Available online at www.sciencedirect.com

SCIENCE DIRECT®

International Journal of Food Microbiology 95 (2004) 177–187

INTERNATIONAL JOURNAL OF Food Microbiology

www.elsevier.com/locate/ijfoodmicro

Application of a molecular beacon—real-time PCR technology to detect *Salmonella* species contaminating fruits and vegetables ☆

Samantha Hu Liming [1], Arvind A. Bhagwat *

Produce Quality and Safety Laboratory, Henry A. Wallace Beltsville Agricultural Research Center, Agricultural Research Service, USDA, 10300 Baltimore Avenue, Building 002, Room 117, BARC-W, Beltsville, MD 20705-2350, USA

Received 2 November 2003; received in revised form 29 January 2004; accepted 20 February 2004

Abstract

An oligonucleotide probe that becomes fluorescent upon hybridization to the target DNA (molecular beacon; MB) was evaluated in a real-time polymerase chain reaction (PCR) assay to detect the presence of *Salmonella* species. As few as 1–4 colony-forming units (CFU) per PCR reaction could be detected. The capability of the assay to detect *Salmonella* species from artificially inoculated fresh-cut produce such as cantaloupe, mixed-salad, cilantro, and alfalfa sprouts was demonstrated. A comparison of two commercially available kits utilizing MB-PCR (iQ-Check, Bio-Rad Laboratories) and conventional Association of Official Analytical Chemists (AOAC)-approved PCR (BAX, Dupont Qualicon) was performed on artificially inoculated produce. As few as 4 CFU/25 g of produce were detected after 16 h of enrichment in buffered peptone broth. These assays could be carried out entirely in sealed PCR tubes, enabling a rapid and high-throughput detection of *Salmonella* species in a large number of food and environmental samples. This is the first report of the application of MB probe being used for real-time detection of *Salmonella* species in whole and fresh-cut fruits and vegetables.

Keywords: Microbial food safety; Ready-to-eat produce; Foodborne pathogens

1. Introduction

The detection of *Salmonella* species by regulatory agencies is still primarily based on traditional microbiological culture methods that may take several days to complete. The incidence of foodborne infections caused by members of the genus *Salmonella* continues to be a problem in the U.S. (Anonymous, 2000; Brackett, 1999; Buck et al., 2003; Thunberg et al., 2002). Several outbreaks of salmonellosis in the U.S. have involved fresh produce, mainly melons, cantaloupes, cilantro, and alfalfa sprouts (Backer et al., 2000; Campbell et al., 2001; Long et al., 2002; Mead et al., 1999; Mohle-Boetani et al., 1999; Wells and Butterfield, 1997). The results of surveys to determine the prevalence of *Salmonella* on melons established that cut melons meet the U.S. Food and Drug Administration (FDA) model food code criteria for

☆ Mention of brand names does not constitute an endorsement by the U.S. Department of Agriculture above others of a similar nature not mentioned.

* Corresponding author. Tel.: +1-301-504-5106; fax: +1-301-504-5107.

E-mail address: bhagwata@ba.ars.usda.gov (A.A. Bhagwat).

[1] Permanent address: School of Life Sciences and Chemical Technology, Ngee Ann Polytechnic, 535 Clementi Road, 599489, Singapore.

0168-1605/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2004.02.013

potentially hazardous food that they should be subjected to code time and temperature requirements (Beuchat et al., 2001; Beuchat and Ryu, 1997; Brackett, 1994; Mead et al., 1999). In the past decade, increased consumption of fresh produce, especially fresh-cut produce (cut and packaged mixed salads, peeled and cut melon cubes, etc.), has been accompanied by an associated increased frequency of outbreaks of foodborne pathogen illness (Buck et al., 2003; Hodge, 1999).

Determining the precise source of contamination is crucial when devising strategies to reduce future outbreaks. However, only 2 of the 27 outbreak investigations on fresh produce clearly identified a point of contamination which underscores the importance and need for rapid and accurate pathogen identification methods (NACMCF, 1999). New methods based on polymerase chain reactions (PCR) have been increasingly used for the rapid, sensitive, and specific detection of foodborne pathogens (Feng, 1997; Fratamico et al., 1995; Fratamico and Strobaugh, 1998; Hill, 1996). Most PCR assays rely on visualizing the amplification product by ethidium bromide staining of agarose gels (Feng, 1997; Ferretti et al., 2001; Hill, 1996). In order to increase the specificity of detection, a number of PCR assays utilize either scoring of the target DNA or post-PCR hybridization-capture methods (Chen and Griffiths, 2001; Cocolin et al., 1998; Rijpens et al., 1999; Weagant et al., 1999). These approaches have met with limited success, as these modifications make the overall pathogen detection procedure labor intensive, time consuming and difficult to automate.

In order to address some of these concerns we recently modified a commercially available, Association of Official Analytical Chemists (AOAC)-approved, PCR-based method (Bailey, 1998; Mrozinski et al., 1998) for real-time monitoring by including the fluorescent dye SYBR Green I (Bhagwat, 2003, 2004). Although this modification enabled simultaneous and rapid detection of three foodborne pathogens in a high-throughput format, the fluorogenic reporter dye lacked specificity for the desired target molecule. Therefore, post-PCR melting curve analysis of the amplified product was incorporated in the protocol, and spurious amplification products were easy to distinguish (Bhagwat, 2003). However, a new fluorogenic PCR-based format has been recently developed, which utilizes an internal fluorogenic probe that is specific to the target gene (Chen et al., 2000; Hoorfar and Radstrem, 2000). During the PCR assay, the target gene is amplified and simultaneously recognized and monitored by the fluorescent probe moiety (Tyagi and Kramer, 1996).

