JEB™
Website : www.jeb.co.in
E-mail : editor@jeb.co.in
Journal of Environmental Biology
ISSN: 0254-8704 (Print)
ISSN: 2394-0379 (Online)
CODEN: JEBIDP

Rapid propidium monoazide cPCR assay for exclusive quantification of viable *Salmonella* spp. cells

N. M. Sunar[1]*, D.I. Stewart[2], L.A. Fletcher[2], E.I. Stentiford[2] and Muhammad Aqeel Ashraf[3,4]

[1]Department of Civil Engineering Technology, Faculty of Engineering Technology, Universiti Tun Hussein Onn Malaysia, 86400, Batu Pahat, Johor, Malaysia

[2]Pathogen Control Engineering (PaCE) Institute, School of Civil Engineering, University of Leeds, Leeds, LS2 9JT, United Kingdom.

[3]Faculty of Science & Natural Resources, University Malaysia Sabah 88400 Kota Kinabalu, Sabah, Malaysia

[4]Department of Environmental Science and Engineering, School of Environmental Studies, China University of Geosciences, 430074 Wuhan, P. R. China

*Corresponding Author E-mail: **shuhaila@uthm.edu.my**

Publication Info

Paper received:
10 December 2015

Revised received:
27 April 2016

Re-revised received:
5 May 2016

Accepted:
23 June 2016

Abstract

Combination of pretreatment with propidium monoazide by competitive polymerase chain reaction (cPCR) was evaluated to enumerate the viability of *Salmonella* spp. The results showed that PMA treatment was effective in preventing the cPCR detection of target sequences from non-viable cells. In less than 5 hrs, this method generated a signal from viable but nonculturable (VBNC) *Salmonella* spp. The standard culture method gave approximately 1-2 log^{10} cfu ml^{-1} less as compared to the PMA-cPCR results. These results provided evidence to support the VBNC state, whereas, the viable cells failed to be cultured by SCM. The proposed method did not detect DNA from dead *Salmonella* spp. but recognizes the infectious potential of the VBNC state and is thereby, able to assess the effect of control strategies and provide trustworthy data for risk assessment.

Key words

Competitive PCR, Pathogen indicator, Propidium monoazide, *Salmonella* spp.

Introduction

Salmonellosis is an enteric pathogen that has frequently been reported for causing illness in many industrialized countries. *Salmonella* can be found in humans and in various animal species, and is generally shed in their feces. The operations that take place in slaughterhouses, feed mills, food production units, and with herds of livestock are the most common sources of *Salmonella* that pose a threat to human health (Yang *et al.,* 2012; Elizaquível *et al.,* 2012). *Salmonella* strains are responsible for most cases of gastroenteritis, enteric fever, septicemia, and are capable of surviving outside their host for various periods of time (Sunar *et al.,* 2009a, 2009b).

The standard cultivation methods typically take around 24 hours or more to quantify the amount of *Salmonella* in samples (Sunar *et al.,* 2010a). These methods have several disadvantages apart from being time-consuming (Jacobsen & Bech 2012; Sunar *et al.,* 2009a). They are not always specific, they omit organisms that are viable in their natural environment but cannot be cultured (VBNC) as these sometimes fail to detect *Salmonella* spp. when present (Trevors, 2012).

The PCR is well known as an effective tool for detecting bacteria in environmental samples; however, PCR can only provide qualitative results. Competitive PCR (cPCR) is a powerful technique for quantifying DNA that

