhibitors present in food samples. Thus, a PMA-IAC-mPCR was employed for concurrent detection of viable *Salmonella* spp., *E. coli*, *Staphylococcus aureus, E. coli* O157:H7, and *Shigella* spp., and its real time applicability were evaluated in low moisture foods such as peanut butter, chocolate, milk powder and egg powder.

2. Materials and methods

2.1. Materials

All the media components including supplements were procured from HiMedia Laboratories (Mumbai, India). All the media preparation was done as per manufacturer's instruction. *Taq* DNA polymerase, dNTPs were purchased from Sigma (St. Louis, MO). All the primers used in this study were synthesized in Eurofins Genomics Pvt. Ltd, (Bangalore, India). Twenty millimolar PMA stock in distilled water was procured from Biotium, Inc. (Hayward, CA).

2.2. Bacterial strains and growth conditions

All the strains used in this study are given in (Table 1). All bacterial strains were cultured in Brain Heart Infusion (BHI) broth at 37 °C in a rotary shaker (180 rpm) for 14–16 h. *E. coli* ATCC 10536, *E. coli* O157:H7 USDA 303, *Salmonella* Typhimurium ATCC 14028, *Staphylococcus aureus* ATCC 43300, *Shigella boydii* ATCC 9207 were used as reference strains to standardize the mPCR assay.

To determine the viable colony count, 100 μL of ten-fold serial dilutions of all target organisms in sterile physiological saline were plated onto Plate Count Agar by following Pour plate method. Colonies were enumerated after 18–24 h incubation at 37 °C. To prepare non-viable cells of these target pathogens, aliquots of required serial dilution of these pathogens were heated at 95 °C for 30 min (Wang, Li, & Mustapha, 2009).

2.3. PMA treatment and genomic DNA extraction

Samples were processed as described previously by (Nkuipou-Kenfack, Engel, Fakih, & Nocker, 2013) with slight modifications. Prior to use, 20 mM PMA stock was diluted with sterile distilled water to 1 mM. Later, 10 μL of 1 mM PMA solution was added to 1 mL suspension to a final concentration of 10 μM. After the addition of PMA, samples were incubated at 40 °C for 30 min in dark with occasional mixing. This was followed by photo activation with 500-W halogen light source for 5 min at 20 cm distance. During this step, the samples were placed on the ice above the rocking platform in order to ensure homogeneous light exposure and to avoid excessive heating. After a 5 min exposure, the cell suspension was centrifuged at 12,000 × *g* for 5 min and washed twice with PBS to remove free PMA prior to DNA extraction from the cell pellet.

DNA used for sensitivity checking and PMA-IAC-mPCR from artificially inoculated food samples were purified using Nucleospin Tissue Kit (Macherey-Nagel GmbH & Co., Duren, Germany) according to manufacturer's instructions.

2.4. Multiplex PCR optimization

All the primers used in this study are given in (Table 2). Firstly, monoplex PCR with a gradient of temperatures ranging from 52 °C to 58 °C were tested to determine the optimum annealing temperature for all target pathogens. This was carried out with 5 pmol of each primer pair using standard concentrations of all PCR components. A non-competitive internal amplification control was incorporated in all PCR reactions without any modification (Nagaraj, Ramlal, Sripathy, & Batra, 2014). Each 30 μL PCR reaction included 25 ng of template DNA, 10 pg of PUC18 DNA, 1× PCR buffer, 2 mM $MgCl_2$, 200 μM dNTPs and 1.5U of *Taq* polymerase. With the