There are two types of fluorogenic PCR-based detection methods. One is based on a linear fluorogenic probe and requires the 5′-3′-nuclease activity of the DNA polymerase (Hoorfar and Radstrem, 2000; Kimura et al., 1999; Nogva and Lillehaug, 1999) (also known as TaqMan assays), while the other utilizes a fluorogenic probe which has flanking GC-rich arm sequences complementary to one another (Chen et al., 2000; McKllip and Drake, 2000; Tyagi and Kramer, 1996) (also known as molecular beacon, MB). In both types of real-time PCR probes, a fluorescent moiety is conjugated to one end of the sequence, and a quencher moiety is attached to the other end of the sequence. In the absence of target DNA sequences, the MB assumes a hairpin conformation, with the two arms hybridizing to each other, thus bringing the quencher into close proximity to the fluorophore (which results in no or low background fluorescence). When the target DNA is present, the sequence in the loop region hybridizes, the hairpin of the MB opens, and the fluorophore and the quencher separate. In the open conformation, the fluorophore of the MB emits a detectable signal that is directly correlated with the quantity of the target template present in the PCR assay (Higuchi et al., 1993; Tyagi and Kramer, 1996). The Taq-Man assay differs from the MB method in that the generation of the fluorophore signal is dependent upon 5′-3′-nuclease activity to cleave the reporter dye from the linear probe (Chen et al., 1997; Kimura et al., 1999; Nogva and Lillehaug, 1999).

Irrespective of the reporter technology employed in the PCR assay, its successful application to food samples, particularly to fresh produce, has been hindered by the lack of a convenient and relatively simple method for preparation of PCR-amplifiable DNA (Heller et al., 2003; Hill, 1996; Liao and Shollenberger, 2003; Miller, 2001; Shearer et al., 2001). We and others have reported the presence of inhibitory compounds of plant origin that interfered with PCR biochemistry, resulting in false-negative data (Bhagwat, 2003; Liao and Shollenberger, 2003; Shearer et al., 2001). In this manuscript, we have evaluated and compared MB probe PCR method with other PCR

and conventional microbiology methods to detect *Salmonella* species from fresh and fresh-cut produce (cantaloupe, mixed-salad, cilantro, and alfalfa sprouts), which was artificially contaminated at the level of 4 colony-forming units (CFU)/25 g. This is the first report of the application of MB probe being used for real-time detection of *Salmonella* species in fresh-cut fruits and vegetables.

2. Materials and methods

2.1. Experimental design

This study examined the sensitivity and efficacy of two PCR-based methods (iQ-Check *Salmonella* kit, Bio-Rad Laboratories, Hercules, CA, and BAX-PCR *Salmonella* kit, Qualicon, Wilmington, DE) and microbiological culture methods using pure cultures of *Salmonella typhimurium* as well as a mixture of seven different serovars of *Salmonella*. Each experiment was performed two to three times, and each experiment contained three subsamples per produce. Artificially inoculated fresh produce (alfalfa sprouts, cilantro, cantaloupe, and pre-packed mixed-salad) at low inoculum dose of 3–4 CFU/25 g was used in this investigation.

2.2. Bacterial strains and media

S. typhimurium ATCC 14028s has been described previously (Fang et al., 1992; Gawande and Bhagwat, 2002a,b). Other *Salmonella* strains [Serovars Agona (SARB 1), Anatum (SARB 2), Dublin (SARB 12), Haifa (SARB 21), Choleraesuis (SARB 4), Pullorum electrophoretic types Pu3 (SARB 51) and Pu4 (SARB 52), and Paratyphi A (SARB 42)] were obtained from the *Salmonella* Genetic Stock Center (Calgary, Alberta, Canada). Cultures were routinely started from freezer stocks for growth on Luria–Bertani (LB) agar medium. Following overnight incubation at 37 °C, a single colony was selected and inoculated into 10 ml LB broth in a 125-ml Erlenmeyer flask. Cells were grown for 20–22 h at 37 °C with shaking at 200 rpm to obtain stationary-phase cultures. Cultures from the stationary growth phase were harvested by centrifugation at 4000 × *g* for 10 min, washed once with 3 vol of saline (0.9% NaCl), and suspended in saline at a cell density of 10^8 cells ml^{-1}; cells were further diluted in saline to get the desired cell density. Final cell numbers were confirmed by determining viable cell counts on LB agar plates.

2.3. Inoculation of fresh produce with Salmonella strains

Alfalfa sprouts, cilantro, cantaloupes, and pre-packed mixed-salad (made up of approximately 80% leaf lettuce, 10% red cabbage, and 10% carrot by weight) were obtained from local grocery stores. Fresh produce (25 g) was inoculated with approximately 4 cells in 50 μl saline using either pure cultures or mixtures of *Salmonella* species and processed within 10 min for pre-enrichment. For each experiment un-inoculated produce was used as a control and was found to be free of *Salmonella* species.

2.4. Enrichment procedures

Two different enrichment protocols were followed, which were specific for the two PCR methods.

To analyze artificially contaminated produce samples by BAX-PCR (Qualicon) the manufacturer's recommended enrichment protocol was followed. Briefly, 25 g of produce was combined with 225 ml of lactose broth (Administration, 1995) in sterile stomacher bags and pummeled for 2 min in a stomacher 400 Lab Blender (Seaward, London, UK). After stomaching, the pH was adjusted to 6.8 ± 0.2 with 5 N HCl or 1 M NaOH as necessary, and the samples were incubated for 24 h at 37 °C. The next day, samples were pummeled once again for 2 min in a stomacher and 1 ml of the slurry was transferred to 9 ml of pre-warmed (37 °C) brain heart infusion broth (Difco Laboratories, Detroit, MI) and further incubated for 3 h at 37 °C. After this short enrichment period, a 5-μl sample was taken for DNA isolation.

To analyze artificially contaminated produce samples by iQ-Check *Salmonella* kit, one-step enrichment protocol recommended by the manufacturers was followed (Bio-Rad Laboratories). Briefly, 25 g of produce was combined with 225 ml of buffered-peptone water broth (Difco Laboratories) in sterile stomacher bags and pummeled for 2 min in a stomacher 400 Lab Blender (Seaward). The samples were

incubated for 18 ± 2 h without shaking at 37 °C. Following the incubation period, 1 ml of the sample was withdrawn from the top without disturbing the food debris and processed for DNA isolation.

