Ann Microbiol (2016) 66:883–888
DOI 10.1007/s13213-015-1178-y

ORIGINAL ARTICLE

Evaluation of bacteriophage amplification assay for rapid detection of *Shigella boydii* in food systems

Lae-Seung Jung[1] · **Juhee Ahn**[1]

Received: 6 June 2015 / Accepted: 28 October 2015 / Published online: 7 November 2015

Abstract This study was designed to investigate the possibility of using bacteriophages for the detection of viable *Shigella boydii* in food products. A *Shigella* bacteriophage belonging to a member of the *Siphoviridae* family was isolated from swine fecal samples. The free bacteriophages were highly stable against pH 4.0 to 9.0 and temperature change (z-value=17.1 °C). The bacteriophage amplification assay was able to selectively detect *S. boydii* in a bacterial mixture of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* Typhimurium. The number of *S. boydii* bacteriophages enumerated by the amplification assay was highly correlated with the number of viable *S. boydii* in single (r=0.987) and mixed (r=0.969) cultures. The bacteriophage-based detection of *S. boydii* was highly reproducible in lettuce (6.3 log CFU/ml and 4.9 log PFU/ml) and cooked chicken breasts (6.1 log CFU/ml and 6.0 log PFU/ml). These results suggest that the bacteriophage amplification assay can be used as an alternative method for rapid, selective, and cost-effective detection of *S. boydii* in food products, and provides useful information for designing a quick and simple detection kit.

Keywords *Shigella boydii* · Bacteriophage · Amplification assay · Lettuce · Chicken breast

✉ Juhee Ahn
juheeahn@kangwon.ac.kr

[1] Department of Medical Biomaterials Engineering and Institute of Bioscience and Biotechnology, Kangwon National University, Chuncheon, Gangwon 200-701, Republic of Korea

Introduction

As microbiological safety continues to be a top priority in the food industry, research endeavors over the last few decades have led to the development of rapid, sensitive, and selective detection methods (Gracias and McKillip 2004; Mandal et al. 2011). The timely determination of bacterial contamination in food is critically important for appropriate treatment and effective prevention of serious bacterial contamination. The traditional pathogen detection platforms are generally time-consuming, labor-intensive, and costly, and require several steps including bacterial enrichment, biochemical identification, and serological confirmation (Lleo et al. 2005; Javed et al. 2013). A number of immunological, genomic, and proteomic techniques have been applied for the accurate detection and identification of bacterial pathogens (Mandal et al. 2011; Riahi et al. 2011). Although advanced detection tools have been proven powerful in terms of sensitivity and selectivity for the identification of pathogens contaminating foods, they require extensive training, technical experience, expensive instruments, and substantial sample preparation (McNerney et al. 1998; Oliveira et al. 2012; Javed et al. 2013). Therefore, the development of a simple and practical detection tool for microbiological analysis has been an area of interest for many researchers.

Bacteriophage-based detection technologies using engineered, reporter, and whole-type bacteriophages have recently received great attention due to their high specificity and low cross-reactivity (Smartt et al. 2012). Unlike engineered bacteriophage-based detection, the bacteriophage amplification assay is a relatively simple and easy method, enabling the quantitative detection of viable pathogens within a few hours (Stewart et al. 1998; Park et al. 2003; Botsaris et al. 2010). This technique is based on the principle of specific interactions between bacteriophage and host bacteria, and

consists of four steps: bacteriophage infection of the target host, inactivation of free bacteriophages, neutralization, and amplification (de Siqueira et al. 2006). Small-scale food processing facilities commonly have difficulty implementing new technologies due to a lack of technical experience and resources. The simple protocol of the bacteriophage amplification assay can be applied in small-scale food processing units as an easy and rapid method for confirming foodborne pathogens in food systems. Therefore, the objective of this study was to evaluate bacteriophage amplification as a practical approach for detecting and confirming the presence of *Shigella boydii* in lettuce and cooked chicken breasts.