Online Copy

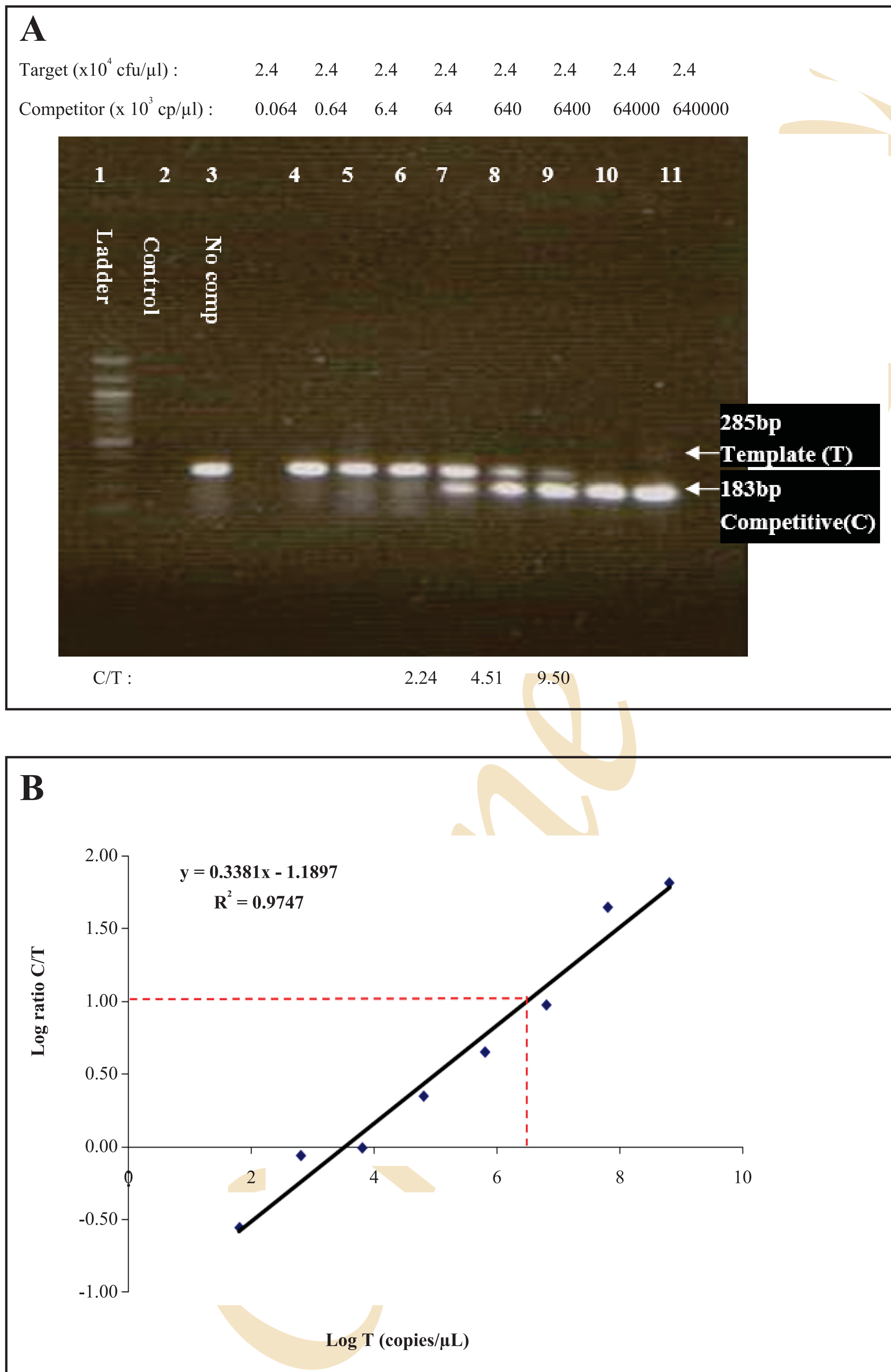

Fig. 1 : The outcome from cPCR. (Panel A) The amplification of a fixed amount of *Salmonella* spp DNA (2.4 x 104 cfu/µl) with increasing amounts of competitor DNA from 64 copies/µl (Lane 4) to 6.4E+10^8 copies/µl (Lane 11). (Panel B) cPCR validation. The quantification of template/competitor ratio after being quantified by Image J software.

overcomes the limitations of conventional PCR by co-amplifying the target DNA fragment with a known amount of competitor DNA fragment. If the competitor fragment is selected, as it shares most of its sequence with the target particularly the primer annealing sites, then the two templates will be subject to the same predictable and unpredictable variables that affect the amplification rate (Zentilin and Giacca, 2007). Thus, the ratio of the target and competitor after PCR reflects the relative initial amount of two templates. Often the competitor is selected to be slightly shorter than the target so that the two fragments can be readily distinguished after PCR (e.g., by agarose gel electrophoresis). Quantification is more straightforward if the series concentration of target and competitor are similar; thus, a cPCR experiment usually involves reactions containing the same amount of target and a serial dilution of a known amount of competitor.

The technique of cPCR targets the DNA sequence that co-amplifies with known amount of a competitor DNA, which shares most of its sequence with the target. As the target sequence and competitor fragment are amplified at the same rate, their relative abundance in the PCR product is a measure of their initial relative abundance.

As a matter of fact, though a PCR-based technique, such as cPCR, is considered to have potential as a fast alternative method to detect pathogenic bacteria in samples but unfortunately it can not differentiate between live or dead cells as the DNA of both would be amplified together. This could result in the overestimation of (pathogenic) organisms, particularly in samples that have undergone some form of sanitization treatment. Thus, differentiation between live and dead cells is an important challenge in microbial enumeration methods.

Recently, to solve this limitation, propidium monoazide and ethidium monoazide have been reported to be useful in determining viable DNA using PCR technique (Elizaquível *et al.*, 2012). Some researchers indicated that ethidium monoazide-PCR was likely to be the most useful method to differentiate between viable and dead cells (Blooi *et al.*, 2013). However, other reports indicated that propidium monoazide was more advantageous than ethidium monoazide (Lee and Levin, 2009).

Propidium monoazide only penetrates into dead cells, whereas ethidium monoazide has been shown to be incorporated in living cells as well, leading to a substantial loss of DNA (Fittipaldi *et al.*, 2012). Propidium monoazide functions as a DNA-intercalating dye with groups to allow covalent binding of the chemical to DNA upon exposure to bring visible light, known as photoactivation (Wang *et al.,* 2014). Propidium monoazide can penetrate comprised membranes (that generally occur in dead cells), after which they bind to DNA after photo-induction of azide group. DNA covalently bound to propidium monoazide cannot be subsequently amplified by PCR and free propidium monoazide is deactivated during photo-activation. The process renders the DNA insoluble and it will be removed during the DNA extraction protocol (Frankenhuyzen *et al.,* 2013). Thus, only DNA from viable cells is amplified in a PCR on a template solution that has been treated with propidium monoazide. This paper reports the development of a novel cPCR protocol that is combined with propidium monoazide pre-treatment. According to existing knowledge, propidium monoazide has never been coupled with a cPCR method to test the viability of *Salmonella* spp. The aim of the present study was to examine the potential usefulness of propidium monoazide-cPCR to quantify viability of *Salmonella* DNA in aqueous samples. With this aim, the first

Fig. 2 : *Salmonella* spp. DNA cell results for samples that contained live cells, dead cells, and mixture of live and dead cells. Lane 1: Ladder (100bp New England Bio); Lane 2: Negative control; Lane 3: Positive control; Lane 4: 100% of dead cells of *Salmonella* spp.; Lane 5: 100% of live cells of *Salmonella* spp.; Lane 6: Mixture of 50% dead and 50% live *Salmonella* spp. cells.

propidium monoazide-cPCR was evaluated and compared with those derived from plate counts. This improved technique will be useful as an essential tool to differentiate between viable and dead cells of pathogen counts, especially in waste, food and environment samples.

Materials and Methods

Bacterial cell culture and culture media : *Salmonella enteritidis* culti-loops, ATTC 13076 (Remel, Lenexa, KS) were suspended in a tryptone soya broth (Oxoid). Tryptone soya broth (TSB) was prepared earlier by suspending 30g in one liter of distilled water in a clean flask. The flask containing the broth was then sterilized by autoclaving at 121°C for 15 mins. The cell was grown in tryptone soya broth approximately after 24 hrs in the fridge. The cell culture was enumerated by plating appropriate serial dilutions using a sterile ringer solution on tryptone soya agar from Oxoid. It was prepared by suspending 40g of tryptone soya agar in one liter of purified water and boiled until it dissolved completely; it was then sterilized at 121°C for 15 mins. The cells grown in the TSB were counted by plating out on TSA immediately after incubation for 24 hours at 37°C. Approximately, 500µl of *Salmonella* spp. solution was then distributed into tubes with three replicates. This cell culture, known as the template that would be used in cPCR works. The TSA was also used for enumerating the colony forming unit in an aqueous sample immediately after propidium monoazide treatment. This procedure was to corroborate the enumeration provided by cPCR after propidium monoazide treatment.

Selection of primers : PCR primers were selected that targeted a 285 bp segment of the *invA* gene of *Salmonella* (the *invA* gene of *Salmonella* is a component of the cell invasion apparatus) (Rahn *et al.,* 1992). These primers were *invA*139 (5' gtg aaa tta tcg cca cgt tcg ggc aa 3') and *invA*141 (5' tca tcg cac cgt caa agg aac c 3'), which target locations 287-312 and 571-550 within the *invA* gene respectively (Rahn *et al.,* 1992). These primers showed excellent specificity for *Salmonella*, detecting 99.4% of *Salmonella* strains without false positives when tested against 630 *Salmonella* strains and 142 non-*Salmonella* strains (Rahn *et al.,* 1992).