2.5. DNA extraction procedures

For the BAX-PCR method, 5 μl of the sample from the brain heart infusion broth was transferred to an Eppendorf tube containing 200 μl of BAX cell lysis reagent. DNA was then isolated according to the manufacturer's protocol. Briefly, cells were incubated in the BAX-lysis buffer for 20 min at 37 °C and then for 10 min at 95 °C (to inactivate the proteases in the lysis buffer). For the iQ-Check protocol, 1-ml samples from the buffered peptone water broth were centrifuged for 12,000 × *g* for 5 min, the pellet was suspended in 200 μl of lysis reagent (iQ-Check *Salmonella*, Bio-Rad Laboratories) and vortexed. Lysis was carried out by incubating the suspension at 100 °C for 15 min.

For isolating DNA from *S. typhimurium* pure cell suspensions or mixtures of serovars, known quantities of viable cells (measured as CFU ml^{-1}) were mixed in individual tubes containing lysis buffer and used as standards in the respective PCR assays.

2.6. Detection of Salmonella strains by PCR

For BAX-PCR, 50 μl of DNA preparations was transferred to *Salmonella* BAX-PCR tubes containing the lyophilized pellet of all PCR reagents except target DNA. The thermocycler was programmed for 94 °C for 2 min (94 °C for 15 s, 72 °C for 3 min) × 35 cycles, and 72 °C for 7 min. Fifteen microliters of loading dye was added and mixed to each PCR tube. The PCR mixture was processed by performing agarose (2.0%) gel electrophoresis and the 800-bp target DNA was visualized by ethidium bromide staining (Bailey, 1998).

A modified protocol of the BAX-PCR that enables real-time detection (Bhagwat, 2003, 2004) of amplified target DNA was also performed using a pure culture of *S. typhimurium*.

For the iQ-Check PCR protocol, samples were examined in duplicate at two concentrations of template DNA. For each sample, 5 μl of 1:10 and 1:25 diluted DNA was mixed with 40 μl of amplification mixture and 5 μl of fluorogenic oligonucleotide molecular beacon probe labeled with FAM at the 5′-end and DABSYL at the 3′-end as the quencher. The fluorogenic MB probe from iQ-Check kit (Bio-Rad Laboratories) targets the *iagA* (*i*nvasion *a*ssociated *g*ene), which is highly specific to *Salmonella* species (Miras et al., 1995). To monitor successful DNA amplification in each reaction tube, kit provides a synthetic DNA (at suboptimal concentration) as a part of the reaction mixture which works as an "internal control." This control DNA was amplified with a specific probe at the same time as the *Salmonella* target DNA sequence and detected by a second fluorophore (Texas red). The thermocycler was programmed for 50 °C for 2 min, 95 °C for 5 min (95 °C for 20 s, 55 °C for 30 s, 72 °C for 30 s) × 50 cycles, and 72 °C for 5 min. Data were collected after each annealing step (i.e., after 55 °C for 30 s) using an excitation wavelength of 490 nm and an emission wavelength of 530 nm as specified by Bio-Rad Laboratories.

2.7. Detection of Salmonella strains by conventional microbiology methods

Protocol recommended for *Salmonella* strains by the U.S. FDA-BAM protocol was followed (Administration, 1995). After enrichment, the identity of *Salmonella* strains was confirmed by the occurrence of black colonies on *Salmonella–Shigella* (SS) agar and black colonies with a bright metallic sheen on Bismuth sulphite (BS) agar (Bopp et al., 1999).

3. Results

3.1. Detection of Salmonella strains by conventional PCR and molecular beacon real-time PCR

We examined the sensitivity of MB probe, real-time PCR methodology with reference to conventional PCR. As a first step, a pure culture of *S. typhimurium* grown to stationary phase in LB broth was serially diluted 10-fold in saline; viable counts were confirmed by growing aliquots on LB agar medium. DNA isolation was performed from samples with varying quantities of cells ranging from 10^8 to 10^0 CFU ml^{-1}. A no-template control, in

which sterile saline was substituted for template DNA, was used in each experiment. This control was used to subtract any fluorescence that was not directly related to amplification. Fig. 1 shows the normalized fluorescence measurement from the MB probe versus PCR cycle collected in real time (Fig. 1). Fluorescence from the MB probe increased as the target DNA (*iagA* gene) accumulated at the end of each successive round of amplification. All data collected during the 30-s annealing cycle were used in the analysis and for quantifying the amplification of target DNA. Using the MB probe with real-time detection, it was possible to detect up to 4 CFUs of *S. typhimurium*.

The conventional AOAC-approved PCR method was performed using reagents specific to *Salmonella* in the BAX-PCR kit, and the amplification product was examined after agarose gel electrophoresis. Using this protocol, the detection limit appears to be 10^3 CFU per PCR assay when the agarose gels were stained with ethidium bromide and examined under UV light (Fig. 2a). Recently, we modified this AOAC-approved PCR protocol in order to achieve real-time detection by including a fluorescent reporter dye, SYBR Green I (Bhagwat, 2003) in the PCR assay. Using this modified protocol, it was possible to collect data in real time. However, there was no change in the sensitivity of the assay and the lower detection limit remained at 10^3 CFU per PCR assay (Fig. 2b).