Materials and methods

Bacterial strains and bacteriophage isolation

Strains of *Escherichia coli* O157:H7 KACC 11598, *Shigella boydii* KACC 10792, and *Listeria monocytogenes* KACC 12671 were kindly provided by the Korean Agricultural Culture Collection (KACC; Suwon, Korea). The *Salmonella* Typhimurium KCCM 40253 strain was obtained from the Korean Culture Center of Microorganisms (KCCM; Seoul, Korea). The strains were cultured in Trypticase soy broth (TSB; Becton, Dickinson and Company [BD], Franklin Lakes, NJ, USA) at 37 °C for 20 h and harvested by centrifugation at 3000 × *g* for 20 min at 4 °C. To isolate *Shigella* bacteriophages, swine fecal samples (10 g each) were mixed with 20 ml of TSB, and the mixtures were inoculated with the bacterial host *S. boydii* (10^6 CFU/ml) and incubated at 37 °C for 20 h. After centrifugation at 5000 × *g* for 10 min, the supernatant was filtered using a 0.2-μm membrane filter to completely eliminate fecal particles. The filtrates (100 μl each) were gently suspended in TSB with 0.5 % agar and poured on the lawn of *S. boydii*. The plates were incubated at 37 °C for 24 to 48 h. *Shigella* bacteriophages were isolated from the clear zone on the top agar.

Bacteriophage preparation and plaque assay

The isolated bacteriophages were propagated at 37 °C for 20 h in TSB containing the *S. boydii* bacterial host strain. After propagation, the cell-free culture supernatants were collected by centrifugation at 3000 × *g* for 20 min and then filtered through a 0.2-μm sterilized filter. The filtrates were further purified by polyethylene glycol (PEG) precipitation and cesium chloride (CsCl) gradient ultracentrifugation. The purified phage stocks were enumerated by a soft-agar overlay method (Bielke et al. 2007). In brief, the phages were serially (1:10) diluted with phosphate buffered saline (PBS, pH 7.2) and gently mixed with *S. boydii* cells in 0.5 % TSB soft-agar. The mixtures were poured onto the surface of pre-warmed 1.5 % TSB base agar. After 24 h of incubation at 37 °C, the numbers of lytic phages were expressed as plaque-forming units (PFU/ml).

Microbiological analysis

Duplicate samples were aseptically mixed with 0.1 % buffered peptone water (BPW) and then serially (1:10) diluted with 0.1 % BPW. Each dilution (50 μl) was plated on trypticase soy agar (TSA) using an Autoplate® Spiral plating system (Spiral Biotech, Inc., Norwood, MA, USA). The plates were incubated at 37 °C for 24 to 48 h, and bacterial host cells were then enumerated using a QCount® Colony Counter (Spiral Biotech, Inc.).

pH and thermal stability of Shigella bacteriophages

The pH susceptibility of *Shigella* bacteriophages was evaluated in TSB adjusted to various pH values (2–12) by adding 1 M HCl or NaOH. *Shigella* bacteriophages (4.5×10^6 PFU/ml) were inoculated in TSB at different pH values and then incubated at 37 °C for 30 min. The titers of bacteriophages were determined using a soft-agar overlay assay. Thermal inactivation of *Shigella* bacteriophages was determined at 50, 55, 60, 65, and 70 °C for 30 min. For isothermal treatment, 0.1 ml of *Shigella* bacteriophages (1.1×10^6 PFU/ml) was inoculated into a glass test tube containing 4.9 ml of PBS preheated to each target temperature using a 10-L oil bath. After the thermal treatments, each glass test tube was immediately immersed in an ice-water bath to avoid further inactivation. Viable bacteriophage counts were determined using a soft-agar overlay assay. *Escherichia coli* O157:H7 bacteriophages were used as control. The decimal reduction time (D-value) was estimated using the equation:

$$\log\frac{N_t}{N_0} = -\frac{t}{D}$$

where N_0 and N_t are the number of bacteriophages at the beginning and at time t (min) of thermal treatment, respectively. The z-values (temperature required for 1 log reduction in D-value) were estimated by plotting log D against temperature.

Morphological assay

The morphological characteristics of *Shigella* bacteriophages were evaluated by transmission electron microscope (TEM, LEO 912AB Omega; Carl Zeiss NTS GmbH, Oberkochen, Germany) in the Korea Basic Science Institute. (KBSI; Gangwon, Korea). The bacteriophages were negatively stained with 5 % aqueous uranyl acetate (pH 4.0) and then dried on a carbon-coated copper grid. The stained

bacteriophages were examined with TEM (120 kV accelerating voltage, ×125,000 magnification).