Polymerase chain reaction carried out (PCR) procedure : The original PCR experiments were on cell cultures in order to validate the functionality of the reagents and primers. Cells were taken from the growing colonies on media plates using a sterile toothpick and were suspended in 100µl of sterile distilled water. Samples were heated at 99°C for 5 minutes, and centrifuged at top speed in a micro-centrifuge for one minute to remove cell debris. The supernatant was then transferred into a new tube that was used as a source of DNA.

The PCR reaction mixture contained 2.5µl of purified DNA, 5 units of GoTaq reaction buffer from Promega Corp., USA, 1 x PCR reaction buffer at approximately 10µl, 1.5mM $MgCl_2$ (already in the GoTaq reaction buffer), 10 mM PCR nucleotide mix (Promega Corp., USA) and 1.5 µM DNA primer in a final volume of 50 µl. The reaction mixtures were incubated at 95°C for 2 min, and then cycled 30 times for another three steps: denaturing (95°C, 30 sec); annealing (50°C, 30 sec); and finally, primer extension (72°C, 45 sec). This was followed by the final extension step at 72°C for 7 mins. Amplification product sizes were verified by

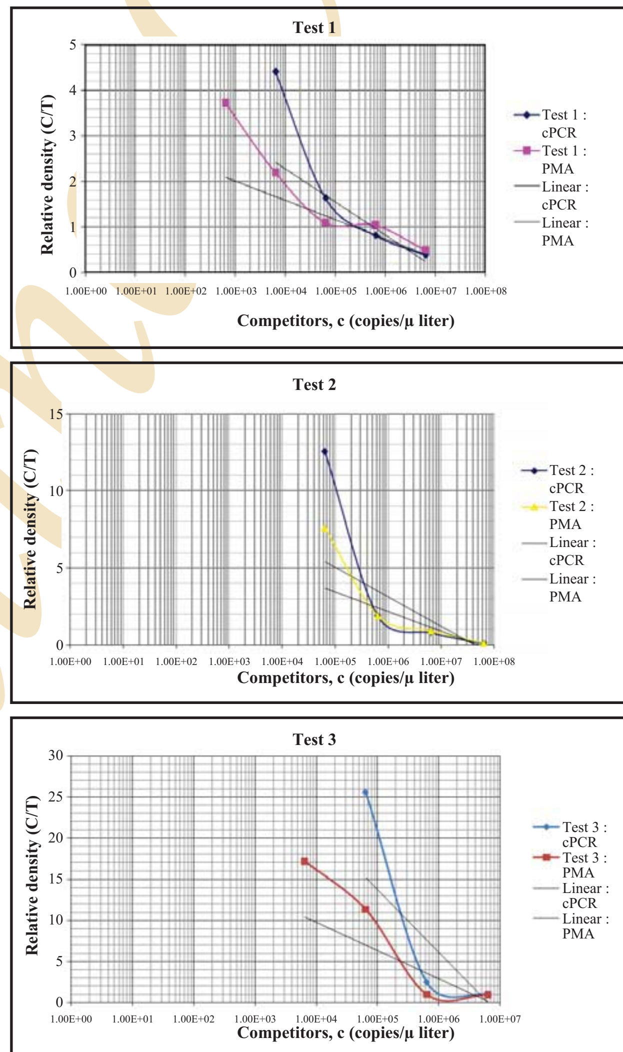

Fig. 3 : Analysis of average relative density of template and competitor fragment (n=3) measured by ImageJ software for Tests 1, 2 and 3

Online Copy

Table 1 : Calibration curves estimation for PMA-cPCR validation values by quantification of competitor/template ratio against dilution of the competitor series.

PMA-cPCR test	*Validation value (R^2)*
Test 1	0.97
Test 2	0.96
Test 3	0.96

electrophoresis of 10 µl samples in a 1.0% agarose Tris borate ethylenediaminetetraacetic acid gel with ethidium bromide staining.

PCR reaction conditions using salmonella spp. DNA : PCR with *invA* genes primers was then conducted with DNA from *Salmonella enteritidis* (ATCC13076) supplied from Remel Europe Ltd. in culti-loops. A suspension of *Salmonella* cells in sterile distilled water was lysed by heating to 99ºC for 5 mins. Cell debris was then removed by centrifugation. A PCR reaction was set-up containing 2.5 µl of DNA solution, 5 units of GoTaq DNA polymerase (Promega Corp., USA) 1× GoTaq PCR reaction buffer (containing 1.5mM $MgCl_2$), 0.2mM PCR nucleotide mix (Promega Corp., USA) and 0.6 µM DNA primers in a final volume of 50 µl. This reaction mixture (and a sterile control) was incubated at 95ºC for 2 mins and then cycled 30 times in three steps: i) denaturing (95ºC, 30 sec); annealing (50ºC, 30 seconds), iii) primer extension (72ºC, 45 sec). This was followed by a final extension step at 72ºC for 7 mins. The PCR product was purified using agarose gel electrophoresis and a QIAquick Gel Extraction Kit (QIAGEN Ltd., UK). The PCR product, which was just under 300bp long, was then sent for direct DNA sequencing (ABI 3100*xl* Capillary Sequencer) using both the *invA*139 and *invA*141 primers.

DNA sequence analysis : The specificities of all the probes and primers at species level were verified using NCBI-BLAST2 (Basic Local Alignment Search Tool) produced by EMBL Nucleotide Sequences Database (European Molecular Biology Laboratory) from the European Bioinformatics Institute (Wéry *et al.,* 2008). Default settings were used for the BLAST parameters (match/mismatch scores 2,-3, open gap penalty 5, gap extension penalty 2). The default settings used the drop down menu showing the hits found in BLAST that locate all common three-letter words between the sequence of interest and the hit sequence, or sequences from the database.

Competitive fragment for cPCR : The DNA fragment competitor with a small deletion was utilized from the DNA fragment produced by the *Salmonella* spp. primers *invA*139 and *invA*141, following the outline protocol developed by Sunar *et al.* (2010b). The third 'internal' primer required, which comprises a ~20bp sequence that will anneal to a site within the DNA template attached to the 3 end of one of the primers that produced the template (Zentilin and Giacca, 2007). The design of this 20bp sequence was undertaken from the target sequence using Primer3 (Rozen and Skaletsky, 2000) to produce a competitor fragment approximately ~100bp shorter than the target for easy identification by agarose gel electrophoresis. Two suitable 20bp sequences were used (5 ctg ttt acc ggg cat acc at 3 and 5 ggg cat acc atc cag aga aa 3) and two primers (Sal-I and Sal-I-V1, respectively) attached to these sequences to the 3 end of *invA*141. These primers were manufactured by Eurofins MWG Operon, Germany.