3.2. Quantitation of Salmonella strains based on the target DNA amplification

The ability of the MB probe PCR assay to detect various *Salmonella* serovars was investigated with pure DNA templates obtained from individual serovars (data not shown). Since for artificial inoculation, experiments were performed with mixtures of different serovars, detection limits of the MB probe PCR assay were determined using two different mixtures of *Salmonella* serovars: (1) a mixture of five diverse serotypes (Agona, Anatum, Dublin, Haifa, and Choleraesuis), and (2) a mixture of serovars that do not appear to be responsive to attachment-mediated acid tolerance (comprised of serovars Pullorum electrophoretic types Pu3, and Pu4, and Paratyphi A) (Gawande and Bhagwat, 2002a,b). The MB probe was able to detect both mixtures of different serovars as effective-

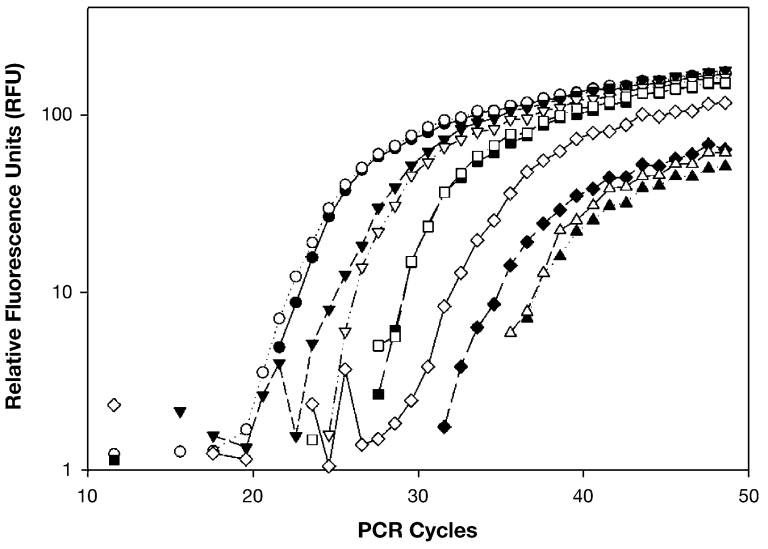

Fig. 1. Amplification plot of a 10-fold serial dilution series of *S. typhimurium*. Sample replicates (open and closed symbols) with cells per assay 10^0 (△, ▲); 10^1 (◇, ◆); 10^2 (□, ■); 10^3 (▽, ▼); and 10^4 (○, ●) using a molecular beacon probe and real-time PCR assay. Real-time detection was done by measuring fluorescence of FAM during the annealing step of each PCR cycle (*X*-axis). Relative fluorescence units (RFU) are plotted on the *Y*-axis.

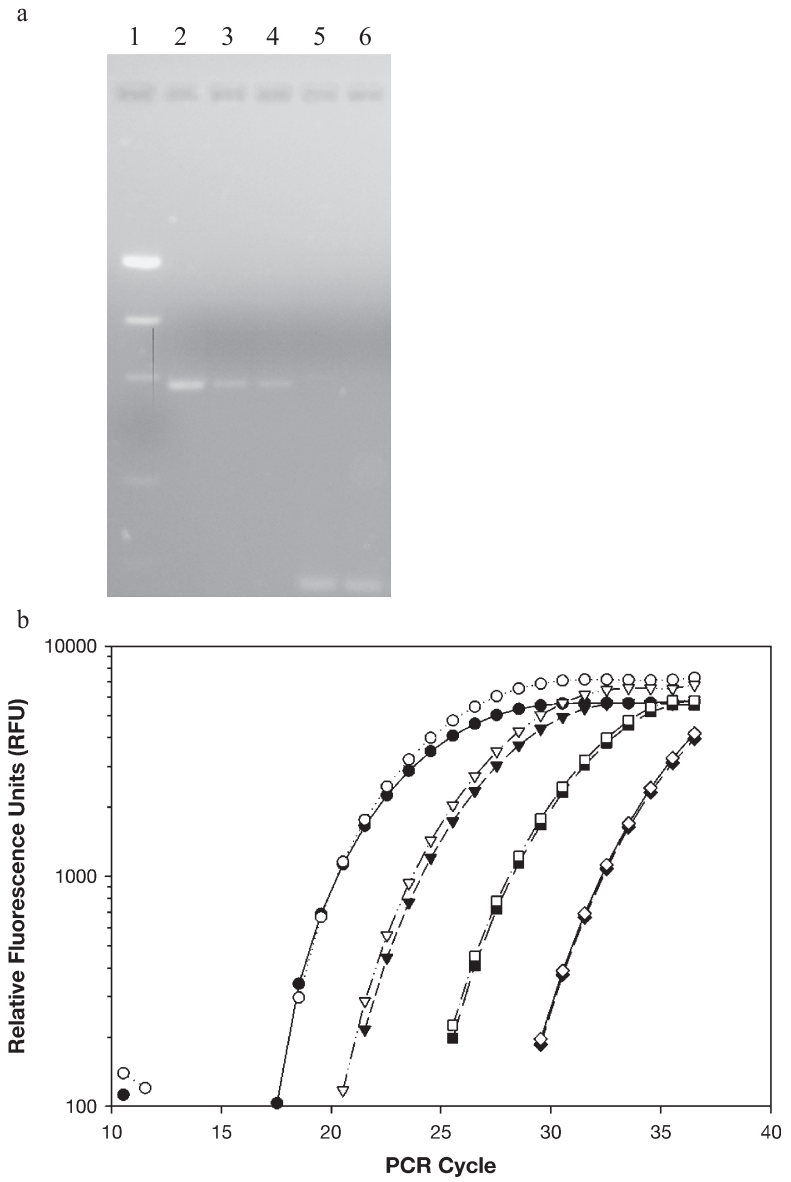

Fig. 2. Amplification plot of a 10-fold serial dilution series of *S. typhimurium* using the conventional AOAC-PCR assay. The target DNA was detected either by agarose gel electrophoresis (a) or in real time (b). (a) Two percent agarose gel prestained with ethidium bromide photographed under UV illumination. A fluorescent band at the 800-bp level indicates a positive result. Cells per PCR assay: 10^6, lane 2; 10^5, lane 3; 10^4, lane 4; 10^3, lane 5; and 10^2, lane 6, a DNA marker ladder covering 100–2000 bp is shown for reference in lane 1. (b) Real-time detection by measuring fluorescence of SYBR Green I dye after each amplification cycle (*X*-axis). Relative fluorescence units (RFU) are plotted on the *Y*-axis. Sample replicates (open and closed symbols) with cells per assay: 10^6 (◇, ◆); 10^5 (□, ■); 10^4 (▽, ▼); and 10^3 (○, ●) in the PCR assay.

ly as serovar Typhimurium, and the relative fluorescence from the MB probe increased with number of PCR cycles (Figs. 1 and 3, respectively). However, the efficiency of detection (measured as threshold cycle value, C_t) varied slightly among serovar mixtures (see below).