Experimental design

The experiment for evaluating the phage amplification assay was carried out in two steps: 1) application of the phage amplification assay for media and 2) validation of the phage amplification assay in challenging food matrices. For the culture medium study, the sensitivity and selectivity of the bacteriophage amplification assay were evaluated in single (*S. boydii*) and mixed (*E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium, and *S. boydii*) cultures, respectively, over a range of 10^7 to 10 CFU/ml. For the food challenge study, the ability of the phage amplification assay to detect *S. boydii* was evaluated in lettuce and cooked chicken inoculated with 2×10^6 CFU/cm^2 and 2×10^6 CFU/g of *S. boydii*, respectively.

Virucide susceptibility assay

The susceptibility of phages and host bacterial cells to ferrous ammonium sulfate (FAS) was evaluated to determine the FAS concentration required to effectively destroy free phages without affecting host cell viability (McNerney et al. 1998; Park et al. 2003). The freshly prepared FAS solution was serially (1:2) diluted with TSB from 32 to 0.5 mM. The host bacterial cells (10^8 CFU/ml) or phages (10^8 PFU/ml) were treated with different concentrations of FAS in 96-well flat-bottom polystyrene microtiter plates (BD Falcon; BD Biosciences, San Jose, CA, USA) and incubated at 37 °C for 15 min. Total bacteriophages and host cells were enumerated by a soft-agar overlay method and an Autoplate® Spiral Plating System (Spiral Biotech, Inc., Norwood, MA, USA), respectively.

Phage amplification assay in culture media

The *S. boydii* bacterial host cells were serially (1:10) diluted with TSB to achieve a range of approximately 10^8 to 10^2 CFU/ml. Each diluent (100 μl) was gently mixed with 100 μl of bacteriophage (10^8 PFU/ml) and then incubated at 37 °C for 15 min. The optimal adsorption time was previously determined using a bacteriophage adsorption assay. The mixtures were treated with 150 μl of FAS at 37 °C for 3 min to eliminate free bacteriophages, and neutralized with 150 μl of 2 % Tween 80. The host cell control was prepared by substituting 100 μl of TSB for the bacteriophage, and proceeding in the same manner as described in the bacteriophage amplification assay. Total bacteriophages and host cells were enumerated by a soft-agar overlay method and an Autoplate® Spiral Plating System (Spiral Biotech, Inc., Norwood, MA, USA), respectively. To evaluate the selectivity of the bacteriophage amplification assay, a bacterial mixture (*E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium, and *S. boydii*) was treated using the same procedure as described above.

Phage amplification assay in foods

Fresh lettuce and cooked chicken breasts were purchased from a local supermarket. Lettuce cut into pieces 5 × 5 cm in size and 25-g portions of cooked chicken were inoculated with approximately 2×10^6 CFU/cm^2 and 2×10^6 CFU/g of *S. boydii*, respectively. The inoculated samples were separately packaged in sterile Nasco Whirl-Pak bags (Fisher Scientific, St. Louis, MO, USA) and aseptically mixed with 25 ml of PBS. The mixtures were homogenized for 2 min using a stomacher laboratory BagMixer® 400 (Interscience Lab. Inc., Rockland, MA, USA). The numbers of *S. boydii* inoculated in lettuce and cooked chicken were determined using a bacteriophage amplification assay as described above.

Statistical analysis

All analyses were performed in duplicate on three replicates. Data were analyzed using SAS statistical software (SAS Institute Inc., Cary, NC, USA). The general linear model (GLM) and least significant difference (LSD) procedures were used to determine significant mean differences among strains and culture conditions at $p<0.05$.

Results and discussion

Because infectious enteric diseases are largely associated with the consumption of foods contaminated with foodborne pathogens, the development of methods for the rapid and early detection of foodborne pathogens remains a high priority in small-scale food processing establishments. In this context, this study aimed to explore the possibility of using a bacteriophage amplification assay for the detection of *S. boydii* in lettuce and cooked chicken breasts.