Competitor fragments were acquired by a PCR reaction using the *invA*139 and either the Sal-I or Sal-I-V1 primers. Each PCR contained 5 µl of DNA solution, 5 units of GoTaq DNA polymerase (Promega Corp., USA), 1× GoTaq PCR reaction buffer (containing 1.5mM $MgCl_2$), 0.2mM PCR nucleotide mix (Promega Corp., USA) and 0.6 µM

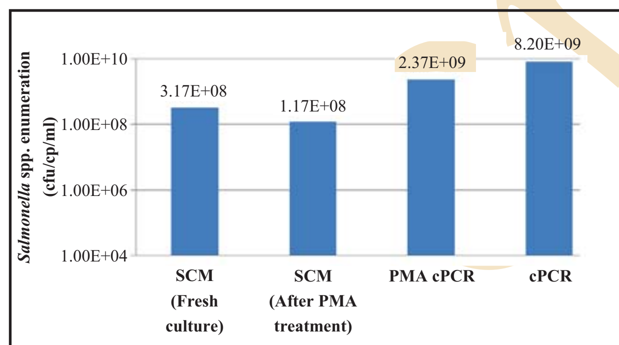

Fig. 4 : Comparison of different enumeration methods for *Salmonella* spp. using aqueous sample

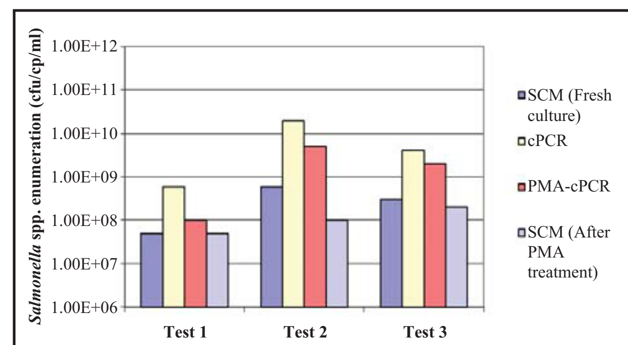

Fig. 5 : Overall average values for enumeration of *Salmonella* spp. using different methods

DNA primers in a final volume of 50 µl. The reaction mixtures (and a sterile control) were incubated at 95 ºC for 2 minutes, and then cycled 30 times through three steps: denaturing (95 ºC, 30 seconds), annealing at 64 ºC (De Clercq *et al.,* 2007) for 30 seconds, primer extension (72ºC, 45 seconds). This was followed by a final extension step at 72ºC for 7 mins. The PCR products were visualized by agarose gel electrophoresis and the gel bands representing the desired products were subsequently excised and purified using QIAquick Gel Extraction Kit (QIAGEN Ltd., UK).

The PCR products were ligated into a standard cloning vector (p-GEM-T Easy supplied by Promega), transformed into *E. coli* cells (XL1-Blue super-competent cells from Stratagene) and colonies were grown on LB-agar plates containing ampicillin (100 g ml^{-1}) surface dressed with IPTG and X-gal (as per the Stratagene protocol) for blue-white color screening. Colonies containing the insert were re-streaked on LB-ampicillin agar plates and single colonies from these plates were then incubated overnight in liquid LB-ampicillin. Plasmid DNA was extracted using a QIAprep Spin Miniprep Kit (QIAGEN Ltd., UK) and sent for automated DNA sequencing (ABI 3100*xl* Capillary Sequencer) using the T7P primer.

Following confirmation that the plasmid contained the correct insert, the cells derived from the same original single colony were then incubated overnight in liquid LB-ampicillin, so as to achieve the extraction of larger amounts of plasmid DNA (Sunar *et al.,* 2010b). This concentration of plasmid in the solution was measured by NanoDrop ND-1000 UV-spectrophotometer (Thermo-Fisher Scientific Inc., USA). It was then converted into an approximate number of plasmid copies assuming that 1g of 1,000bp DNA contains 9.1 $x10^{11}$ molecules (*New England Biolab*) after correcting the length of the plasmid formed by pGEM-T Easy and the insert.

cPCR reaction conditions : A cell culture from *Salmonella enteritidis* (ATCC13076) was lysed (99 ºC for 5 mins), centrifuged to remove the cell debris, and the supernatant diluted by a factor of 10 was used as target DNA solution. Each experiment comprised a number of PCR reactions containing an equal amount of target DNA and one member of dilution series of the competitor DNA (64, 640, 6400, 64000, 640000, 6400000, 64000000, 6.4E+08 copies/L). Each PCR reaction mixture contained 10 µl of target DNA solution, 2l of competitor DNA solution, 5 units of GoTaq DNA polymerase (Promega Corp., USA), 1× PCR reaction buffer (containing 1.5mM $MgCl_2$), 0.2mM PCR nucleotide mix (Promega Corp., USA) and 0.6 µM DNA primers (*invA*139 and *invA*141) in a final volume of 50 µl. The reaction mixtures were incubated at 94ºC for 4 mins and then cycled 40 times in three steps: denaturing (94ºC, 30 seconds); annealing (53C, 30 seconds) and primer extension (72ºC, 60 seconds). This was followed by a final extension step at 72ºC for 7 mins. The PCR products were then imaged via electrophoresis of 10 µl samples in 1.5% agarose TBE gel with ethidium bromide staining.

Analysis of cPCR : In order to quantify the DNA target, competitive PCR was utilized by running the amplification according to the cycling profiles, much the same as the competitor amplification. At this stage, DNA subjected to be quantified was combined with scalar amount of competitor DNA. The PCR products were then resolved by gel electrophoresis and stained with ethidium bromide. The intensity of two bands, i.e., competitor and target, was quantified by ImageJ (downloaded from the Research Service Branch of the National Institute of Mental Health of NIH) (Zentilin and Giacca, 2007). The competitor and target bands were easily distinguished by their different mobility after electrophoresis. The ratio between the amplification products (competitor/target) was plotted against the competitor concentrations. This plotted graph provided reading as an initial amount of target DNA.

Preparation of propidium monoazide solution : The propidium monoazide (phenanthridium,3-amino-8 azido-5-[3-(diethylmethyl-ammonio)propyl]-6-phenyldichloride (Biotium, Hayward, CA) solution was prepared by diluting one mg of propidium monoazide with 19.6µl of 100% DMSO (anhydrous dimethyl sulfoxide) (Biotium, Hayward, CA). This produced 100mM of propidium monoazide solution in 100% of DMSO. This step was important as propidium monoazide was only dissolved in DMSO instead of using water for the initial step of dilution. This solution was then distributed into dark centrifuge tubes with 4 µl of solution in each tube. The stock concentration of propidium monoazide was then prepared by diluting five-folds, by adding 16 µl of ddH_2O directly into each tube. This resulted the stock concentration of 20mM of propidium monoazide solution in 20% of DMSO. All the tubes were labeled and stored at -20°C in dark.