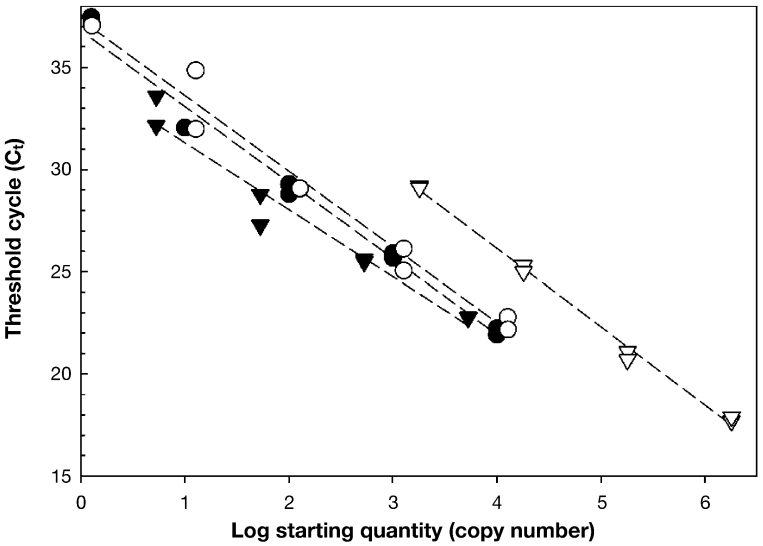

Fig. 3. Standard curve for a 10-fold serial dilution series of *Salmonella* strains (10^6 to 10^0 cells per assay, in duplicate) plotted as the threshold cycle (C_t) on the *Y*-axis using *S. typhimurim*. Symbols: ○ (data from Fig. 1) and ▽ (data from Fig. 2b). Data were also obtained from molecular beacon probe experiments using the mixture of serovars Agona, Anatum, Dublin, Haifa, and Choleraesuis (●); or serovars Pullorum electrophoretic types Pu3, and Pu4, and Paratyphi A (▼). The target copy number per assay is on the *X*-axis.

The threshold cycle (C_t) is defined as the cycle at which a significant increase in fluorescence is first recorded. The C_t value increases as the initial number of the available template molecules decreases. Thus, C_t values can potentially be used to quantify input target molecules. For MB real-time PCR assays using *Salmonella* serovar Typhimurium, C_t values decreased linearly with increasing target quantity 10^0 to 10^4 CFU per PCR assay (Fig. 3), with a correlation coefficient of 0.93. The variability for detection and quantitation between various serovar mixtures was minimal and correlation coefficient values were 0.98 and 0.94 for serovar mixtures 1 and 2, respectively. Modification of the BAX-PCR method with inclusion of SYBR Green I dye allowed the quantitative detection of the amplified target in the range 10^3 to 10^6 CFU per PCR assay, and had a high correlation coefficient of 0.99.

3.3. Evaluation of detection limits using artificially contaminated produce

The ability to detect *Salmonella* sp. from fresh produce was tested using artificially contaminated cantaloupe, cilantro, mixed salad, and alfalfa sprouts. The two PCR assays and conventional microbiological protocols were performed in parallel for each produce sample (Table 1). Uninoculated produce samples served as controls. MB probe real-time PCR assays and microbial selective media (*Salmonella–Shigella* agar and Bismuth sulphite agar) were able to detect contamination of *Salmonella* serovar Typhimurium from four different types of produce and at a low level of contamination in all samples tested (approximately 4 CFU/25 g produce). BAX-PCR assays were also able to detect *Salmonella* contamination in all samples of cantaloupe and mixed salad. However, five out of nine samples of alfalfa sprouts and one out of eight cilantro samples gave false-negative results even though the sample tested positive when analyzed using selective growth media.

Interestingly, when the MB probe detection protocol was followed, the C_t values for the detection of *Salmonella* in alfalfa and cilantro were much higher (25.37 ± 4.6 and 32.66 ± 2.86, respectively) than the C_t values observed for cantaloupe and mixed-salad (17.74 ± 0.28 and 19.66 ± 2.02, respectively), even though all produce was inoculated at 3–4 CFU/25 g. It took approximately 3–4 days to obtain definitive results from microbial selective media, about 18

Table 1
Comparative analyses of detection frequencies of *S. typhimurium* from artificially inoculated fresh produce by two PCR methods

Type of fresh produce	Contamination level (CFU/25 g)	Detection of *S. typhimurium*					
		One-step enrichment (18 h)			Two-step enrichment (24 and 3 h)		
		Detection frequency (selective media)	Molecular beacon PCR		Detection frequency (selective media)	Conventional PCR	
			Detection frequency (iQ-Check)	Quantitation (C_t value[a])		Detection frequency (BAX-PCR)	Quantitation
Alfalfa sprouts	3.7 ± 1.2	9/9	9/9	25.37 ± 4.6	9/9	4/9	NA[b]
Cantaloupe	3.4 ± 1.5	6/6	6/6	17.74 ± 0.28	6/6	6/6	NA
Mixed salad	3.3 ± 1.2	6/6	6/6	19.16 ± 2.02	6/6	6/6	NA
Cilantro	4.1 ± 2.0	8/8	8/8	32.66 ± 2.86	8/8	7/8	NA

[a] C_t value is defined as the cycle at which a significant increase in fluorescence is first recorded.
[b] NA, not applicable.

h using MB probe real-time PCR and 27 h using the BAX-PCR protocol.