Morphological and physiological properties of bacteriophage isolated from swine fecal samples

The morphological characteristics of the *S. boydii* bacteriophage isolated from swine fecal samples were determined using TEM (Fig. 1). The bacteriophage has a polyhedral structure (70 nm) with a long tail (142 nm) typical of the *Siphoviridae* family. The pH sensitivity of the *S. boydii* and *E. coli* O157:H7 bacteriophages was compared across a range of different pH values from 2 to 12 (Fig. 2a). *Shigella boydii* and *E. coli* O157:H7 bacteriophages were highly stable when inoculated in broth at pH 4 to 9 and pH 5 to 11, respectively, showing no significant reduction in the number of viable

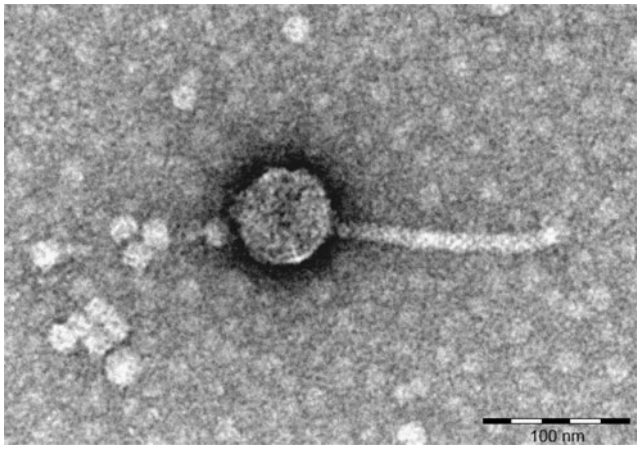

Fig. 1 TEM image of *Shigella boydii* bacteriophages isolated from swine fecal sample

bacteriophages. The stability of *S. boydii* bacteriophages was significantly reduced at pH below 3 and at pH 12. In general, most bacteriophages are stable between pH 5.0 and 8.0 (Mishra et al. 2012). Following heat treatment for 30 min, the D-values of *S. boydii* and *E. coli* O157:H7 bacteriophages decreased with increasing temperature, showing that D_{50}, D_{55}, D_{60}, D_{65}, and D_{70} were 66.9, 24.0, 11.6, 6.6, and 4.2 min for *S. boydii* bacteriophages and 147.3, 71.0, 31.6, 7.6, and 4.1 min for *E. coli* O157:H7 bacteriophages, respectively. Thermal stability was evaluated using the *z*-values calculated for *S. boydii* and *E. coli* O157:H7 bacteriophages heated at 50 °C, 55 °C, 60 °C, 65 °C, and 70 °C (Fig. 2b). *Shigella boydii* bacteriophages (*z*-value=17.1 °C) were less sensitive to temperature changes than *E. coli* O157:H7 bacteriophages (*z*-value=12.3 °C).

Selectivity of bacteriophage amplification assay for *Shigella* detection in single and mixed cultures

The relationship between the colony (CFU/ml) and plaque (PFU/ml) counts was evaluated in *S. boydii* alone and in mixed cultures of *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium, and *S. boydii* (Fig. 3). FAS at a working concentration of 10 mM demonstrated effective inactivation of bacteriophages without affecting bacterial viability. Standard curves were generated by plotting the number of *S. boydii* enumerated using the plate count method against the number of *S. boydii* bacteriophages detected by the bacteriophage amplification assay. The number of *S. boydii* bacteriophages was highly correlated with the number of *S. boydii* in single (r=0.987) and mixed (r=0.969) cultures (p<0.001). The number of plaques was consistently lower than that of colonies due to the bacteriophage adsorption rate. This observation also suggests that the bacteriophage-infected *S. boydii* was not lysed until plating was performed (McNerney et al. 1998). No significant differences were observed between single and mixed cultures in the corresponding numbers of plaques and colonies. This indicates that the bacteriophage amplification assay was able to selectively detect *S. boydii* in a broth system without interference from other bacteria, *E. coli* O157:H7, *L. monocytogenes*, and *S.* Typhimurium. The phylogenetic characteristics of pathogenic *E. coli* are closely related to those of *Shigella* spp. belonging to the Enterobacteriaceae family, including *S. boydii*, *S. dysenteriae*, *S. flexneri*, and *S. sonnei* (Wyckoff et al. 1998; Li et al. 2009; Wang and Chen 2012), which is a direct cause of difficulty in detecting *Shigella* spp. using traditional identification methods such as culture media, phenotyping, and serotyping. Therefore, bacteriophage-based detection of *Shigella* provides many advantages in terms of speed and selectivity.