Propidium monoazide cross-linking procedure : The propidium monoazide was dissolved in water to make a stock concentration of 5 mg/ml and stored at -20°C in the dark. After a brief vortex of DNA samples, propidium monoazide was added to the samples at a final concentration of 100 µg

Online Copy

ml^{-1}. It has been reported earlier that this is a suitable concentration, resulting in highest signal reduction (Nogva *et al.,* 2003). Following an incubation period of 5 mins in dark with the occasional flipping at room temperature (Nocker *et al.,* 2007), the sample was then exposed to light for 3 mins using a 500 W halogen light source (Varma *et al.*, 2009), which was placed 20 cm from the sample tubes. During the exposure, the sample was placed on ice in order to avoid excessive heating. Following the photo-induced cross-linking process, cells were pelleted at 5,000 x g for 5 mins prior to DNA isolation.

Propidium monoazide-cPCR reaction conditions : A propidium monoazide-cPCR with *invA* primer was conducted with DNA solution immediately after the propidium monoazide cross-linking procedure. The dilution series of the competitor DNA used was 64, 640, 6400, 64000, 640000, 6400000, 64000000, 6.4E+08 copies/L. Each PCR reaction mixture contained 10 µL of target DNA solution, 2L of competitor DNA solution, 5 units of GoTaq DNA polymerase (Promega Corp., USA), 1× PCR reaction buffer (containing 1.5mM $MgCl_2$), 2mM PCR nucleotide mix (Promega Corp., USA) and 0.6 µM DNA primers (*invA*139 and *invA*141) in a final volume of 50 µl. The reaction mixtures were incubated at 94°C for 4 mins, and then cycled 40 times through three steps: i) denaturing (94°C, 30 second), ii) annealing (53°C, 30 second), iii) primer extension (72°C, 60 second). This was followed by a final extension step at 72°C for 7 mins. The PCR products were imaged by electrophoresis with 1.5% agarose TBE gel with ethidium bromide straining.

Results and Discussion

cPCR validation for *Salmonella* spp. : The 40-cycle program used for cPCR takes approximately 2.5 hrs to run. Different bands on agarose gel produced by the target and competitor DNA were easily distinguishable on a normal size gel after 1.5 hrs. Thus, the entire protocol look less than 5 hrs. Other reactions with very low target DNA copy numbers (not reported here) showed that 40 copies of the competitor were readily detected by PCR as a bright band on agarose gel and significantly fewer than 40 copies of the target DNA (*i.e.* a target concentration that produced no band when 40 copies of the competitor fragment were present) can be detected in the absence of the competitor fragment. Thus, provided a cPCR dilution series is always one PCR reaction with no competitor fragment, it is possible to detect extremely low copy numbers (possibly one or two copies) of the target.

Different dilution series of competitor were tested in cPCR to develop the calibration curve as shown in Fig. 1. This calibration curve for cPCR was plotted according to the competitor/template ratio. The bands of the amplification products corresponding to the competitor and target (*Salmonella* spp. DNA from aqueous sample) of the gel were quantified by Image J software. The number of DNA copies of the competitor added to the sample before starting amplification was plotted against the competitor/template ratio. The experimental points were fitted by a straight line (r''= 0.97), as expected from the theory of competitive PCR. Fig. 1 shows the reaction conditions using *invA* gene as a primer.

The upper and lower arrows shown in Panel A indicate the amplification products for the template and competitor, respectively. The amount of target amplification product (T, corresponding to *invA* gene primer) and the competitor product (C) were evaluated using Image J software. The ratio between the two values (C/T) has been reported at the bottom of gel image. The graph in Fig. 1 (Panel B) shows quantification of template/competitor ratio after being quantified by Image J software.

Relationship between the C/T ratio and the input amount of competitor is shown as a straight line fit (correlation coefficient, R^2= 0.97). The equation describing this straight line is shown above the line. According to this equation, the number of competitor DNA copies at C/T ratio, r''=1 refers to the number of target quantifications in the aliquot of the sample during PCR amplification.

The PCR program used in the study for cPCR was deliberately selected to maximize the probability of obtaining a product from very low copy numbers. Features included a relatively low annealing temperature of 53ºC, and a large number of thermal cycles. The former would maximize the chances of primers annealing to the DNA template, but may allow non-specific annealing (annealing at a site where there is some degree of mismatch). The outcome of such a non-specific annealing would be non-specific PCR products, which were usually easy to distinguish from the desired product due to their difference in length (it is extremely improbable that non-specific annealing of the primer to either an incorrect site on the *Salmonella* template or to non-*Salmonella* DNA will produce a product of exactly 183bp). Experiments carried out till date with *Salmonella* as only template do not indicate any issues with non-specific amplification, but further work with different samples is required to completely eliminate this possibility. A PCR

Online Copy

program involving 40 thermal cycles is sufficient to reach the plateau phase of amplification for most systems, and thus the products will be readily detectable with rapid, low-cost, easy to use techniques like gel electrophoresis.

Viability of *Salmonella* spp. DNA (propidium monoazide-PCR) : This developed method is highly significant and useful, particularly when applied in the sanitizing process in environmental samples. In the beginning, the propidium monoazide procedure was combined with PCR to determine the *Salmonella* spp. DNA target with live and dead cells (known as propidium monoazide-PCR). The result is shown in Fig. 2.

In this experiment, the *Salmonella* spp. DNA cells were separately prepared as 100% dead cells (Lane 4), 100% live cells (Lane 5), and a mixture of 50% live cells and 50% dead cells (Lane 6). Propidium monoazide was added to all the samples containing dead, live and a combination of live and dead cells. This test showed the success of the propidium monoazide cross-linking procedure as a pre-treatment to the PCR method. The light exposure (500 watts) for 5 mins led to covalent binding and inactivation of free propidium monoazide.