4. Discussion

4.1. Advantages of real-time detection capability

Since the inception of PCR over 15 years ago, PCR methods for the detection of foodborne pathogens have been recognized as increasingly valuable diagnostic tools. The availability of commercial PCR kits for detection of specific foodborne pathogens has alleviated problems typically associated with new technology, such as reproducibility and accuracy of data in comparison with traditional microbiology procedures. The BAX-PCR method has AOAC approval status for *Salmonella* detection in milk, meat, and poultry (Bailey, 1998). Data are lacking, however, on the application of PCR detection systems for detection of bacterial pathogens in fresh fruits and vegetables (Shearer et al., 2001). Several studies have shown that plant tissue contains substances that inhibit nucleic acid isolation and the PCR reaction (Bhagwat, 2003; Liao and Shollenberger, 2003; Shearer et al., 2001). Secondly, in most PCR-based methods, identification of a specific amplicon (and thereby a respective pathogen) is achieved by determining the molecular weight of the amplified product by performing agarose-gel electrophoresis (Fratamico et al., 1995; Fratamico and Strobaugh, 1998; Shearer et al., 2001), a method not particularly suitable for large sample sizes.

The fluorogenic detection of the target DNA provides the possibility of real-time quantitative detection of a specific pathogen directly in the PCR tube, thus making it compatible for a high-throughput format (Higuchi et al., 1993; Tyagi and Kramer, 1996). Many studies have used fluorogenic PCR for the rapid detection of *Salmonella* spp. from culture, as well as from meat and poultry products (Chen et al., 1997; Kimura et al., 1999; Sharma and Carlson, 2000). Using fluorogenic linear probe targeting of the *sipB* gene of *Salmonella* strains and end-point (instead of real-time) detection, Sharma and Carlson (2000) reported a detection limit of 250 CFU/25 g of meat.

4.2. Evaluation of molecular beacon probes in detection of various Salmonella serovars

In the present study, we examined the efficacy of MB probes to detect *Salmonella* spp. from whole and fresh-cut produce. The MB beacon probe used in this study was able to detect *Salmonella* spp. from variety of fresh and fresh-cut produce at a very low level of contamination (i.e., at 1–3 CFU/25 g of produce) (Table 1). Attachment of *Salmonella* sp. to food matrices plays a crucial role in their virulence and enables to cause disease at lower their infection dose (Waterman and Small, 1998). We reported that low infection dose of *Salmonella* sp., which is observed when the bacteria are associated with food, can be explained on the basis of attachment-mediated increased acid tolerance (Gawande and Bhagwat, 2002b). However, certain serovars such as Pullorum (electrophoretic type Pu3 and Pu4) and Paratyphi A

appear to be nonresponsive to attachment-mediated acid tolerance (Gawande and Bhagwat, 2002a). Moreover, attachment also plays a crucial role in recovery of pathogens from food sources and hence may significantly influence the sensitivity of PCR-based detection protocols. The MB probe PCR assay was equally sensitive when tested with attachment-responsive and nonresponsive serovar mixtures (Fig. 3). The assay also enabled the quantitation of different *Salmonella* serovars with a high correlation coefficient. Recently, we modified the conventional AOAC-approved PCR assay (BAX-*Salmonella* kit) to enable real-time detection in a high-throughput compatible format (Bhagwat, 2003, 2004). However, as our data indicate (Fig. 2b), mere fluorogenic detection by itself was not sufficient to increase sensitivity. In fact, ethidium bromide staining and SYBR Green I fluorescence detection had identical ranges of sensitivity (Fig. 2a and b).

4.3. Factors influencing real-time sensitivity and detection limits

The occurrence of false-negative results, mainly due to the presence of DNA polymerase inhibitors in plant tissue, poor quality of target DNA, or insufficient enrichment of the target pathogen has been observed in the past (Bhagwat, 2003; Heller et al., 2003; Liao and Shollenberger, 2003; Miller, 2001; Shearer et al., 2001). However, enrichment in BPW and taking samples *without* disturbing the media at the end of enrichment helped keep inhibition by plant pigments to a minimum (comparative data for with and without shaking the enrichment broth prior to sampling for DNA isolation is not shown). Further the dilution of the DNA template (1:10 and 1:25) before the assay and presence of a suspended gel-matrix in the lysis buffer helped to reduce the concentration of inhibitory compounds to a level that did not affect DNA amplification and allowed detection of *Salmonella*. Recently, Heller et al. (2003) compared different DNA isolation methods to detect Shiga toxin-producing *Escherichia coli* O157:H7 isolates from various foods by fluorogenic linear probe (TaqMan) real-time PCR and reported a detection limit of 5.3×10^3 CFU/g of salad green or ground beef, which translates to 1.3×10^5 CFU/25 g of food. Fortin et al. (2001) reported the InstaGene matrix to be superior to conventional (phenol–chloroform) DNA isolation procedures, and reported a sensitivity of 1 CFU of *E. coli* O157:H7 per milliliter of raw milk or apple juice by MB probe real-time PCR, which is similar to the detection limits observed in this study. Both studies utilized fluorogenic probes, and the combination of superior DNA isolation and sensitive MB probes apparently achieved a better pathogen surveillance capability.

Although automated real-time PCR is rather complicated and requires costly equipment, an increasing number of reference laboratories are converting traditional gel-based detection PCR to real-time, fluorescence-based detection in order to conduct microbial quality control and handle a high sample volume (Daum et al., 2002; Hoorfar and Radstrem, 2000). This study illustrates for the first time the feasibility of using MB probe real-time PCR detection technology for detection and/or surveillance of *Salmonella* involving whole and fresh-cut fruits and vegetables.