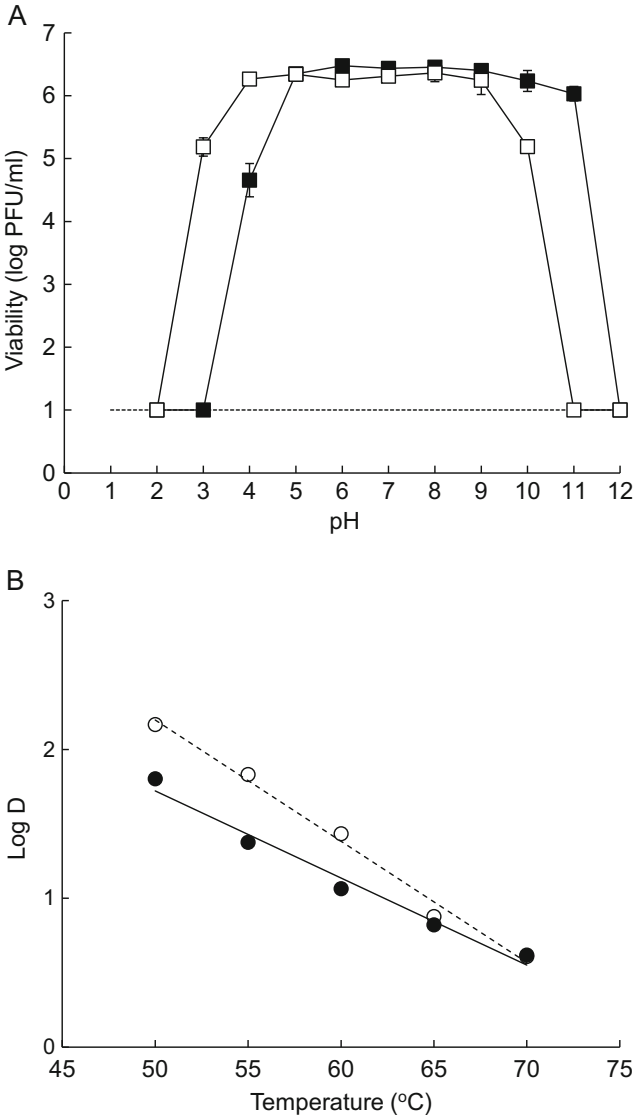

Fig. 2 pH stability (**a**) of *Shigella boydii* bacteriophages (■) and *Escherichia coli* O157:H7 (□) bacteriophages, and heat sensitivity (**b**) of *S. boydii* (●, ——) and *E. coli* O157:H7 (○, – – –) bacteriophages. The dotted line (, A) indicates the limit of detection of method used (10 PFU/ml)

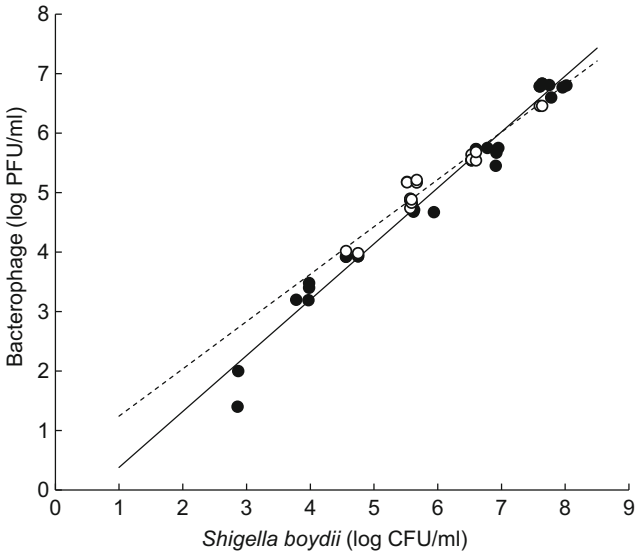

Fig. 3 Relationship between the number of *Shigella boydii* enumerated using the plate count method (CFU/ml) and the number of *S. boydii* bacteriophages detected by the bacteriophage amplification assay (PFU/ml) in single (*S. boydii*; ●, ——, r=0.987, n=33) and mixed cultures (*S. boydii*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* Typhimurium; ○, – – –, r=0.969, n=17) cultures