Propidium monoazide-cPCR validation for *Salmonella* spp. : Propidium monoazide combined successfully with cPCR to utilize the quantitative PCR for enumerating viable *Salmonella* spp. DNA. From the results, it can be seen that the developed cPCR method was successfully combined with propidium monoazide procedure (in this study it is known as propidium monoazide-cPCR). A wide range of dilution series was applied in order to perform the cPCR test (Test 1, Test 2 and Test 3). This result highlights the trend of enumeration of known samples of viable DNA shown by the lines getting brighter with decreasing amounts of competitor fragment. Validation result of propidium monoazide-cPCR was analyzed with Image J software to determine relative density of the bands. The density ratio (C/T) for propidium monoazide-cPCR was plotted against dilution of the competitor series, following the same procedure as shown in Fig. 1 (validation of cPCR). The competitor copy number was calculated when the C/T ratio was 1 (Zentilin and Giacca, 2007). All validation experimental points were nearly fitted by a straight line ($R^2 \sim 1$), as shown in Table 1.

Quantification of viable DNA of *Salmonella* spp (propidium monoazide-cPCR) : The method of enumeration of *Salmonella* spp. using an aqueous sample of *Salmonella* spp. in sterile culture broth was conducted. All the tests (Tests 1, 2, and 3) used the same source of fresh *Salmonella* spp. DNA that was prepared in a culture broth solution. The tests (Tests 1, 2 and 3) were not conducted on the same day. Each test that involved a set of cPCR and propidium monoazide-cPCR was carried out on the same day. The standard cultivation method was used for every enumeration and to ensure that the *Salmonella* spp. source was not less than 1-2 $\log_{10}$ cfu/ml in the original stock.

Fig. 3 shows the result of statistical analysis produced from relative density of bands measured by Image J software. The analysis involved an average of relative density measurements (n=3), which were then plotted against a series of competitor fragments. As shown in Fig. 3, the amount of initial target template was read from the point on the curve where the amount of competitive (C) and target (T) are equal (Raeymaekers, 1995), whereas, in the study the value point for relative density (C/T) was 1.

The enumeration methods used for *Salmonella* spp. is shown in Fig. 4. Fig. 5 shows additional tests pertaining to standard cultivation technique that was applied to enumerate the culture before and after the propidium monoazide pre-treatment. These results proved that the method developed for propidium monoazide treatment did not affect the viability of live *Salmonella* spp. during photo-activation procedure. Fig. 4 and 5 show that the amount of *Salmonella* spp. in cfu before and after propidium monoazide pre-treatment did not decrease significantly, as all figures were maintained at the same order or magnitude. This is in agreement with the previous reports that show that propidium monoazide does not affect DNA yield from live cells (Nkuipou-Kenfack *et al.,* 2013; Nocker *et al.,* 2007).

The results of the standard culturing method (standard culture method) in Fig. 4 for fresh culture showed the typical bacterial growth phase, as all the tests (Tests 1, 2 and 3) were performed on different days. Beginning with Test 1 the culture broth was approximately 5.0×10^7 cfu ml^{-1}. Then, it slightly increased indicating that the cells in the broth culture grew at log-phase as measured in Tests 2 and 3, which gave approximately 6.0×10^8 cfu ml and 3.0×10^8 cfu ml^{-1}, respectively. However, the standard culture method only measures viable cells in samples. The study showed that cPCR gave a higher enumeration figure for each test as compared to standard culture method.

This can be explained as the enumeration figures provided by cPCR included both viable and non-viable DNA. Standard culture method only enumerated viable culturable

Online Copy

cells. The propidium monoazide-cPCR, only enumerated viable DNA, thus, showing slightly lower figures as compared to cPCR.

Standard culture method gave approximately 1-2 $\log_{10}$ cfu ml^{-1} less in comparison to the propidium monoazide-cPCR results. These results provide evidence to support the VBNC state; whereas, the viable cells failed to be cultured by the standard culture method. This is a significant advantage of DNA-molecular based quantifying methods, such as cPCR, when applied with propidium monoazide pre-treatment. In Fig. 5, all values were used to determine an average figure to summarize all the tests based on the different methods.

In this study, it was found that enumeration technique using propidium monoazide-cPCR provided rapid enumeration for viable DNA. The conventional culture method required 24 hrs to provide the enumeration result; whereas, the cPCR methods combined with propidium monoazide treatment required approximately 5 hrs to obtain the results. The cPCR used in the study showed that they could provide a potential method for accurately quantifying *Salmonella* spp. in samples. cPCR was more accurate than real-time PCR in all experimental conditions, especially when determining differences in the nucleic acid abundance of less than two to four folds (Zentilin and Giacca, 2007).

The propidium monoazide-cPCR was developed to turn the qualitative PCR method into an improvised one, which gave both quantitative results and differentiate between live and dead. Whilst, in this case, conventional PCR only showed presence or absence of target microorganisms, not their concentrations. The results obtained were considered important because the method also gave numerical results for *Salmonella* spp. as these are needed elsewhere when assessing any environmental quality. A cPCR method was developed in order to provide quantitative results for the target microorganisms. The previous study on deletion method of pathogen-specific competitor fragments for use in cPCR (Sunar *et al.,* 2009c) was suitably utilized in this propidium monoazide-cPCR development. In this study, cPCR technique was utilized to enumerate *Salmonella* spp. in aqueous samples. The primers that produced 284 bp fragment from *invA* gene proved to be effective for detection of *Salmonella* spp. by PCR owing to its high specificity. A clear and single band imaged in 2% agarose gel electrophoresis produced a good match; thus, it was highly significant in the cPCR and propidium monoazide-cPCR methods developed in this study.

The results demonstrated that cPCR has potential to enumerate *Salmonella* spp. species in an aqueous solution. The PCR program used in this study for cPCR was selected in order to maximize the probability of obtaining a product from very low copy numbers. Features include a relatively low annealing temperature of 53°C, and a large number of thermal cycles. The former maximized the chance of primer annealing to the DNA template, but may ultimately allow non-specific annealing, i.e., annealing at a site where there is some degree of mismatch. The outcome of such non-specific annealing would be non-specific PCR products, which are usually easy to distinguish from the desired product owing to their difference in length. It is extremely improbable that non-specific annealing of a primer to either an incorrect site on the *Salmonella* spp. template or to a non-*Salmonella* spp. DNA would produce a product of exactly 183bp. Importantly, a PCR program involving 40 thermal cycles was sufficient to reach the plateau phase of amplification for most systems, and the products would be readily detectable with rapid, low-cost, easy-to-use techniques, such as gel electrophoresis.

The development of cPCR to enumerate *Salmonella* spp. was highly significant and suitable to be adapted to any aqueous and solid environmental samples. Furthermore, cPCR detects the presence of pathogen specific DNA and is able to identify the presence of VBNC cells, which cannot be enumerated through traditional culturing methods (Higgins *et al.,* 2007). On this basis there may appear a role for conventional cPCR in environmental monitoring. The cPCR procedure, as presented, does not differentiate between viable and dead cells, which means it could overestimate the number of viable cells in a system. This might be a serious limitation for a test applied to the sanitizing process, as the dead cells may rethidium monoazidein after the waste is safe. Therefore, the propidium monoazide pretreatment step as DNA-intercalating dye was used in the cPCR protocol to provide viable/dead cell differentiation known as propidium monoazide-cPCR.