Acknowledgements

We thank Ingrid Berlanger and Irving Newman for excellent technical assistance. SHL was supported by the Overseas Industrial Attachment Program of the School of Life Sciences and Chemical Technology, Ngee Ann Polytechnic, Singapore. We also thank William Conway, Kenneth Gross, and Pina Fratamico for critically reading the manuscript and helpful suggestions.

References

Administration, U.S.F.D., 1995. Bacteriological Analytical Manual, 8th ed. Association of Official Analytical Chemists International, Gaithersburg, MD.

Anonymous, 2000. FDA survey of imported fresh produce. No. 2000. U.S. Food and Drug Administration, http://vm.cfsan.fda.gov/~dms/.

Backer, H.D., Mohle-Boetani, J.C., Werner, S.B., Abbott, S.L., Farrar, J., Vugia, D.J., 2000. High incidence of extra-intestinal infections in a *Salmonella havana* outbreak associated with alfalfa sprouts. Public Health Rep. 115, 339–345.

Bailey, J.S., 1998. Detection of *Salmonella* cells within 24 to 26 hours in poultry samples with the polymerase chain reaction BAX system. J. Food Prot. 61, 792–795.

Beuchat, L.R., Ryu, J.H., 1997. Produce handling and processing practices. Emerg. Infect. Dis. 3, 459–465.

Beuchat, L.R., Harris, L.J., Ward, T.E., Kajs, T.M., 2001. Development of a proposed standard method for assessing the efficacy of fresh produce sanitizers. J. Food Prot. 64, 1103–1109.

Bhagwat, A.A., 2003. Simultaneous detection of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* strains by real-time PCR. Int. J. Food Microbiol. 84, 217–224.

Bhagwat, A.A., 2004. Rapid detection of *Salmonella* from vegetable rinse-water using real-time PCR. Food Microbiol. 21, 73–78.

Bopp, C.A., Brenner, F.W., Wells, J.G., Strockbine, N.A., 1999. *Escherichia*, *Shigella* and *Salmonella*. In: Murray, P.R.E., Baron, E.J., Pfaller, M.A., Tenover, F.C., Yolken, R.H. (Eds.), Manual of Clinical Microbiology. American Society for Microbiology, Washington, DC, pp. 459–474.

Brackett, R.E., 1994. Microbiological spoilage and pathogens in minimally processed fruits and vegetables. In: Wiley, R.C. (Ed.), Minimally Processed Refrigerated Fruits and Vegetables. Van Nostrand Reinhold, New York, pp. 269–312.

Brackett, R.E., 1999. Incidence, contributing factors, and control of bacterial pathogens in produce. Postharvest Biol. Technol. 15, 301–311.

Buck, J.W., Walcot, R., Beuchat, L.R., 2003. Recent trends in microbial safety of fruits and vegetables. No. 2003. Plant Health Progress.

Campbell, J.V., Mohle-Boetani, J., Reporter, R., Abbott, S., Farrar, J., Brandl, M., Mandrell, R., Werner, S.B., 2001. An outbreak of *Salmonella* serotype *Thompson* associated with fresh cilantro. J. Infect. Dis. 183, 984–987.

Chen, J., Griffiths, M.W., 2001. Detection of *Salmonella* and simultaneous detection of *Salmonella* and Shiga-like toxin producing *Escherichia coli* using the magnetic capture hybridization polymerase chain reaction. Lett. Appl. Microbiol. 32, 7–11.

Chen, S., Yee, A., Griffiths, M., Larkin, C., Yamashiro, C.T., Behari, R., Paszko-Kolva, C., De Grandis, S.A., 1997. The evaluation of a fluorogenic polymerase chain reaction assay for the detection of *Salmonella* species in food commodities. Int. J. Food Microbiol. 35, 239–250.

Chen, W., Martinez, G., Mulchandani, A., 2000. Molecular beacons: a real-time polymerase chain reaction assay for detecting *Salmonella*. Anal. Biochem. 280, 166–172.

Cocolin, L., Manzano, M., Cantoni, C., Comi, G., 1998. Use of polymerase chain reaction and restriction enzyme analysis to directly detect and identify *Salmonella typhimurium* in food. J. Appl. Microbiol. 85, 673–677.

Daum, L.T., Barnes, W.J., McAvin, J.C., Neidert, M.S., Cooper, L.A., Huff, W.B., Gaul, L., Riggins, W.S., Morris, S., Salem, A., Lohman, K.L., 2002. Real-time detection of *Salmonella* in suspect foods from a gastroenteritis outbreak in Kerr county, Texas. J. Clin. Microbiol. 40, 3050–3052.

Fang, F.C., Libby, S.J., Buchmeier, N.A., Loewen, P.C., Switala, J., Harwood, J., Guiney, D.G., 1992. The alternative sigma factor katF (rpoS) regulates *Salmonella* virulence. Proc. Natl. Acad. Sci. U. S. A. 89, 11978–11982.

Feng, P., 1997. Impact of molecular biology on the detection of foodborne pathogens. Mol. Biotechnol. 7, 267–278.

Ferretti, R., Mannazzu, I., Cocolin, L., Comi, G., Clementi, F., 2001. Twelve-hour PCR-based method for detection of *Salmonella* spp. in food. Appl. Environ. Microbiol. 67, 977–978.

Fortin, N.Y., Mulchandani, A., Chen, W., 2001. Use of real-time polymerase chain reation and molecular beacon for the detection of *Escherichia coli* O157:H7. Anal. Biochem. 289, 281–288.

Fratamico, P., Strobaugh, T.P., 1998. Simultaneous detection of *Salmonella* spp. and *Escherichia coli* O157:H7 by multiplex PCR. J. Ind. Microbiol. Biotech. 21, 92–98.

Fratamico, P., Sackitey, S.K., Wiedmann, M., Deng, M., 1995. Detection of *Escherichia coli* O157:H7 by multiplex PCR. J. Clin. Microbiol. 33, 2188–2191.