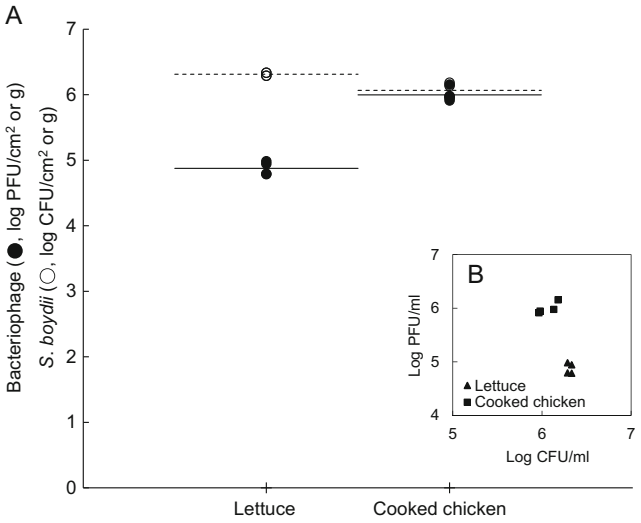

Fig. 4 Quantitative detection of *Shigella boydii* (CFU, ○) and *S. boydii* bacteriophages (PFU, ●) in lettuce and cooked chicken by the bacteriophage application assay. *Lines* indicate the average of *S. boydii* (CFU, – – –) and *S. boydii* bacteriophages (PFU, ——) (n=16)

Practical application for validating the bacteriophage amplification assay in different food matrices

A bacteriophage amplification assay was applied for the detection of *S. boydii* in lettuce and cooked chicken breasts (Fig. 4). Differences between the number of colonies and plaques were consistent in lettuce (6.3 log CFU/ml and 4.9 log PFU/ml) and cooked chicken breasts (6.1 log CFU/ml and 6.0 log PFU/ml) (Fig. 4a), and may be attributed to the adsorption rate of bacteriophages and the interference of food components. As such, further studies with various bacteriophages and foods are needed to establish correction factors for accurately predicting the number of *Shigella* contaminated. The bacteriophage amplification assay was highly reproducible in lettuce and cooked chicken breasts, with repeated measurements closely grouped, as shown in Fig. 4b. The assay was able to precisely estimate the number of *S. boydii* inoculated in both lettuce and cooked chicken breasts. Given that traditional detection methods for *Shigella* spp. in foods commonly produce false-negative results (Thiem et al. 2004; Mokhtari et al. 2012), the bacteriophage amplification assay can be considered an attractive alternative for the simple and accurate detection of viable *Shigella* within a period of a few hours. Furthermore, no specialized instruments are required with this method, which enables the targeting of multiple foodborne pathogens in a single assay.

In conclusion, the most significant finding in this work was the demonstrated capacity of the bacteriophage amplification assay for the selective detection of *S. boydii*, thus resulting in reduced detection time. The application of a highly rapid and selective detection method is essential for enhancing food safety. To this end, the bacteriophage amplification assay is suitable for the routine detection of viable *Shigella* in food products. Bacteriophage-based detection of foodborne pathogens is easily accessible for small-scale food processing facilities. Further study is needed to improve the specificity of the bacteriophage amplification assay through combination with PCR-based detection methods.

Acknowledgments This study was supported by a 2015 Research Grant from Kangwon National University (Grant No. 520150317). The authors also thank Ms. Myeong-Seon Jung of the Korea Basic Science Institute (KBSI, Chuncheon, Korea) for her technical assistance in the analytical field using transmission electron microscopy (TEM).