The propidium monoazide-cPCR method developed was shown to be effective in enumerating the live *Salmonella* spp. DNA. The quantification results from the propidium monoazide-cPCR method for viable and non-viable DNA were analyzed and compared with conventional methods and cPCR. This type of cPCR and propidium monoazide-cPCR procedure for quantifying *Salmonella* spp. showed positive results, especially when utilized with aqueous sample.

Online Copy

Moreover, it also represented non-degradation of *Salmonella* spp. DNA during cross-linking upon bright light exposure. The results were approximately of the same magnitude order at an average 10^8cfu ml^{-1} for measured before and after photo-activation procedure. This proves that propidium monoazide does not penetrate the membranes of live *Salmonella* spp. cells and that it is efficiently taken up by the permeabilized cells (Singh *et al.,* 2013). The propidium monoazide entered the cells intercalating the dye into double-stranded nucleic acids. In the binding process, the photo-induced cross-linkage renders the DNA insoluble and results in its degradation together with cell debris during the DNA extraction procedure. In this test, an aqueous sample containing unknown live and dead *Salmonella* spp. cells was used; therefore, the rethidium monoazideining *Salmonella* spp. DNA from the live cells is amenable to further downstream quantification using cPCR.

Overall, the study successfully upgraded the application of cPCR from DNA-based quantification to limiting diagnostics to only live cells of *Salmonella* spp. by utilizing the propidium monoazide-cPCR methodology. The results show that the propidium monoazide-cPCR method only enumerated the viable DNA of *Salmonella* spp. in the samples. The propidium monoazide-cPCR gives a higher amount of viable DNA in the sample compared to the standard cultivation method, which provides evidence for the VBNC state. Several authors have reported on the VBNC state (Dinu and Bach, 2013; Li *et al.*, 2014; Vendrame *et al.*, 2013), stating that bacterial cells that are in a VBNC state may still have the potential to cause human infection (Josefsen *et al.*, 2010). In this study the reasons for the *Salmonella* spp. entering a VBNC state were not clear and should be addressed in future studies concerning the viability of pathogen indicators. Several possibilities such as sublethal injury, which reduces their ability to grow on selective media, the presence of growth inhibitors; and/or transition into a survival mode with changes in physiology that reduces culturability were reportedly associated with the VBNC state (Higgins *et al.*, 2007).

Several researchers have indicated that the efficiency of propidium monoazide for environmental samples depends on the type of chemical used (Su *et al.*, 2013; Barth Jr. *et al.*, 2012). For example, the soil sample is associated with the turbidity and suspended solids in the samples extracted from the compost material (results not shown). For any environmental samples, it is recommended that turbidity and suspended solids should be measured before propidium monoazide pre-treatment and should be less than approximately 10 NTU. Though it can be easily seen by the naked eye, low turbidity affects and interrupts the propidium monoazide cross-linking photoactivation process (Luo et al., 2010). In this context, Varma et al. (2009) reported that tests with water and biomass samples containing suspended solids appeared to interfere with the ability of the propidium monoazide-qPCR method to specifically detect live cells, thereby suggesting that further optimization of the method is required (Bae and Wuertz, 2009). The developed propidium monoazide-cPCR method presented in this study, even if used effectively for a DNA viability study, may require further optimization before propidium monoazide pre-treatment, as environmental samples usually contain high levels of turbidity and suspended solids (Sunar, 2011). Despite the concerns about the propidium monoazide cross-linking procedure, e.g., the DNA-based quantification also presents challenges with false or negative results that, depending on the elements contained in the environmental samples, require laborious optimization procedures to be eliminated. Therefore, highly competent individuals are needed if the molecular DNA-based amplification is to be used for routine environment sample quality monitoring in the field.

This study has been successful in developing the propidium monoazide-cPCR as a useful molecular technique for pathogen indicator detection and DNA-based quantification. The propidium monoazide-cPCR as DNA based detection and nucleic acid amplification protocols are shown to be practical for a viability study of *Salmonella* spp. This can be applied to produce accurate and reliable data for risk assessment in waste, food and environmental samples. The propidium monoazide-cPCR method recognizes the potential of the VBNC state and is thereby able to assess the outcome and impact of new control strategies.

References

Bae, S. and S. Wuertz: Discrimination of viable and dead fecal Bacteroidales bacteria by quantitative PCR with propidium monoazide. *App. Environ. Microbiol.*, **75**, 2940–4 (2009).

Barth Jr., V.C. Cattani, F. Ferreira, C.A.S. and S.D. de Oliveira: Sodium chloride affects propidium monoazide action to distinguish viable cells. *Analyt. Biochem.*, **428**, 108–110 (2012).

Blooi, M., A. Martel, F. Vercammen and F. Pasmans: Combining ethidium monoazide treatment with real-time PCR selectively quantifies viable Batrachochytrium dendrobatidis cells. *Fun. Biol.*, **117**, 156–162 (2013).

De Clercq, D., A. Ceustermans, M. Heyndrickx, J. Coosethidium monoazidens and J. Ryckeboer: A rapid monitoring assay for the detection of *Salmonella* spp. and *Salmonella senftenberg* strain W775 in composts. *J. App. Microbiol.*, **103**, 2102–12 (2007).

Online Copy

Dinu, L.D. and S. Bach: Detection of viable but non-culturable Escherichia coli O157:H7 from vegetable samples using quantitative PCR with propidium monoazide and immunological assays. *Food Control*, **31**, 268–273 (2013).

Elizaquível, P., G. Sánchez and R. Aznar: Application of propidium monoazide quantitative PCR for selective detection of live *Escherichia coli* O157:H7 in vegetables after inactivation by essential oils. *Inter. J. Food Microbiol.*, **159**, 115–121 (2012).

Fittipaldi, M., A. Nocker and F. Codony: Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. *J. Microbiol. Meth.*, **91**, 276–289 (2012).

Frankenhuyzen, J., J. Trevors, C. Flemming, H. Lee and M. Habash: Optimization, validation and application of a real-time PCR protocol for quantification of viable bacterial cells in municipal sewage sludge and biosolids using reporter genes and *Escherichia coli. J. Ind. Microbiol. Biotechnol.*, **40**, 1251–1261 (2013).