Gawande, P.V., Bhagwat, A.A., 2002a. Inoculation onto solid surfaces protect *Salmonella* spp. during acid challenge: a model study using polyethersulfone membranes. Appl. Environ. Microbiol. 68, 86–92.

Gawande, P.V., Bhagwat, A.A., 2002b. Protective effects of cold temperature and surface-contact on acid tolerance of *Salmonella* spp. J. Appl. Bacteriol. 93, 689–696.

Heller, L.C., Davis, C.R., Peak, K.K., Wingfield, D., Cannons, A.C., Amuso, P.T., Cattani, J., 2003. Comparison of methods for DNA isolation from food samples for detection of Shiga toxin-producing *Escherichia coli* by real-time PCR. Appl. Environ. Microbiol. 69, 1844–1846.

Higuchi, R., Fockler, C., Dollinger, G., Watson, R., 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Bio/Technology 11, 1026–1030.

Hill, W.E., 1996. The polymerase chain reaction: applications for the detection of foodborne pathogens. Crit. Rev. Food Sci. Nutr. 36, 123–173.

Hodge, K., 1999. Microbial safety and quality: a delicate balance in fresh-cut produce. Fresh Cut 9, 1–2.

Hoorfar, J., Radstrem, P., 2000. Automated 5′ nuclease PCR assay for identification of *Salmonella enterica*. J. Clin. Microbiol. 38, 3429–3435.

Kimura, B., Kawasaki, S., Fujii, T., Kusunoki, J., Itoh, T., Flood, S.J.A., 1999. Evaluation of TaqMan PCR assay for detecting *Salmonella* in raw meat and shrimp. J. Food Prot. 62, 329–335.

Liao, C.-H., Shollenberger, L.M., 2003. Detection of *Salmonella* by indicator agar media and PCR as affected by alfalfa seed homogenates and native bacteria. Lett. Appl. Microbiol. 36, 152–156.

Long, S.M., Adak, G.K., O'Beirne, S.J., Gillespie, I.A., 2002. General outbreaks of infectious intestinal disease linked with salad vegetables and fruits, England and Wales, 1992–2000. Commun. Dis. Public Health 5, 101–105.

McKllip, J.L., Drake, M.A., 2000. Molecular beacon polymerase chain reaction detection of *Escherichia coli* O157:H7 in milk. J. Food Prot. 63, 855–859.

Mead, P.S., Slursker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., Tauxe, R.V., 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. 5, 607–625.

Miller, D.N., 2001. Evaluation of gel filtration resins for the removal of PCR-inhibitory substances from soils and sediments. J. Microbiol. Methods 44, 49–58.

Miras, I., Hermant, D., Arricau, N., Popoff, M.Y., 1995. Nucleotide sequence of *iagA* and *iagB* genes involved in invasion of HeLa

cells by *Salmonella enterica* subsp. *enterica* ser. Typhi. Res. Microbiol. 146, 17–20.

Mohle-Boetani, J.C., Reporter, R., Werner, S.B., Abbott, S., Farrar, J., Waterman, S.H., Vugia, D.J., 1999. An outbreak of *Salmonella* serogroup saphra due to cantaloupes from Mexico. J. Infect. Dis. 180, 1361–1364.

Mrozinski, P.M., Betts, R.P., Coates, S., 1998. Performance tested method certification of Bax for screening *Salmonella*: a case study. J. AOAC Int. 81, 1147–1154.

NACMCF (National Advisory Committee on Microbiological Criteria for Foods), 1999. Microbiological safety evaluations and recommendations on fresh produce. Food Control 10, 117–143.

Nogva, H.K., Lillehaug, D., 1999. Detection and quantification of *Salmonella* in pure cultures using 5′-nuclease polymerase chain reaction. Int. J. Food Microbiol. 51, 191–196.

Rijpens, N., Herman, L., Vereecken, F., Jannes, G., Smedt, J.D., Zutter, L.D., 1999. Rapid detection of stressed *Salmonella* spp. in dairy and egg products using immunomagnetic separation and PCR. Int. J. Food Microbiol. 46, 37–44.

Sharma, V.K., Carlson, S.A., 2000. Simultaneous detection of *Salmonella* strains and *Escherichia coli* O157:H7 with fluorogenic PCR and single-enrichment-broth culture. Appl. Environ. Microbiol. 66, 5472–5476.

Shearer, A.E.H., Strapp, C.M., Joerfer, R.D., 2001. Evaluation of a polymerase chain reaction-based system for detection of *Salmonella enteritidis*, *Escherichia coli* O157:H7, *Listeria* spp., and *Listeria monocytogenes* on fresh fruits and vegetables. J. Food Prot. 64, 788–795.

Thunberg, R.L., Tran, T.T., Bennett, R.W., Matthews, R.N., Belay, N., 2002. Microbial evaluation of selected fresh produce obtained at retail markets. J. Food Prot. 65, 677–682.

Tyagi, S., Kramer, F.R., 1996. Molecular beacon: probes that fluoresce upon hybridization. Nat. Biotechnol. 14, 303–308.

Waterman, S.R., Small, P.L.C., 1998. Acid-sensitive enteric pathogens are protected from killing under extremely acidic conditions of pH 2.5 when they are inoculated onto certain solid food sources. Appl. Environ. Microbiol. 64, 3882–3886.

Weagant, S.D., Jagow, J.A., Jinneman, K.C., Omiecinski, C.J., Kaysner, C.A., Hill, W.E., 1999. Development of digoxigenin-labeled PCR amplicon probes for use in detection and identification of enteropathogenic *Yersinia* and Shiga toxin-producing *Escherichia coli* from foods. J. Food Prot. 62, 438–443.

Wells, J.M., Butterfield, J.E., 1997. *Salmonella* contamination associated with bacterial soft rot of fresh fruits and vegetables in the marketplace. Plant Dis. 81, 867–872.