References

Bielke L, Higgins S, Donoghue A, Donoghue D, Hargis BM (2007) *Salmonella* host range of bacteriophages that infect multiple genera. Poult Sci 86:2536–2540

Botsaris G, Slana I, Liapi M, Dodd C, Economides C, Rees C, Pavlik I (2010) Rapid detection methods for viable *Mycobacterium avium* subspecies paratuberculosis in milk and cheese. Int J Food Microbiol 141:S87–S90

de Siqueira RS, Dodd CER, Rees CED (2006) Evaluation of the natural virucidal activity of teas for use in the phage amplification assay. Int J Food Microbiol 111:259–262

Gracias KS, McKillip JL (2004) A review of conventional detection and enumeration methods for pathogenic bacteria in food. Can J Microbiol 50:883–890

Javed MA, Poshtiban S, Arutyunov D, Evoy S, Szymanski CM (2013) Bacteriophage receptor binding protein based assays for the simultaneous detection of *Campylobacter jejuni* and *Campylobacter coli*. PLoS ONE 8:1–10

Li Y, Cao B, Liu B, Liu D, Gao Q, Peng X, Wu J, Bastin DA, Feng L, Wang L (2009) Molecular detection of all 34 distinct O-antigen forms of *Shigella*. J Med Microbiol 58:69–81

Lleo MM, Bonato B, Tafi MC, Signoretto C, Pruzzo C, Canepari P (2005) Molecular *vs* culture methods for the detection of bacterial faecal indicators in groundwater for human use. Lett Appl Microbiol 40: 289–294

Mandal PK, Biswas AK, Choi K, Pal UK (2011) Methods for rapid detection of foodborne pathogens: an overview. Am J Food Technol 6:87–102

McNerney R, Wilson SM, Sidhu AM, Harley VS, Al Suwaidi Z, Nye PM, Parish T, Stoker NG (1998) Inactivation of mycobacteriophage D29 using ferrous ammonium sulphate as a tool for the detection of viable *Mycobacterium smegmatis* and *M. tuberculosis*. Res Microbiol 149:487–495

Mishra CK, Choi TJ, Kang SC (2012) Isolation and characterization of a bacteriophage F20 virulent to *Enterobacter aerogenes*. J Gen Virol 93:2310–2314

Mokhtari W, Nsaibia S, Majouri D, Ben Hassen A, Gharbi A, Aouni M (2012) Detection and characterization of *Shigella* species isolated from food and human stool samples in Nabeul, Tunisia, by molecular methods and culture techniques. J Appl Microbiol 113:209–222

Oliveira IC, Almeida RCC, Hofer E, Almeida PF (2012) Bacteriophage amplification assay for detection of *Listeria* spp. using virucidal laser treatment. Braz J Microbiol 43:1128–1136

Park DJ, Drobniewski FA, Meyer A, Wilson SM (2003) Use of a phage-based assay for phenotypic detection of *Mycobacteria* directly from sputum. J Clin Microbiol 41:680–688

Riahi R, Mach KE, Mohan R, Liao JC, Wong PK (2011) Molecular detection of bacterial pathogens using microparticle enhanced double-stranded DNA probes. Anal Chem 83:6349–6354

Smartt A, Xu T, Jegier P, Carswell J, Blount S, Sayler G, Ripp S (2012) Pathogen detection using engineered bacteriophages. Anal Bioanal Chem 402:3127–3146

Stewart J, Denyer N, Linley D (1998) The specific and sensitive detection of bacterial pathogens within 4 h using bacteriophage amplification. J Appl Microbiol 84:777–783

Thiem VD, Sethabutr O, von Seidlein L, Van Tung T, Canh DG, Chien BT, Tho LH, Lee H, Houng H-S, Hale TL, Clemens JD, Mason C, Trach DD (2004) Detection of *Shigella* by a PCR assay targeting the *ipaH* gene suggests increased prevalence of Shigellosis in Nha Trang, Vietnam. J Clin Microbiol 42: 2031–2035

Wang S-J, Chen JH (2012) A rapid and specific PCR method for the detection of *Shigella* spp. in spiked samples. J Food Drug Anal 20:59–65

Wyckoff EE, Duncan D, Torres AG, Mills M, Maase K, Payne SM (1998) Structure of the *Shigella dysenteriae* haem transport locus and its phylogenetic distribution in enteric bacteria. Mol Microbiol 28:1139–1152