Higgins, M.J., Y.C. Chen, S.N. Murthy, D. Hendrickson, J. Farrel and P. Schafer: Reactivation and growth of non-culturable indicator bacteria in anaerobically digested biosolids after centrifuge dewatering. *Water Res.*, **41**, 665–73 (2007).

Jacobsen, C.S. and T.B. Bech: Soil survival of Salmonella and transfer to freshwater and fresh produce. *Food Res. Int.*, **45**, 557–566 (2012).

Josefsen, M.H., C. Löfström, T.B. Hansen, L.S. Christensen, J.E. Olsen and J. Hoorfar: Rapid quantification of viable *Campylobacter* bacteria on chicken carcasses, using real-time PCR and propidium monoazide treatment, as a tool for quantitative risk assessment. *App. Environ. Microbiol.*, **76**, 5097–104 (2010).

Lee, J.L. and R.E. Levin: A comparative study of the ability of ethidium monoazide and propidium monoazide to distinguish viable from heat killed mixed bacterial flora from fish fillets. *J. Microbiol. Meth.*, **76**, 93–96 (2009).

Li, D., T. Tong, S. Zeng, Y.Lin, S. Wu and M. He: Quantification of viable bacteria in wastewater treatment plants by using propidium monoazide combined with quantitative PCR (propidium monoazide-qPCR). *J. Environ. Sci.*, **26**, 299–306 (2014).

Luo, J.F., W.T. Lin and Y. Guo: Method to detect only viable cells in microbial ecology. *App. Microbiol. Biotechnol.*, **86**, 377–84 (2010).

Nkuipou-Kenfack, E., H. Engel, S. Fakih and A. Nocker: Improving efficiency of viability-PCR for selective detection of live cells. *J. Microbiol. Meth.*, **93**, 20–24 (2013).

Nocker, A., P. Sossa-Fernandez, M.D. Burr and A.K. Camper: Use of propidium monoazide for live/dead distinction in microbial ecology. *App. Environ. Microbiol.*, **73**, 5111-5117 (2007).

Nogva, H.K., S.M. Drømtorp, H. Nissen and K. Rudi: Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5'-nuclease PCR. *Biotechniques*, **34**, 804–8; **810**, 812–3 (2003).

Raeymaekers, L.: A commentary on the practical applications of competitive PCR. *Genome Res.*, **5**, 91–94 (1995)

Rahn, K., S.A. De Grandis, R.C. Clarke, S.A. McEwen, J.E. Galán, C. Ginocchio and C.L. Gyles: Amplification of an invA gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Molec. Cell. Prob.*, **6**, 271–9 (1992).

Rozen, S. and H.J. Skaletsky : Primer3 on the WWW for general users and for biologist programmers. In: Bioinformatics Methods and Protocols (Ed.: M.S. Krawetz): Methods in Molecular Biology. Humana Press, Totowa, NJ, pp. 365–386(2000).

Singh, G., P. Vajpayee, S. Bhatti, N. Ronnie, N. Shah, P. McClure and R. Shanker: Determination of viable *Salmonellae* from potable and source water through propidium monoazide assisted qPCR. *Ecotoxicol. Environ. Saf.*, **93**, 121–127(2013).

Su, C.P., W.N. Jane and H. Wong: Changes of ultrastructure and stress tolerance of *Vibrio parahaemolyticus* upon entering viable but nonculturable state. *Inter. J. Food Microbiol.*, **160**, 360–366 (2013).

Sunar, N.M., D.I. Stewart, E.I. Stentiford and L.A. Flecther: A rapid molecular approach to determining the occurrence of pathogen indicators in compost. In: Proceeding of Twelfth International Waste Management and Landfill. Sardinia 2009 Symposium, pp. 53–54 (2009a).

Sunar, N.M., E.I. Stentiford, D.I. Stewart and L.A. Fletcher : The process ind pathogen behaviour in composting: A Review. In Proceeding UMT-MSD 2009 Post Graduate Seminar 2009. Universiti Malaysia Terengganu, Malaysian Student Department UK & Institute for Transport Studies University of Leeds. pp. 78–87 (2009b).

Sunar, N.M., D.I. Stewart, E.I. Stentiford and L.A. Flecther: Enumeration of *Salmonella* in compost material by a non-culture based method. In: Proceeding of Twelfth International Waste Management and Landfill. Sardinia 2009 Symposium (2009c).

Sunar, N.M., E.I. Stentiford, L.A. Fletcher and D.I. Stewart: Survival of *Salmonella* spp. in composting using vial and direct inoculums technique. In ORBIT 2010 International Conference of Organic Resources in the Carbon Economy. Crete, Greece. (pp. 886–892) (2010a).

Sunar, N.M., E.I. Stentiford, D.I. Stewart and L.A. Flecther: Molecular techniques to characterize the invA genes of *Salmonella* spp. for pathogen inactivation study in composting. In ORBIT 2010 International Conference of Organic Resources in the Carbon Economy (2010b).

Trevors, J.T.: Can dead bacterial cells be defined and are genes expressed after cell death? *J. Microbiol. Meth.*, **90**, 25–28 (2012).

Varma, M., R. Field, M. Stinson, B. Rukovets, L. Wymer and R. Haugland: Quantitative real-time PCR analysis of total and propidium monoazide-resistant fecal indicator bacteria in wastewater. *Water Res.*, **43**, 4790–801(2009).

Vendrame, M., L. Iacumin, M. Manzano and G. Comi: Use of propidium monoazide for the enumeration of viable *Oenococcus oeni* in must and wine by quantitative PCR. *Food Microbiol.*, **35**, 49–57 (2013).

Wang, L., P. Li, Y. Yang, H. Xu, Z.P. Aguilar, H. Xu and H. Wei: Development of an immunomagnetic separation–propidium monoazide–polymerase chain reaction assay with internal amplification control for rapid and sensitive detection of viable *Escherichia coli* O157:H7 in milk. *Inter. Dairy J.*, **34**, 280–286 (2014).

Wéry, N., C. Lhoutellier, F. Ducray, J.P. Delgenès and J.J. Godon: Behaviour of pathogenic and indicator bacteria during urban wastewater treatment and sludge composting, as revealed by quantitative PCR. *Water Res.*, **42**, 53–62 (2008).

Yang, Y., C. Wan, H. Xu, W. Lai, Y. Xiong, F.H. Xu and Wei: Development of a multiplexed PCR assay combined with propidium monoazide treatment for rapid and accurate detection and identification of three viable *Salmonella enterica* serovars. *Food Control*, **28**, 456–462 (2012).

Zentilin, L. and M. Giacca: Competitive PCR for precise nucleic acid quantification. *Nature Protocols*, **2**, 2092–104 (2007).

Online Copy